# Introduction

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A. INTRODUCTION

This document is written to provide guidance to investigators that are interested in developing assays useful for the evaluation of compound collections to identify chemical probes that modulate the activity of biological targets. Originally written as a guide for therapeutic projects teams within a major pharmaceutical company, this manual has been adapted to provide guidelines for:

a. Identifying potential assay formats compatible with High Throughput Screen (HTS), and Structure Activity Relationship (SAR)
b. Developing optimal assay reagents
c. Optimizing assay protocol with respect to sensitivity, dynamic range, signal intensity and stability
d. Adopting screening assays to automation and scale up in microtiter plate formats
e. Statistical validation of the assay performance parameters
f. Secondary follow up assays for chemical probe validation and SAR refinement
g. Data standards to be followed in reporting screening and SAR assay results.

## General definition of biological assays

This manual is intended to provide guidance in the area of biological assay development, screening and compound evaluation. In this regard an assay is defined by a set of reagents that produce a detectable signal allowing a biological process to be quantified. In general, the quality of an assay is defined by the robustness and reproducibility of this signal in the absence of any test compounds or in the presence of inactive compounds. This robustness will depend on the type of signal measured (absorbance, fluorescence, radioactivity etc), reagents, reaction conditions and analytical and automation instrumentation employed. The quality of the HTS is then defined by the behavior of this assay system when screened against a collection of compounds. These two general concepts, assay quality and screen quality, are discussed with specific examples in the chapters of this manual.

Assays developed for HTS can be roughly characterized as cell-free or cell-based in nature. The choice of either biochemical or cell-based assay design and the particular assay format is a balancing act between two broad areas. On one side of the fulcrum is the need to ensure that the measured signal is providing relevant data to the desired biological process. For assays that are to be used in HTS, this must be balanced with the ability of these assays to support reagents that yield robust data in microtiter plate formats where typically $10^5$ to $10^6$ samples are screened in the assay.

## General Concepts in Method (Assay) Development and Validation

The investigator must validate the assay methodology by proceeding through a series of steps along the pathway to HTS. The overall objective of any method validation procedure is to demonstrate that the method is acceptable for its intended purpose. As mentioned above, the purpose can be to determine the biological and or pharmacological activity of new chemical entities. The acceptability of a measurement procedure or bioassay method begins with its design and construction, which can significantly affect its performance and robustness.

This process originates during method development and continues throughout the assay life cycle (Figure 1). Successful completion of validation at an earlier stage increases the likelihood of success at later stages. During method development, assay conditions and procedures are selected that minimize the impact of potential sources of invalidity (e.g. so-called false positives or false negatives) on the measurement of analyte or the biological end point (eg. Gene expression, protein phosphorylation ) in targeted sample matrices or test solutions. There are three fundamental general areas in method development and validation: (a) Pre-study (Pre-screen) validation (b) In-study (In-screen) validation, and (c) Cross-validation or method transfer validation. These stages encompass the systematic scientific steps in assay development and validation cycle.
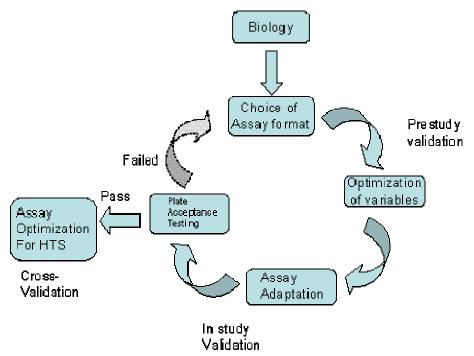


**Figure 1: The Assay Development Cycle**

**Pre-study validation:** The investigator is faced with a number of choices with respect to the assay design and format. For many well characterized target classes there are a number of methods and kits available. At this stage the choice of an assay format is made. Close attention must be paid at this early stage to factors such as the selection of reagents with appropriate specificity and stability. Validation of assay performance at this stage should proceed smoothly if high quality procedures are chosen during method development. This requires the generation and statistical analysis of confirmatory data from planned experiments to document that analytical results satisfy pre-defined acceptance criteria. The choice of detection is made here. If fluorescent labels are chosen, careful attention must be paid to the wavelength to ensure low interference by compounds, compatibility with microtiter plate plastics and that appropriate filters are available on high-throughput plate readers. If available, the assay sensitivity and pharmacology is evaluated using control compounds. Section IV illustrates procedures common to compound evaluation using dose-response curves. Several examples of assay design and optimization are given in the additional sections of this manual for well-studied target classes (Sections V-XI). A complete discussion of design of experiment procedures will be a topic for a future chapter in this manual.

**In-study validation:** These procedures are needed to verify that a method remains acceptable during its routine use. For assays to be run in HTS the assay must be adapted to microtiter plate volumes. Therefore, plate acceptance testing is required where the assay is run in several microtiter plates (at least 96-well plates). From this data, statistical measures of assay performance such as Z-factors are calculated. Some methods may require additional experiments to validate the automation and scale up of an assay that may not have been addressed in earlier stages. The plates should contain appropriate maximum and minimum control samples to serve as quality controls of each run to check the performance. This will allow the investigator to check for procedural errors and to evaluate stability of the method over time. Assaying a randomly selected subset of test samples at multiple dilution levels monitors parallelism of test and standard curve samples. Sections II and III illustrate the procedures typically used to evaluate assay performance in microtiter plates and some of the common artifacts that are observed.

**Cross validation:** This portion includes the assay hand-off from the individual investigator's team to the high-throughput screening center. More broadly, this procedure is used at any stage to verify that an acceptable level of agreement exists in analytical results before and after procedural changes in a method as well as between results from two or more methods or laboratories. Typically, each laboratory assays a subset of compounds and the agreement in results is compared to predefined criteria that specify the allowable performance for HTS. Considerations in adapting assays to automated robotic liquid handling and plate screening protocols are also discussed in the sections of this manual.

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2. Smith WC, Sittampalam GS: Conceptual and statistical Issues in the validation of analytical dilution assays for pharmaceutical applications. *J. Biopharm. Stat., 8, 509-532, 1998*.

# SECTION II

# ASSAY VALIDATION

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# A. Overview

The statistical validation requirements for an assay vary, depending upon the prior history of the assay.  Stability and Process studies (Section B) should be done for all assays, prior to the commencement of the formal validation studies.  If the assay is new, or has never been previously validated, then full validation is required.  This consists of a 3 day Plate Uniformity study (Section C) and a Replicate-Experiment study (Section D).  If the assay has been previously validated in a different laboratory, and is being transferred to a new laboratory, then a 2 day Plate Uniformity study (Section C) and a Replicate-Experiment study (Section C) are required.  An assay is considered validated if it has previously been assessed by all the methods in this section, and is being transferred to a new laboratory without undergoing any substantive changes to the protocol.  If the intent is to store the data with the results of the previous facility's data then an assay comparison study (Section D) should be done as part of the Replicate-Experiment study.  Otherwise only the intra-laboratory part of the Replicate-Experiment study (Section D) is recommended.

If the assay is updated from a previous version run in the same facility then the requirements vary, depending upon the extent of the change.  Major changes require a validation study equivalent to a laboratory transfer.  Minor changes require bridging studies that demonstrate the equivalence of the assay before and after the change.  See Section E for examples of major and minor changes.

These techniques are intended to be applied to $\geq 96$ well primary target binding and functional assays.  You should discuss with a statistician alternatives for assays with significant time, resource or expenditure constraints to properly balance validation requirements with these constraints.

# B. Stability and Process Studies

## B.1. Reagent Stability and Storage Requirements

It is important to determine the stability of reagents under storage and assay conditions.

- Use the manufacturer's specifications if the reagent is a commercial product.

- Identify conditions under which aliquots of the reagent can be stored without loss of activity.

- If the proposed assay will require that the reagent be frozen and thawed repeatedly, test its stability after similar numbers of freeze-thaw cycles.

- If possible, determine the storage-stability of the reagent

- If reagents are combined and aliquoted together, examine the storage-stability of the mixtures.

## B.2. Reaction Stability Over Projected Assay Time

Conduct time-course experiments to determine the range of acceptable times for each incubation step in the assay. This information will greatly aid in addressing logistic and timing issues.

### Reagent Stability During Daily Operations; Use Of Daily Leftover Reagents

The stability studies will require running assays under standard conditions, but with one of the reagents held for various times before addition to the reaction. The results will be useful in generating a convenient protocol and understand the tolerance of the assay to potential delays encountered during screening.

If possible, reagents should be stored in aliquots suitable for daily needs. However, some information pertinent to saving leftover reagents (particularly expensive ones) for future assays should be obtained.

New lots of critical reagents should be validated using the bridging studies

## B.3. DMSO Compatibility

Test compounds are delivered at fixed concentrations in 100% DMSO, thus solvent-compatibility of assays should be determined. Typically, the uninhibited or fully stimulated assay may be performed in the presence of DMSO concentrations spanning the expected final concentration. Typically, DMSO concentrations from 0 to 10% are tested. Note that this study

should be done relatively early in development of the assay because other studies, such as the variability studies, should be performed with the concentration of DMSO that will be used in screening. For cell based assays, it is recommended that the final %DMSO be kept under 1%

# C. Plate Uniformity and Signal Variability Assessment

## C.1. Overview

All assays should have a plate uniformity assessment.  For new assays the plate uniformity study should be run over 3 days to assess uniformity and separation of signals, using DMSO at the concentration to be used in screening.  For assay transfers (See Section A for the definition of an assay transfer) the plate uniformity study need be only 2 days.

The actual variability tests are conducted on three types of signals.

- "Max" signal: This measures the maximum signal.  For agonist assays this would be maximal response of an agonist; for potentiator assays this would be an $EC_{10}$ concentration of a standard agonist (the actual percentage is as per protocol and may not be 10% in some cases) plus maximal concentration of a standard potentiator.  For inhibition type assays this would be a reaction with an $EC_{80}$ concentration of a standard agonist (again the actual percentage is as per protocol, and may not be 80%).  For inverse agonist assays this would be the untreated constitutively active condition in the presence of DMSO alone.

- "Min" signal: This measures the background signal. For agonist assays this is the basal signal.  For potentiator assays this is an $EC_{10}$ concentration of agonist.  For inhibitor assays, including receptor-binding assays, this is an $EC_{80}$ concentration of the standard agonist plus a maximally inhibiting concentration of a standard antagonist (preferred) or unstimulated reaction.

- "Mid" signal: This estimates the signal variability at some point between the maximum and minimum signals. Typically, for agonist assays the mid-point is reached by adding an $EC_{50}$ concentration of a full agonist/activator compound; for potentiator assays it is an $EC_{10}$ concentration of agonist plus $EC_{50}$ concentration of a potentiator; and for inhibitor assays it is an $EC_{80}$ concentration of an agonist plus an $IC_{50}$ concentration of a standard inhibitor to each well.

**N.B.**  If calibration of the signals is required then the concentration levels and all analyses are to be conducted on the calibrated responses and not the raw plate reader counts.  It is a requirement that the raw signals lie within the range of the calibration curve, ie at most 1-2% of the wells lie outside the calibration range (i.e. above the fitted top or below the fitted bottom of the calibration curve).

Two different plate formats exist for the plate uniformity studies:  an *Interleaved-Signal* format where all signals are on all plates, but varied systematically so that over all plates on a given day each signal is observed in each well, and a *Uniform-Signal* plate format where each signal is run uniformly across entire plates.  There are no universal advantages to either format.  The Interleaved-Signal format can be used in all instances and requires fewer plates.  The Uniform-Signal format is easier to run, and more useful for detecting non-uniform signals, but takes more plates in total.  It also should not be used if signals vary across plates on a given day.  See Section C.3.d. for examples of when they should not be used.

## C.2. Interleaved-Signal Format

### C.2.a. Procedure

You should use the following plate layouts, for which Excel analysis templates have been developed. These layouts have a combination of wells producing max, min, and mid signals on a plate with proper statistical design. Use the same plate formats on all days of the test. <u>Do not change the concentration producing the mid point signal over the course of the test.</u> See Section C.2.d. for a further discussion about midpoint accuracy. The trials should use independently prepared reagents and preferably be run on separate days.

Plate 1

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | H | M | L | H | M | L | H | M | L | H | M | L |
| 2 | H | M | L | H | M | L | H | M | L | H | M | L |
| 3 | H | M | L | H | M | L | H | M | L | H | M | L |
| 4 | H | M | L | H | M | L | H | M | L | H | M | L |
| 5 | H | M | L | H | M | L | H | M | L | H | M | L |
| 6 | H | M | L | H | M | L | H | M | L | H | M | L |
| 7 | H | M | L | H | M | L | H | M | L | H | M | L |
| 8 | H | M | L | H | M | L | H | M | L | H | M | L |

H=Max, M=Mid, L=Min

Plate 2

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | L | H | M | L | H | M | L | H | M | L | H | M |
| 2 | L | H | M | L | H | M | L | H | M | L | H | M |
| 3 | L | H | M | L | H | M | L | H | M | L | H | M |
| 4 | L | H | M | L | H | M | L | H | M | L | H | M |
| 5 | L | H | M | L | H | M | L | H | M | L | H | M |
| 6 | L | H | M | L | H | M | L | H | M | L | H | M |
| 7 | L | H | M | L | H | M | L | H | M | L | H | M |
| 8 | L | H | M | L | H | M | L | H | M | L | H | M |

H=Max, M=Mid, L=Min

Plate 3:

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | M | L | H | M | L | H | M | L | H | M | L | H |
| 2 | M | L | H | M | L | H | M | L | H | M | L | H |
| 3 | M | L | H | M | L | H | M | L | H | M | L | H |
| 4 | M | L | H | M | L | H | M | L | H | M | L | H |
| 5 | M | L | H | M | L | H | M | L | H | M | L | H |
| 6 | M | L | H | M | L | H | M | L | H | M | L | H |
| 7 | M | L | H | M | L | H | M | L | H | M | L | H |
| 8 | M | L | H | M | L | H | M | L | H | M | L | H |

H=Max, M=Mid, L=Min

## C.2.b. Summary Signal Calculations and Plate Acceptance Criteria

The points below describe these calculations and acceptance criteria. The overall requirement for the signals is that the raw signals are sufficiently tight and that there is sufficient separation between the max and min signals to conduct screening. Calculations and acceptance criteria are summarized as follows.

1. Outliers should be flagged with an asterisk in the plate input section. The outliers should be "obvious", and the rate of outliers should be less than 2 percent (i.e. on average less than 2 on a 96 well plate, 8 on a 384 well plate).

2. Compute the mean (AVG), SD, and CV (of the mean) for each signal (max, mid, min) on each plate. Note that the CV should be calculated taking into account the number of wells per test compound per concentration that will be used in the production assay. For example if in the production assay duplicate wells will be run for each concentration of each test substance then $CV = \left(SD/\sqrt{2}\right)/AVG$. More generally, if there will be $n$ wells per test compound per concentration then $CV = \left(SD/\sqrt{n}\right)/AVG$. The acceptance criterion are that the CV's of each signal be less than or equal to 20%. Note that the min signal often fails to meet this criterion, especially for those assays whose min signal mean is very low. An alternate acceptance criterion for the min signal is $SD_{min} \leq$ both $SD_{mid}$ and $SD_{max}$. All plates should pass all signal criteria (ie all Max and Mid signals should have CV's less than 20% and all Min signals should either pass the CV criteria or all Min signals should pass the SD criteria).

2. For each of the mid-signal wells, compute a percent activity for agonist or stimulation assay relative to the means of the max and min signals on that plate,

$$\text{i.e., } \%Activity = \frac{well_{mid} - AVG_{min}}{AVG_{max} - AVG_{min}} \times 100 \, .$$

For inhibition assays compute percent inhibition for each mid-signal well, where %Inhibition = 100 - %Activity.

3. Compute the mean and SD for the mid-signal percent activity values on each plate. The acceptance criterion is $SD_{mid} \leq 20$ on all plates.

4. Compute a Signal Window (SW) or Z' factor (Z') for each plate, as described below. The acceptance criterion $SW \geq 2$ or $Z' \geq 0.4$ on all plates (either all SW's $\geq 2$ or all $Z' \geq 0.4$).

The formula for the signal window is
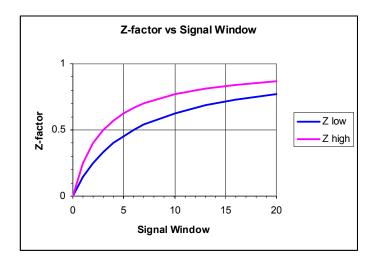
$$SW = \frac{\left(AVG_{max} - 3SD_{max}/\sqrt{n}\right) - \left(AVG_{min} + 3SD_{min}/\sqrt{n}\right)}{SD_{max}/\sqrt{n}} \, ,$$

where $n$ is the number of replicates of the test substance that will be used in the production assay. Instead of the SW the Z' factor can be used to evaluate the signal separation, where the only

difference is the denominator ($AVG_{max} - AVG_{min}$) is used instead of $SD_{max}$. The complete formula is

$$Z' = \frac{\left(AVG_{max} - 3SD_{max}/\sqrt{n}\right) - \left(AVG_{min} + 3SD_{min}/\sqrt{n}\right)}{AVG_{max} - AVG_{min}}$$

If one assumes that the SD of the max signal is at least as large as the SD of the min signal, then the Z' factor will be within a specific range for a given signal window, as illustrated in the following graph. Note that Z' values greater than 1 are possible only if $AVG_{max} < AVG_{min}$, and so the templates also check that all Z' values are less than 1.



Z-Factor interval versus Signal Window
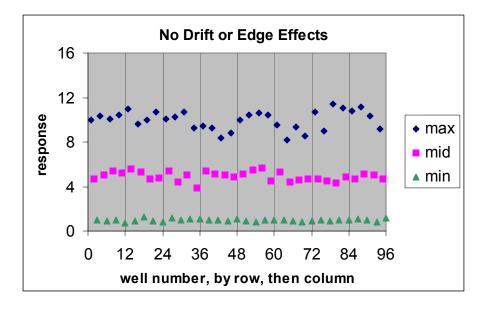
The recommended acceptance criterion is Z' factor $\geq 0.4$, which is comparable to a SW $\geq 2$. Either measure could be used.
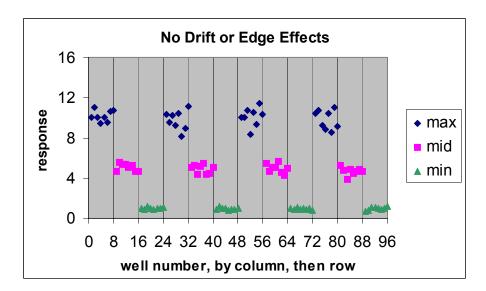

## C.2.c. Spatial Uniformity Assessment

A scatter plot (see examples below) can reveal patterns of drift, edge effects and other systematic sources of variability. The response is plotted against well number, where the wells are ordered either by row first, then by column, or by column first, then by row. The overall requirement is that plates do not exhibit material edge or drift effects. In general drift or edge effects < 20% are considered non-material, and effects seen only on a single or few plates, and not the predominant pattern are also considered non-material. Some guidelines to detecting and dealing with these problems follow.

*No drift or edge effects*
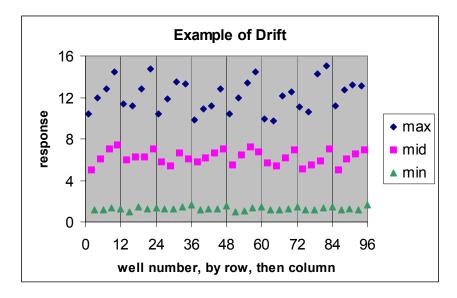
The following two plots (of the same data) show an example where there are no edge effects or drift.

**No Drift or Edge Effects**

(plot: response vs. well number, by row, then column; legend: max, mid, min)



**No Drift or Edge Effects**

(plot: response vs. well number, by column, then row; legend: max, mid, min)
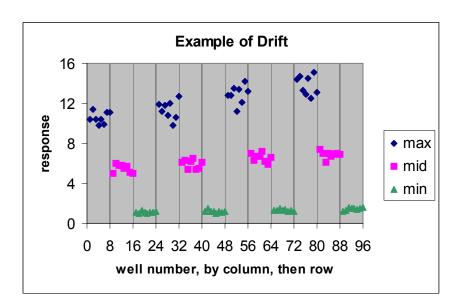
## *Drift*
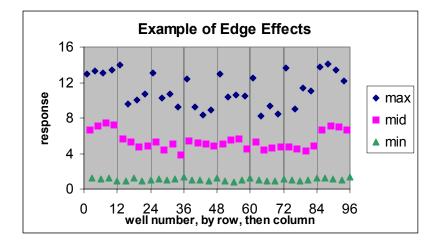
Use the max and mid signals to look for drift. Consider drift associated with the min only if the mean signal is greater than 10% of the maximum signal. Look for significant trends in the signal from left-to-right and top-to-bottom.  If you observe drift that exceeds 20% then you have material drift effects.  In the example below, the mean of column 1 is 10.6, while the mean of column 10 is 13.8, and the overall mean is 12.2.  The drift is 26% [(13.8-10.6)/12.2], and therefore should be investigated.

**Example of Drift**



**Example of Drift**
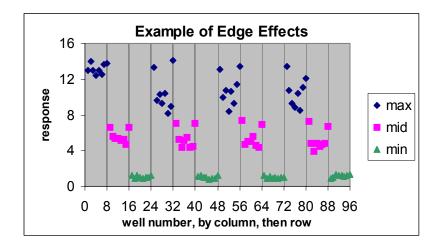


### Edge Effects

Edge effects can contribute to variability, and spotting them can be a helpful troubleshooting technique. Edge effects are sometimes due to evaporation from wells that are incubated for long periods of time. Edge effects can also be caused either by a short incubation time or by plate stacking – these conditions allow the edge wells to reach the desired incubation temperature faster than the inner wells. Edge effects may show up in the data as represented in the following example.

**Example of Edge Effects**



**Example of Edge Effects**



Note:  Because of the vertical axis scale, problems in the min and even mid signals may not be visible.  Adjusting the scale to highlight the min and mid scales may be necessary to properly examine these signals.

## C.2.d. Inter-Plate and Inter-Day Tests

The normalized mid signal should not show any significant shift across plates or days. "Significant" depends to a certain extent on the typical slopes encountered in dose response curves.  Thus plate-to-plate or day-to-day variation in the mid point percent activity needs to be assessed in light of the steepness of the dose-response curves of the assay.  For receptor binding assays, and other assays with a slope parameter of 1, a 15% difference can correspond to a two-fold change in potency. The template will translate the mean normalized mid-signal to potency shifts across plates and days.  There should not be a potency shift >2 between any two plates within a day, or >2 between any two average day mid point %activities.  For functional assays whose slopes may not equal 1 you can enter a "typical" slope into the template.  This should be based on the slope of a dose-response curve for the substance used to generate the mid point signal.

For these calculations to have utility the mid point %Inhibition/Activity should be "near" the midpoint.  Values within the range of 30-70% are ideal.  Studies with mean values outside this range should be discussed with a statistician, especially before any studies are repeated solely for this reason.  Also note that the conditions used to obtain the midpoint should not be changed over the course of the plate uniformity study.

## C.2.e. Summary of Acceptance Criteria

1. **Intra-plate Tests:**  Each plate should have a
   $CV_{max}$ and $CV_{mid} \leq 20\%$,
   $CV_{min} \leq 20\%$ or $SD_{min} \leq \min(SD_{mid}, SD_{max})$,
   Normalized $SD_{mid} \leq 20$,
   $SW \geq 2$ or $Z' \geq 0.4$.

2. **No material edge, drift or other spatial effects.**  Note that the templates do not check this criterion

3. **Inter-plate and Inter-Day Tests:**  The normalized average mid-signal should not translate into a fold shift
   > 2 within days,
   > 2 across any two days.

## C.2.f. 384-well Plate Uniformity Studies

384-well plates contain 16 rows by 24 columns, and one 384-well plate contains the equivalent of four 96-well plates.  Two different formats of interleaved plate uniformity templates have been developed.  The first layout expands the 96-well plate format into 4 squares.  The plate layouts are as follows:

**Plate 1**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 2 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 3 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 4 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 5 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 6 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 7 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 8 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 9 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 10 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 11 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 12 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 13 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 14 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 15 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |
| 16 | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L |

**Plate 2**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 2 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 3 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 4 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 5 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 6 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 7 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 8 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 9 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 10 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 11 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 12 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 13 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 14 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 15 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |
| 16 | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M |

**Plate 3**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 2 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 3 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 4 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 5 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 6 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 7 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 8 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 9 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 10 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 11 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 12 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 13 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 14 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 15 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |
| 16 | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H | M | L | H |

Standard Interleaved 384-well Format Plate Layouts

The second is useful for assays using certain automation equipment such as Tecan and Beckman. In that case column 1 of the 96-well plate corresponds to columns 1 and 2 of the 384-well plate, and is laid out in 8 pairs of columns.  The plate layouts for it are as follows:

**Plate 1**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 2 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 3 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 4 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 5 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 6 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 7 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 8 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 9 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 10 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 11 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 12 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 13 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 14 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 15 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |
| 16 | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L |

**Plate 2**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 2 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 3 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 4 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 5 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 6 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 7 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 8 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 9 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 10 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 11 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 12 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 13 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 14 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 15 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |
| 16 | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M |

**Plate 3**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 | C22 | C23 | C24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 2 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 3 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 4 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 5 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 6 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 7 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 8 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 9 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 10 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 11 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 12 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 13 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 14 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 15 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |
| 16 | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H | M | M | L | L | H | H |

HHMMLL 384-well Plate Uniformity Plate Layouts

The analysis and acceptance criterion are exactly the same as for 96-well format Plate Uniformity Studies.  See Section 2.C.2e for a summary of the acceptance criterion.

## C.3. Uniform-Signal Plate Layouts

Uniform-Signal plate layouts are an alternative format to conduct the plate uniformity studies. Their main advantage is easier execution since all wells on each plate are exactly the same, and together with heat maps provide for a straightforward assessment of spatial properties.  The disadvantages are that this format requires twice as many plates as the Interleaved-Signal format, and that the normalizing calculations are quite artificial in that max and min signals are not on-plate signals and therefore may produce misleading results.  See Section C.3.d for further elaboration of this point.

### C.3.a. Procedure

Max, Mid and Min signals are prepared as defined in Section C.1.  Two plates are run for each signal, making six plates per day.  On each plate all wells are the same, i.e. either all Max, all

Mid, or all Min.  The number of days required is the same as for the Interleaved-Signal layout: three days for new assays, two days for transfers of previously validated assays.

## C.3.b. Summary Calculations and Plate Acceptance Criterion

The actual calculations will be performed by the template.  Details of the calculations are as follows:

1. Compute the mean (AVG), standard deviation (SD) and Coefficient of Variation (CV) for each plate (as per the Interleaved-Signal format the CV's should reflect the number of wells per test-condition envisioned in the production assay).  Requirements are the same as for Interleaved-Signal format:  The CV of each plate should be less than 20%.  For the Min plates having $SD \leq SD_{mid}$ and $SD_{max}$, where $SD_{mid} = \sqrt{\left(SD^2_{mid-1} + SD^2_{mid-2}\right)/2}$ is the combined standard deviation from the two Mid plate SD's, and similarly for the Min and Max signals.

2. For each of the Mid signal plates, compute the percent activity for agonist or stimulation assays, and percent inhibition for antagonist or inhibition assays (including binding assays).  In this format the calculation is

$$\%\text{Activity} = \frac{well_{mid} - AVG_{min}}{AVG_{max} - AVG_{min}} \times 100$$
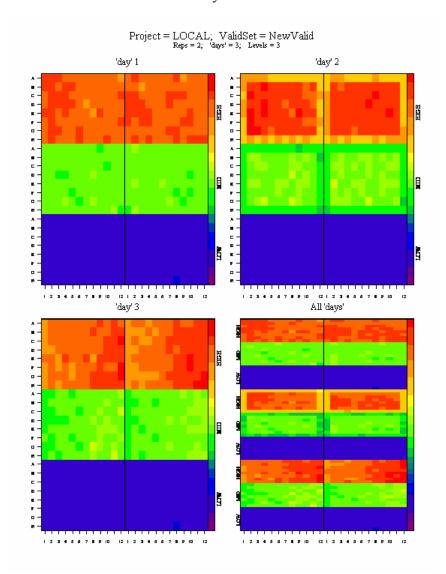
    where $AVG_{min}$ is the average taken over the two Min plate averages, and $AVG_{max}$ is the average taken over the two Max plate averages.  Percent Inhibition = 100 - %Activity.

3. Compute the SD of the normalized signals on each Mid plate.  The acceptance criterion is $SD_{\%mid} \leq 20$.

4. Compute the Z' factor and/or the SW for each day.  The formulas are the same as in Section C.2.b, except that $AVG_{max}$ and $AVG_{min}$ are defined as in point 2 above, and $SD_{max}$ and $SD_{min}$ are defined as in point 1 above.  The acceptance criterion is either all $Z' \geq 0.4$ or all $SW \geq 2$.

## C.3.c. Spatial Uniformity Assessment

The Excel template provides scatterplots of the plate signals combined across plates and days and is interpreted in a similar manner as the Interleaved-Signal format. The criterion for acceptance is the same as for the interleaved format:  No drift or edge effects that exceed 20% of the mean.  Also as in the Interleaved-Signal format the presence of these effects should be apparent as the predominant effect, and not seen just in single isolated plates for the assay to be failed by this criterion.

The following example illustrates a spatially uniform result, an edge effect, and a drift effect. Day 1 shows an acceptably uniform result.  Day 2 shows an assay with a significant edge effect (25% from the mean edge value to the mean of the interior), and Day 3 shows an assay with significant drift (25% change in mean value from left to right as compared to the average in the middle).  If patterns are similar or worse than those depicted in Day 2 or Day 3 then the assay does not pass the spatially uniform requirement.

Project = LOCAL;  ValidSet = NewValid
Reps = 2;  'days' = 3;  Levels = 3

## C.3.d. Inter-Plate and Inter-Day Tests
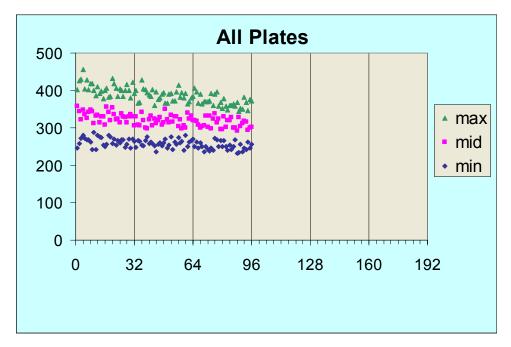
The Inter-plate and inter-day tests are exactly the same as in Section C.2.d, except the definitions of %Activity and %Inhibition defined above (Section C.3.a) are used in the tests.

## C.3.e. Impact of Plate Variation on Validation Results
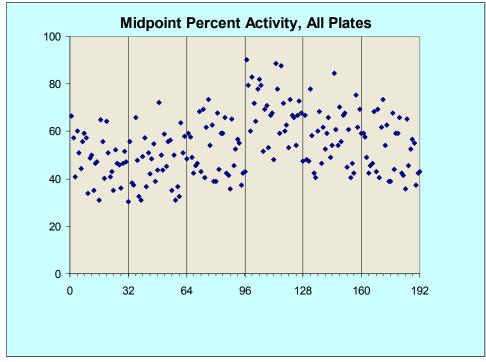
The Uniform-Signal format does make the assumption that plate variation within each run day is negligible.  If this assumption is not correct then many of the diagnostic tests described here will be misleading, and the Interleaved-Signal format should be used instead.  In particular, Z' factors and/or Signal Windows may be incorrect in either direction, and the Inter-plate and Inter-Day tests could possibly fail acceptable assays.

The following example illustrates the problem.  The raw signals of one day of an Interleaved-Signal format Plate Uniformity Study are shown on the left in Panel A.  The Max and Mid raw signals vary across the 3 plates (Panel A, Plates 1-3), but note that the %Activity is very stable across the 3 plates (Panel B, Plates 1-3).  The maximum fold shift across plates is 1.2.  The

Midpoint Percent Activity plot (Panel B) shows what can happen if you don't have on-plate Max and Min controls.  The three left-hand panels show the plates normalized to their own controls while, to mimic the Uniform-Signal protocol with its off-plate controls, the right hand columns of Panel B show each plate's mid signal normalized to the plate 3 controls, i.e. "Plate 1" shows the actual plate 1 mid signal normalized to the plate 3 Max and Min signals, "Plate 2" shows the actual plate 2 mid signals normalized to the plate 3 Max and Min signals and "Plate 3" is the plate 3 mid signals normalized to their own controls.  In the presence of plate variation the off-plate controls do not effectively normalize the assay.  As Panel B shows, plate-to-plate variation in the raw signals can induce the appearance of significant mid-point variation when in fact there is little variation in signals properly normalized to on-plate controls.  In this example using off-plate controls Plates 1-3 have a max fold shift of 2.0 which does not pass the inter-plate acceptance criterion.



Panel A.  Raw data values for 3 plates of an Interleaved-Signal Plate Uniformity Study.  Plates 1-3 show the actual plate values obtained on one day of the test.

**Midpoint Percent Activity, All Plates**

Panel B. Normalized midpoint values for 3 plates of a Interleaved-Signal Plate Uniformity Study. Plates 1-3 show the actual plate midpoints normalized to the on-plate controls. Plates 4-6 show the same mid points all normalized to the Plate 3 Min and Max controls.

# D. Replicate-Experiment Study

## D.1. Overview

It is important to verify that the assay results are reproducible, i.e. that the variability of key end points of the assay are acceptably low. In addition, if the assay is to report results with those previously reported by another assay then it should be verified that the two labs produce equivalent results. In this section, we define how to quantify assay variability and determine assay equivalence. It is important to read the entire section below to understand the rationale for the statistical methods employed in calculating reproducibility of potency and efficacy. We strongly recommend consultation with a statistician before designing experiments to estimate variability described below.

## Rationale

Replicate-Experiment studies are used to formally evaluate the *within-run* assay variability and formally compare the new assay to the existing (old) assay. They also allow a preliminary assessment of the *overall* or *between-run* assay variability, but two runs are not enough to adequately assess overall variability. Post-production methods (Section III) are used to formally evaluate the overall variability in the assay. Note that the Replicate-Experiment study is a diagnostic and decision tool used to establish that the assay is ready to go into production by showing that the endpoints of the assay are reproducible over a range of potencies. It is not intended as a substitute for post-production monitoring or to provide an estimate of the *overall* Minimum Significant Ratio (MSR).

It may seem counter-intuitive to call the differences between two independent assay runs "within-run". However, the terminology results from the way those terms are defined. Experimental variation is categorized into two distinct components: *between-run* and *within-run* sources. Consider the following examples:

- If there is variation in the concentrations of buffer components between 2 runs then the assay results could be affected. However, assuming that the same buffer is used with all compounds within the run, each compound will be equally affected and so the difference will only show up when comparing one run to another run, i.e. in two runs one run will appear higher on average than the other run. This variation is called *between-run* variation.

- If the concentration of the compound in the stock plate varies from the target concentration then all wells where that compound is used will be affected. However, wells used to test other compounds will be unaffected. This type of variation is called *within-run* as the source of variation affects different compounds in the same run differently.

- Some sources of variability affect both within- and between-run variation. For example, in a FLIPR assay cells are plated and then incubated for 24-72 hours to achieve a target cell density taking into account the doubling time of the cells. For example, if the doubling time equals the incubation time, and the target density is 30,000 cells/well, then 15,000 cells/well are plated. But even if exactly 15,000 cells are placed in each well there won't be exactly

30,000 cells in each well after 24 hours.  Some will be lower and some will be higher than the target.  These differences are *within-run* as not all wells are equally affected.  But also suppose in a particular run only 13,000 cells are initially plated.  Then the wells will on average have fewer than 30,000 cells after 24 hours, and since all cells are affected this is *between-run* variation.  Thus cell density has both within- and between-run sources of variation.

The total variation is the sum of both sources of variation.  When comparing two compounds across runs, one must take into account both the within-run and between-run sources of variation.  But when comparing two compounds in the same run, one must only take into account the within-run sources, since, by definition, the between-run sources affect both compounds equally.

In a Replicate-Experiment study the between-run sources of variation cause one run to be on average higher than the other run.  However, it would be very unlikely that the difference between the two runs were exactly the same for every compound in the study.  These individual compound "differences from the average difference" are caused by the within-run sources of variation.  The higher the within-run variability the greater the individual compound variation in the assay runs.

The analysis approach used in the Replicate-Experiment study is to estimate and factor out between-run variability, and then estimate the magnitude of within-run variability.

## D.2. Procedure (Steps)

All assays should have a reproducibility comparison (Steps 1-3).  If the assay is to replace an existing assay and combine the data then an assay comparison study should also be done (Steps 4 and 5).

1.  Select 20-30 compounds that have potencies covering the concentration range being tested and, if applicable, efficacy measures that cover the range of interest.  The compounds should be well spaced over these ranges.

2.  All of the compounds should be run in each of two runs of the assay.

3.  Compare the two runs as per Section D.3-D.6.

4.  All compounds should be run in a single run of the previous assay.

5.  Compare the results of the two labs by analyzing the first run of the new assay with the single run of the previous assay.

## D.3. Analysis (Potency)

For the reproducibility comparison paste potency values from the two runs into the Run 1 and Run 2 data columns.  All tests are conducted by the spreadsheet, and there are additional plots and diagnostics available to assist in judging the results.  For the assay comparison study paste the potency values for the first run of the new assay into the Run1 column and the potency values

for the (single) run of the previous assay into the Run 2 column.  Potency values should be calculated according to the methods of Section III.

The points below describe and define the terms used in the template and the acceptance criterion discussed in the Diagnostic Tests section below.

1.  Compute the difference in log-potency (= first – second) between the first and second run for each compound.  Let $\bar{d}$, and $s_d$ be the sample mean and standard deviation of the difference in log-potency.  Since ratios of EC$_{50}$ values (relative potencies) are more meaningful than differences in potency (1 and 3, 10 and 30, 100 and 300 have the same ratio but not the same difference), we take logs in order to analyze ratios as differences.

2.  Compute the **Mean-Ratio**: $MR = 10^{\bar{d}}$.  This is the geometric average fold difference in potency between two runs.

3.  Compute the **Ratio Limits**: $RLs = 10^{\bar{d} \pm 2s_d / \sqrt{n}}$, where $n$ is the number of compounds.  This is the 95% confidence interval for the Mean-Ratio.

4.  Compute the **Minimum Significant Ratio**: $MSR = 10^{2s_d}$.  This is the smallest potency ratio between two compounds that is statistically significant.

5.  Compute the **Limits of Agreement**: $LsA = 10^{\bar{d} \pm 2s_d}$.  Most of the compound potency ratios (approximately 95%) should fall within these limits.

6.  For each compound compute the **Ratio** (=first/second) of the two potencies, and the **Geometric Mean** potency: $GM = \sqrt{\text{first} \times \text{second}}$.

Items 2-6 can be combined into one plot: the Ratio-GM plot.  An example is in Figure 1.  The points represent the compounds; the blue-solid, green long-dashed and red short-dashed lines represent the MR, RLs and LsA values respectively.

Figure 1 shows the desired result of pure chance variation in the difference in activities between runs.  The blue solid line shows the geometric mean potency ratio, i.e. the *average* relationship between the first and second run.  The green long-dashed lines show the 95% confidence limits of the mean ratio. These limits should contain the value 1.0, as they do in this case.  The red short-dashed lines indicate the limits of agreement between runs.  They indicate the *individual compound* variation between the first and second run.  You should see all, or almost all, the points fall within the red dashed lines.  The lower line should be above 0.33, while the upper line should be below 3.0, which indicates a 3-fold difference between runs in either direction. The MSR should be less than 3.0, as it is in this example.
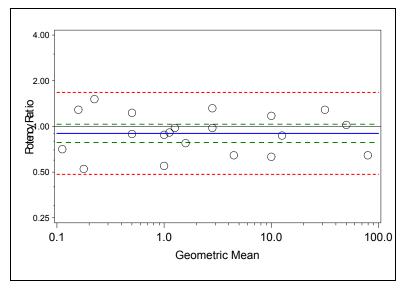
Figure 1.  Potency Ratio versus GM Potency.  This is a typical example
for an acceptable assay: The MR=0.90, RLs=(0.78-1.03) [contains
the value 1.0], MSR=1.86 [under 3.0], LsA=(0.48-1.67) [between
0.33 and 3.0].

## D.4. Diagnostic Tests and Acceptance Criterion (Potency)

1.  If the MSR $\geq$ 3 then there is poor **individual** agreement between the two runs.  This problem
    occurs when the *within-run* variability of the assay is too high.  See Figure 2(a) below for an
    illustration.  An assay meets the MSR acceptance criterion if the (within-run) **MSR < 3.**

2.  If Ratio limits do not contain the value 1, then there is a statistically significant **average**
    difference between the two runs.  Within a lab (Step 3) this is due to high *between-run* assay
    variability.  Between labs (Step 4), this could be due to a systematic difference between labs,
    or high between-run variability in one or both labs.  See Figure 2(b) below for an illustration.
    Note that it is possible with a very "tight" assay (i.e. one with a very low MSR) or with a
    large set of compounds to have a statistically significant result for this test that is not very
    material, i.e., the actual MR is small enough to be ignorable.  If the result is statistically
    significant then examine the MR.  If it is between 0.67 and 1.5 then the average difference
    between runs is less than 50% and is deemed immaterial.  However, in Figure 2(b) the
    MR=2.01, indicating a 101% difference between runs, which is too high to be considered
    "equivalent".  Note that there is no direct requirement for the MR, but values that are this
    extreme are unlikely to pass the Limits of Agreement criterion in step 3 below.

3.  The MR and the MSR are combined into a single interval referred to as the **Limits of
    Agreement**.  An assay that either has a high MSR and/or an MR different from 1 will tend to
    have poor agreement of results between the two runs.  An assay meets the Limits of
    Agreement acceptance criterion if both the upper and lower limits of agreement are between
    0.33 and 3.0.  Note that assays depicted in both Figures 2a and 2b do not have Limits of
    Agreement inside the acceptance region and thus do not meet the acceptance criterion.

**PANEL A**                              **PANEL B**
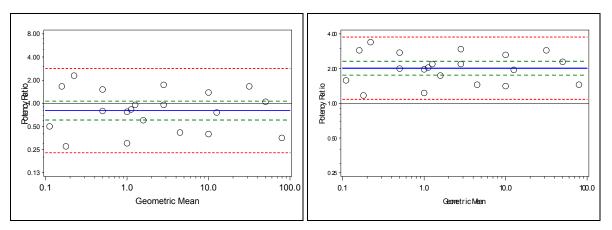


Figure 2.  Potency Ratio vs. GM Potency.  (A) Shows a case where the within-run variability is too large (MR= 0.8, RLs= (0.61-1.07), MSR= 3.54, and LsA= (0.23-2.84), and (B) shows a case where the LsA are outside the acceptable range because the Mean Ratio is too large, i.e., there is a tendency for the activity values in run 1 to be larger than in run 2 (MR= 2.01, RL= (1.75-2.32), MSR= 1.86, and LsA= (1.08-3.75).  In both cases the reason(s) for these conditions should be investigated.

## D.5. Analysis (Efficacy)

The points below describe and define the terms used in the template and the acceptance criterion discussed in the Diagnostic Tests section.  Note that the methods described here are intended for functional full/partial assays and non-competitive antagonist assays.  Some potentiator assays, as well as assays normalized by fold stimulation may best be analyzed with the techniques described in the potency section rather than the methods described here.  Consult a statistician for the best method of analysis.

1.  Compute the difference in efficacy (= first – second) between the first and second run for each compound.  Let $\bar{d}$, and $s_d$ be the sample mean and standard deviation of the difference in efficacy.

2.  Compute the **Mean-Difference**: $MD = |\bar{d}|$.  This is the average difference in efficacy between the two runs.

3.  Compute the **Difference Limits**: $DLs = \bar{d} \pm 2s_d / \sqrt{n}$, where $n$ is the number of compounds. This is a 95% confidence interval for the Mean-Difference.

4.  Compute the **Minimum Significant Difference**: $MSD = 2s_d$.  This is the smallest efficacy difference between two compounds that is statistically significant.

5.  Compute the **Limits of Agreement**: $LsAd = \bar{d} \pm 2s_d$.  Most of the compound efficacy differences should fall within these limits (approximately 95%).

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6.  For each compound compute the **Difference** (= first-second) of the two efficacies, and the **Mean** efficacy (average of first and second).

Items 2-6 can be combined onto one plot: the Difference-Mean plot (not shown).  The plot is very similar to the Ratio-GM plot except that both axes are on the linear scale instead of the log scale.

## D.6. Diagnostic Tests (Efficacy)

Generally the same two problems discussed under potency need to be judged for efficacy as well.  However, a general acceptance criterion for efficacy has not been established as there is not a consensus on efficacy standards, and for most projects potency is the primary property of interest.  As guidelines, the MD should be less than 5 (i.e., less than 5% average difference between runs) and the MSD should be less than 20 (e.g., 20% activity).  More importantly, the MD and MSD should be used to judge the appropriateness of any efficacy CSF's a project may have.  For example, if the CSF for efficacy is >80%, and the MSD is 30%, then the assay will fail too many efficacious compounds - a 90%-active compound would fall below the CSF 25% of the time.  A more appropriate CSF in this situation would be 70 or even 60%.

## D.7. Summary of Acceptance Criteria

1.  In Step 3 conduct reproducibility and equivalence tests for potency comparing the two runs in the new lab.  The assay should pass both tests (MSR < 3 and both Limits of Agreement should be between 0.33 and 3.0).

2.  In Step 5 conduct reproducibility and equivalence tests for potency comparing the first run of the new lab to the single run of the old lab.  The assays should pass both tests to be declared equivalent (Limits of Agreement between 0.33 and 3.0).

3.  For full/partial agonist assays and non-competitive antagonist assays, repeat points 1 and 2 for efficacy.  Use the informal guidelines discussed above, and project efficacy CSFs to judge acceptability of results.

## D.8. Notes

1.  If a project is very new, there may not be 20-30 unique active compounds (where active means some measurable activity above the minimum threshold of the assay).  In that case it is acceptable to run compounds more than once to get an acceptable sample size.  For example, if there are only 10 active compounds then run each compound twice.  However, when doing so, (a) it is important to biologically evaluate them as though they were different compounds, including the preparation of separate serial dilutions, and (b) label the

compounds "a", "b" etc. so that it is clear in the test-retest analyses which results are being compared across runs.

2.  Functional assays need to be compared for both potency ($EC_{50}$) and efficacy (%maximum response).  This may well require a few more compounds in those cases.

3.  In binding assays, it is best to compare $K_i$'s, and in functional antagonist assays it is best to compare $K_b$'s.

4.  An assay may pass the reproducibility assessment (Steps 1-3 in the procedure [Section D.2.]), but may fail the assay comparison study (Steps 4-5 in the procedure [Section D.2]).  The assay comparison study may fail either because of a MR different from 1 or a high "MSR" in the assay comparison study.  If it's the former then there is a potency shift between the assays.  You should assess the values in the assays to ascertain their validity (e.g. which assay's results compare best to those reported in the literature?).  If it fails because the Lab Comparison study is too large (but the new assay passes the reproducibility study) then the old assay lacks reproducibility.  In either case, if the problem is with the old assay, then the team should consider rerunning key compounds in the new assay to provide comparable results to compounds subsequently run in the new assay.

# E. How to Deal with High Assay Variability

## E.1. High Variation in Single Concentration Determinations

The table below can be used as a reference to determine the number of replicates necessary for assays with high variability. For a given CV of the raw data values based on 1 well, it shows the number of replicates needed for the CV of a mean to be less than or equal to 10 or 20%. This table does not indicate how the $IC_{50}/K_i/K_b$ variability will be affected (See Section E.2 for high variation in $IC_{50}/K_i/K_b$ responses).

| CV using 1 well | Number of Wells so that CV < 10% | Number of Wells so that CV < 20% |
|---|---|---|
| <10 | 1 | 1 |
| 10.1-14.1 | 2 | 1 |
| 14.2-17.3 | 3 | 1 |
| 17.4-20.0 | 4 | 1 |
| 20.1-22.3 | 5 | 2 |
| 22.4-24.4 | 6 | 2 |
| 24.5-26.4 | 7 | 2 |
| 26.5-28.2 | 8 | 2 |
| 28.3-30.0 | 9 | 3 |
| 30.1-31.6 | 10 | 3 |
| 31.7-33.1 | 11 | 3 |
| 33.2-34.6 | 12 | 3 |
| 34.7-36.0 | 13 | 4 |
| 36.1-37.4 | 14 | 4 |
| 37.4-38.7 | 15 | 4 |
| 38.8-40.0 | 16 | 4 |

Adding replicates to reduce variability will also reduce the capacity (i.e., throughput) of the assay to test compounds. Further optimization of the assay could reduce variability and maintain or increase its capacity. The decision to further optimize or add replicates will have to be made for each assay.

## E.2. Excess Variation in Concentration-Response Outcomes (EC$_{50}$, IC$_{50}$, K$_i$, or K$_b$)

If in Section D the assay fails either test (MSR > 3 or Limits of Agreement outside the interval 1/3-3) then the variability of the assay is too high. The following options should be considered to reduce the assay variability:

1. Optimizing the assay to lower variability in the signal (see Section V) of the raw data values Check that the dose range is appropriate for the compound results.  Adding doses and/or replicates may improve the results.  A minimum of 8 doses at half-log intervals is recommended.  In general, it is better to have more doses (up to 12) rather than more replicates.

2. Consider adding replicates as discussed below.  Note that the impact of adding replication may be minimal, and so the Replicate Experiment Study should be used to assess whether increasing the number of replicates will achieve the objective.

3. Adopt as part of the standard protocol to re-run results.  For example, each compound may be tested once per run on 2 or more runs.  Then averaging the results will reduce the assay variability (**NB**.  In such cases the individual run results are stored in the database and then the data mining/query tools are used to average the results).

To investigate the impact of adding replicate wells in the concentration-response assay you should conduct the Replicate-Experiment study with the maximum number of wells contemplated (typically 3-4 wells / concentration).  To examine the impact of replication compute the MSR versus number-of-replicates curve.  To construct this curve, make all data calculations using just the first replicate of each concentration to evaluate the MSR and Limits of Agreement for 1 well per concentration.  Then repeat all calculations using the first two replicates per concentration, and so on until you are using all replicates.  If the assay does not meet the acceptance criterion when all replicates are used then replication will not sufficiently impact the assay to warrant the replication.  If it does meet the criterion using all replicates ascertain how many replicates are needed by noting the smallest number of replicates that are required to meet the Replicate-Experiment acceptance criterion.  Two examples below will help illustrate the steps.

A binding assay was run using 1 well per concentration and the Replicate-Experiment study did not meet the acceptance criterion.  To examine if replication would help a new Replicate-Experiment study was conducted using 4 wells per concentration.  Using just the first replicate from each concentration, the results were normalized, curves fit and $K_i$'s were calculated for each concentration-response curve.  The MSR and LsA were evaluated.  The entire calculation steps were repeated using the first 2 replicates, first 3 replicates and all 4 replicates, with the following results:

| Reps | MSR | LsA |
|------|-----|-----|
| 2 | 3.62 | 0.35-4.59 |
| 3 | 3.32 | 0.43-4.74 |
| 4 | 2.44 | 0.53-3.16 |

From the table we can see that it takes all 4 replicates to meet the MSR acceptance criterion, and no amount of replication (up to 4 replicates) will meet LsA acceptance criterion.

In a second study, a pair of uptake inhibition assays (the project had two targets, each measured by one assay) the Plate Uniformity Study indicated two replicates would be required to meet the Plate Uniformity Signal acceptance criteria in Assay 2.  However, plate uniformity criteria concerning replication do not readily translate to dose-response requirements, and so the

requirements were investigated in both assays.  The Replicate-Experiment Study was conducted using two replicates.  The calculations were performed using both replicates, and the re-calculated using just the first replicate.  The MSR and LsA are summarized in the following table

| Replicates Used | Assay 1 | | Assay 2 | |
|---|---|---|---|---|
| | MSR | LsA | MSR | LsA |
| Rep 1 Only | 2.27 | 0.44-2.27 | 3.30 | 0.28-3.08 |
| Both Reps | 1.71 | 0.57-1.67 | 2.15 | 0.44-2.03 |

Using two replicates both assays meet all acceptance criterion.  Using just a single replicate Assay 1 still meets all criteria, while assay 2 does not.  Note that in this instance both assays benefited from increased replication.  However, assay 1 is a very tight assay and hence this benefit is not really needed in that case.  So in this case the replication requirements were the same for both single dose screening and dose-response studies, but in general this will not be the case.

# F. Bridging Studies for Assay Upgrades and Minor Changes

## F.1. Overview

Sections C and D cover the validation of entirely new assays, or assays that are intended to replace existing assays.  The replacement assays are "different" from the original assay, either because of facility changes, personnel differences, or substantively different detection and automation equipment.  Assay upgrades and changes occur as a natural part of the assay life cycle.  Requiring a full validation for every conceivable change is impractical and would serve as a barrier to implementing assay improvements.  Hence full validation following every assay change is not recommended.  Instead bridging studies or "mini-validation" studies are recommended to document that the change does not degrade the quality of the data generated by the new assay.

The level of validation recommended has 3 tiers, from a small plate uniformity study (Tier I), to just the assay comparison portion of the Replicate-Experiment study (Tier II) to the full validation package of Sections C and D (Tier III).  Examples of changes within each Tier are given below, along with the recommended validation study for that tier.  Note that if the study indicates the change will have an adverse impact on assay quality (i.e. the study indicates there are problems), then the cause should be investigated and a full (Tier III) validation should be done.  If the results from that study indicate the assays are not equivalent, but the new assay has to be implemented, then a the results should not be combined into one set.

The following applies principally to changes in biological components of the protocol.  If changes are made to the data analysis protocol then these can ordinarily be validated without generating any new data, by comparing the results using the original and new data analysis protocols on a set of existing data.  Discuss any changes with a statistician.  If changes are made to both the data analysis and biological components of the protocol then the appropriate tier should be selected according to the severity of the biological change as discussed below.  The data analysis changes should be validated on the new validation data and any additional validation work may be needed as judged by the statistician.

## F.2. Tier I:  Single Step Changes to the Assay

Tier I modifications are single changes in an assay such as a change to a reagent, instrumentation, or assay condition that is made either to improve the assay quality or increase the capacity without changing the assay quality.  The changes can also be made for reasons unrelated to assay throughput or performance (e.g. change of a supplier for cost savings).  Examples of such change are

- Changes in detection instruments with similar or comparable optics and electronics. E.g.: plate readers, counting equipment, spectrophotometers. A performance check for signal dynamic range, and signal stability is recommended prior to switching instruments.

- Changes in liquid handling equipment with similar or comparable volume dispensing capabilities. Volume calibration of the new instrument is recommended prior to switching

instruments. [Note that plate and pipette tip materials can cause significant changes in derived results (IC50, EC50). This may be due to changes in the adsorption and wetting properties of the plastic material employed by vendors. Under these conditions a full validation may be required].

The purpose of the validation study is to document the change does not reduce the assay quality.

## Protocol
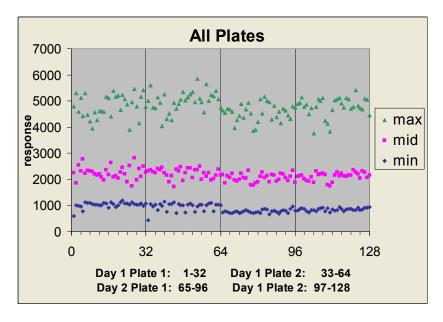
Conduct a 4 plate Plate Uniformity Study using the layouts in the "2 Plates per Day" tab of the Plate Uniformity Template (the layouts are the same as Plates 1 and 2 of Section C.2. Plates 1 and 2 should be done using the existing protocol, and Plates 3 and 4 done using the new protocol on the same day using the same reagents and materials (except for the intentional change). Use the 2 Day / 2 Plates per Day template to conduct the analysis.
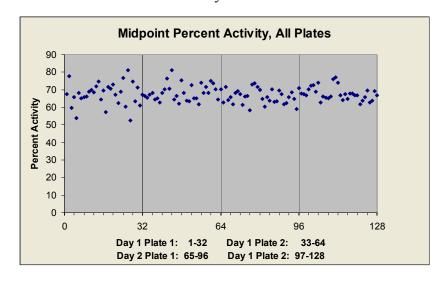
## Analysis

The main analysis is a visual inspection of the "all plates" plots to ensure that the signals have not changed in either in magnitude and/or variability. The mean and SD calculations for each plate can help, but visual inspection is usually sufficient.

## Example

An assay was changed by replacing a manual pipetting step with a multidrop instrument. A 4-plate Plate Uniformity study was run as per the protocol, with the manual pipetting done in plates 1 and 2, and the multidrop in plates 3 and 4. The results show that the mean percent activity is the same, and the multidrop's varability superior (i.e. lower) to the manual pipetting.

Tier I Validation study comparing manual pipetting (plates 1 and 2) versus Multidrop pipetting (plates 3 and 4) in GTPγS assay

## F.3. Tier II:  Minor Assay Changes

Tier II changes are more substantive than Tier I changes, and have greater potential to directly impact EC50/IC50 results.  Examples of such changes are

- Changes in dilution protocols covering the same concentration range for the concentration–response curves. A bridging study is recommended when dilution protocol changes are required.

- Lot changes of critical reagents such as a new lot of receptor membranes or a new lot of serum antibodies.

- Assay moved to a new laboratory without major changes in instrumentation, using the same reagent lots, same operators and assay protocols.

- Assay transfer to an associate or technician within the same laboratory having substantial experience in the assay platform, biology and pharmacology.  No other changes are made to the assay.

### Protocol and Analysis

Conduct the assay comparison portion of the Replicate Experiment Study discussed in Section D, i.e. compare one run of 20-30 compounds of the assay using the existing assay to one run of the assay under the proposed format and compare the results.  If the compound set used in the original validation is available then one need to only run the set again in the new assay protocol, and compare back to Run 1 of the original Replicate-Experiment Study.  The acceptance

criterion is the same as for the assay comparison study:  Both Limits of Agreement should be between 1/3 and 3.0.

## F.4. Tier III:  Substantive Changes

Substantive changes requiring full assay validation: When substantive changes are made in the assay procedures, measured signal responses, target pharmacology and control compound activity values may change significantly. Under these circumstances, the assay should be re-validated according to methods described in Sections IIC and IID. The following changes constitute substantive changes, particularly when multiple changes in factors listed below are involved:

- Changes in assay platform: e.g.: Filter binding to Fluorescence polarization for kinase assays.

- Changes in assay reagents (including lot changes and supplier) that produce significant changes in assay response, pharmacology and control activity values. For example, changes in enzyme substrates, isozymes, cell-lines, label types, control compounds, calibration standards, (radiolabel vs. fluorescent label), plates, tips and bead types, major changes in buffer composition and pH, co-factors, metal ions, etc.

- Transfer of the assay to a different laboratory location, with distinctly different instrumentation, QB practices or training.

- Changes in detection instruments with significant difference in the optics and electronics. For example, plate readers, counting equipment, spectrophotometers.

- Changes in liquid handling equipment with significant differences in volume dispensing capabilities.

- Changes in liquid handling protocol with significant differences in volume dispensing methods.

- Changes in assay conditions such as shaking, incubation time, or temperature that produce significant change in assay response, pharmacology and control activity values.

- Major changes in dilution protocols involving mixed solvents, number of dilution steps and changes in concentration range for the concentration-response curves.

- Change in analyst/operator running the assay, particularly if new to the job and/or has no experience in running the assay in its current format/assay platform.

- Making more than one of the above-mentioned changes to the assay protocol at any one time.

Substantive changes require full validation, i.e. a three day Plate Uniformity Study and Replicate Experiment Study.  If the intent is to report the data together with the previous assay data then an assay comparison study should be conducted as part of the Replicate Experiment study.

# G. References

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7.      Eastwood, BJ, Chesterfield, AK, Wolff MC, and Felder CC:  Methods for the Design and Analysis of Replicate-Experiment Studies to Establish Assay Reproducibility and the Equivalence of Two Potency Assays, in Gad, S (ed):  *Drug Discovery Handbook*, John Wiley and Sons, New York, 2005, 667-688

# SECTION III

# ASSAY OPERATIONS
# FOR
# SAR SUPPORT

# Table of Contents

## SECTION III:  ASSAY OPERATION FOR SAR SUPPORT

# ASSAY OPERATION FOR SAR SUPPORT

## A. Determination of EC50/IC50

### Models and Curve Fitting Guidelines

For competition binding assays and functional antagonist assays the most common summary measure of the dose-response curve is the $IC_{50}$, the concentration of substance that provides 50% inhibition. For agonist/stimulator assays the most common summary measure is the $EC_{50}$, the concentration giving 50% of that compound's maximal response. Substantial variation in the methodology used to derive these values exists, and this variation has been shown to substantially impact overall assay variability. This section discusses important issues to consider and provides some guidelines on how to proceed. They are a based on the Data Standardization for Results Management document (Section XI of this manual). Consult that document for the specifics for each assay type. Consult a statistician to see if these guidelines are appropriate for your assay, and if other outcomes such as AUC or a threshold dose should be used.

Before fitting a dose-response curve to obtain the $EC/IC_{50}$, each well should be converted to either percent activity or percent inhibition with respect to positive and negative controls (note: for simplicity all text below is stated for determining $IC_{50}$'s; determining $EC_{50}$'s is identical). Then all replicate wells from a given run (including multiple plates per run) for a given concentration should be averaged either by taking the mean, or preferably, taking the median. Outliers less influence the latter when there are 3 or more replicates. Thus only one point per concentration per run is used to fit the dose-response equation to the data. This is because replicate wells on either the same or different plates are often correlated with each other and, thus, do not provide true replication of the experiment.

The four parameter logistic model (4PL), also called the Hill-Slope model, is the most common equation fit to in vitro dose-response data. One form of the equation is

$$y = bot + \frac{(top - bot)}{1 + (x/\text{IC}_{50})^{slope}},$$

where $y$ is the percent activity and $x$ is the corresponding concentration. The fitted $IC_{50}$ parameter is the **relative** $IC_{50}$, and is defined as the concentration giving a response half way between the fitted top and bottom of the curve. Some software, such as Activity/Base, also provides the **absolute** $IC_{50}$, which is defined as the concentration giving exactly a 50% response. The relative $IC_{50}$ is recommended for most assays. You should also report the **fitting error**, which is usually called the standard error by most software packages (we use the term fitting error to differentiate it from the standard error of the mean [SEM] derived from multiple determinations of a compound).

The 4PL model is the best model for dose-response data, but there are cases where it should not be used. In some cases, due to the potency of the compound falling outside
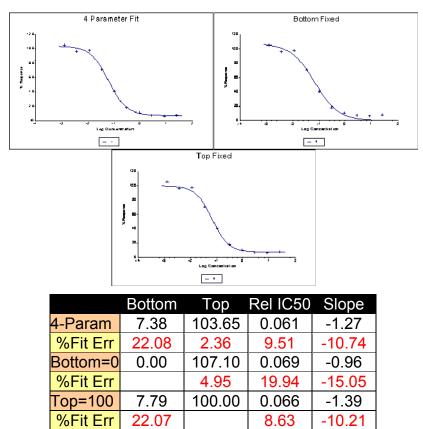
the dosing range, the data may not fully describe the bottom or top asymptote of the curve.  In those cases, respectively, the bottom (3PLFB) or top (3PLFT) can be fixed to improve the curve fit.  If you observe a substantial reduction in the %Fitting Error, and a better dose-response plot of the fitted curve with respect to the actual data then you should switch to either the 3PLFB or 3PLFT model as appropriate.

### *Examples*

All examples below are from receptor binding data fitting %Activity versus concentration (expressed by Activity/Base as log-concentration in the plots).  For this type of assay, the top, bottom and slope parameters should in theory by 100, 0 and −1 respectively.

*Example 1* is a dose-response best fit by the 4PL model.  Both asymptotes are defined by the data, and the fitting error is approximately the same with all 3 models.  Note that even though the fitting error is smallest with the top fixed (8.63% versus 9.51%), the reduction is not small enough to warrant the fixed top model, nor is there any material change in the $IC_{50}$.  The fixed bottom model is clearly inappropriate as the data clearly defines a bottom >0.



| | Bottom | Top | Rel IC50 | Slope |
|---|---|---|---|---|
| 4-Param | 7.38 | 103.65 | 0.061 | -1.27 |
| %Fit Err | 22.08 | 2.36 | 9.51 | -10.74 |
| Bottom=0 | 0.00 | 107.10 | 0.069 | -0.96 |
| %Fit Err | | 4.95 | 19.94 | -15.05 |
| Top=100 | 7.79 | 100.00 | 0.066 | -1.39 |
| %Fit Err | 22.07 | | 8.63 | -10.21 |
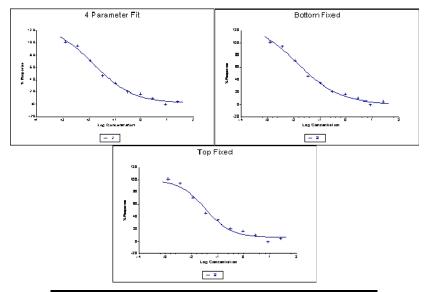
Example 1 Curve fit Results for a dose-response best fit by a 4PL model

The fitting error is expressed here as a percentage of the fitted parameter value.  For example, if the $IC_{50}$ is 0.061 and its fitting error is 0.058, then the %Fit Error is 9.51%.

*Example 2* is best fit by the fixed top (3PLFT) model.  The data does not define a top asymptote, and the fitted top (128.32) and slope (-0.58) from the 4PL model are

**4**
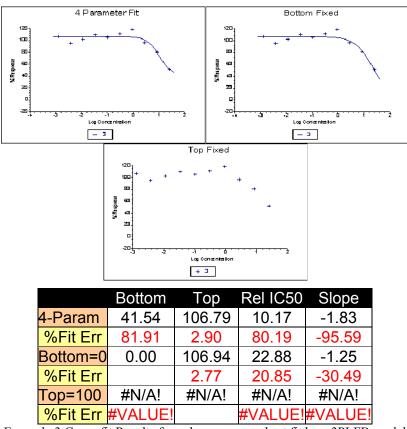
inappropriate for this (binding) data.  By fixing the top at 100% the fitting error is reduced from 57.54 to 21.55%, and the $IC_{50}$ increases by more than two-fold.  Thus the 3PLFT model should be selected over the 4PL.



|          | Bottom | Top    | Rel IC50 | Slope  |
|----------|--------|--------|----------|--------|
| 4-Param  | 2.01   | 128.32 | 0.015    | -0.58  |
| %Fit Err | 202.85 | 14.82  | 57.54    | -22.56 |
| Bottom=0 | 0.00   | 134.33 | 0.014    | -0.53  |
| %Fit Err |        | 12.42  | 57.21    | -12.91 |
| Top=100  | 6.21   | 100.00 | 0.034    | -0.87  |
| %Fit Err | 57.08  |        | 21.55    | -16.09 |
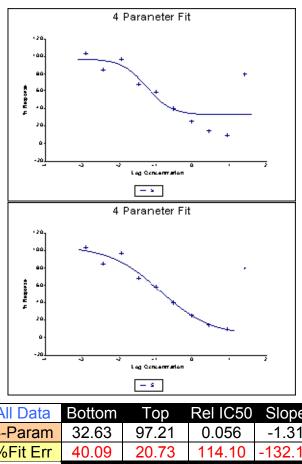
Example 2 Curve fit Results for a dose-response best fit a by a 3PLFT model

*Example 3* is best fit by a fixed bottom (3PLFB) model.  Note that the data does not define the bottom asymptote, and the fitted bottom (41.54) and fitted slope (-1.83) from the 4PL are inappropriate for binding data.  The fixed bottom model reduces the fitting error from 80.19% to 20.85%, while the IC50 increases by more than two-fold.  The fitted IC50 (20.88nM) is inside the dose-range (0.001-25nM), and so it is appropriate to report this value.  Note in this case Activity Base was unable to fit a fixed top model.



| | Bottom | Top | Rel IC50 | Slope |
|---|---|---|---|---|
| 4-Param | 41.54 | 106.79 | 10.17 | -1.83 |
| %Fit Err | 81.91 | 2.90 | 80.19 | -95.59 |
| Bottom=0 | 0.00 | 106.94 | 22.88 | -1.25 |
| %Fit Err | | 2.77 | 20.85 | -30.49 |
| Top=100 | #N/A! | #N/A! | #N/A! | #N/A! |
| %Fit Err | #VALUE! | | #VALUE! | #VALUE! |

Example 3 Curve fit Results for a dose-response best fit by a 3PLFB model
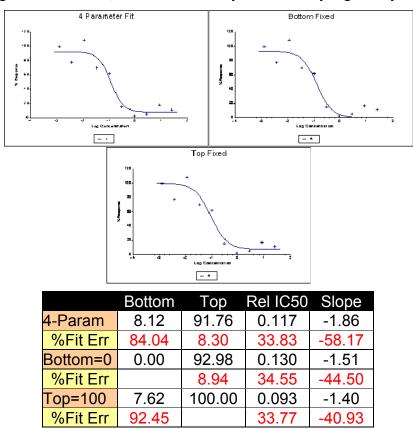
*Example 4* illustrates the definition and effect of outliers (left panel).  Outliers are single, vertically isolated points that are clearly inappropriate.  The point is "obviously" erroneous. The effect of the outlier in this case is to bias the estimate of the bottom upwards, pulling it away from the other points of the data.  In general, outliers can bias either the top, bottom or slope parameter depending upon where they occur in the dose-response.  It is appropriate to remove the outlier (right panel) and refit the points.  Fixing top or bottom did not materially improve the curve fit (not shown).



| All Data | Bottom | Top | Rel IC50 | Slope |
|---|---|---|---|---|
| 4-Param | 32.63 | 97.21 | 0.056 | -1.31 |
| %Fit Err | 40.09 | 20.73 | 114.10 | -132.18 |
| Outlier Rem | Bottom | Top | Rel IC50 | Slope |
| 4-Param | 2.25 | 104.78 | 0.130 | -0.63 |
| %Fit Err | 611.06 | 11.29 | 53.38 | -40.98 |

Example 4 curve fit results for a dose-response containing an outlier

*Example 5* illustrates the effect of high assay variation.  No single point stands out as "obviously erroneous", and therefore it would be inappropriate to remove any points from the curve fit.  Fixing top or bottom does not materially improve the curve fit, and so the 4PL model should be used.  Note that the estimates themselves are not implausible, but the fitting error is 33.83%, which is caused by the relatively high assay variation.



|          | Bottom | Top    | Rel IC50 | Slope  |
|----------|--------|--------|----------|--------|
| 4-Param  | 8.12   | 91.76  | 0.117    | -1.86  |
| %Fit Err | 84.04  | 8.30   | 33.83    | -58.17 |
| Bottom=0 | 0.00   | 92.98  | 0.130    | -1.51  |
| %Fit Err |        | 8.94   | 34.55    | -44.50 |
| Top=100  | 7.62   | 100.00 | 0.093    | -1.40  |
| %Fit Err | 92.45  |        | 33.77    | -40.93 |

Example 5 Curve fit Results for a dose-response with high assay variability, but no outliers

**Notes:**

1.  This equation can be fit to the data using Activity/Base, Bravo/Curve fit, JMP, Graphpad/Prism or Sigma/Plot.  Note that the form of the equation varies from one software package to the next.  Some, such as Graphpad/Prism, fit Log-$IC_{50}$ instead of $IC_{50}$, and the equation looks quite different, but the results are the same as that shown above.

2.  The terms **absolute** and **relative** $IC_{50}$ are not universal.  Both are usually just called the "$IC_{50}$", and it's left unstated which value is actually used.

3.  If the software tool you are using reports Log-$IC_{50}$ then you must convert both the estimate and the % fitting error (%FE) according to the formulas

$$IC_{50} = 10 \wedge \text{Log-IC}_{50} \text{ and } \%FE\left(IC_{50}\right) = FE\left(\text{Log-IC}_{50}\right) \times \ln(10) \times 100$$

4.  There should be at least one point on both sides of the reported $IC_{50}$, i.e. the reported $IC_{50}$ should lie inside the dose-range used in the assay.  The intent of this rule is to make the $IC_{50}$ estimate an interpolation of generated data and not an extrapolation of generated data.  Cases not satisfying this rule should not have an $IC_{50}$ reported or reported with a comment that indicates the value is extrapolated.  If a value is reported, it should be "<X" or ">Y", as appropriate, where X is the lowest concentration and Y is the largest concentration included in the analysis.

5.  The fitting error of the $IC_{50}$ should be no more than 40% of the $IC_{50}$.  Estimates not satisfying this rule should be flagged in the database.  A fitting error of 40% of the $IC_{50}$ corresponds to an MSR of 3-fold.

6.  It is a good idea to remove obvious outliers and then refit the curve without the outliers.  Note that if it isn't obvious, it isn't an outlier.  See examples 4 and 5 above to distinguish high variability from outliers.

7.  For competition assays, such as radioligand binding assays and competitive inhibition assays, the fitted slope should be within 2 (slope) fitting errors of the value 1, and slope estimates outside this range indicate assay problems that need to be investigated.


## B.  Production Monitoring

Production assays can be monitored in two basic ways: running control (reference) compounds and retrospective studies of compounds that have repeat evaluations that accumulate as part of the normal SAR process.  Of the two methods, running control compounds is definitely better as it allows problems to be identified prospectively and corrected, whereas retrospective studies are limited to verification of past activity, be it acceptable or unacceptable.  However, retrospective studies can be useful supplements, especially when conducted prior to important milestones such as Program Sanction where demonstration of "valid biological assays" is a requirement.  Below are comments on the setup/selection of controls and the analysis of retrospective studies, and the use of bridging studies to verify that changes to assay protocols have no effect on the assay results.


### *Control Compounds*

Key assays in a project and assays where problems are suspected should have two control compounds, a primary and a secondary (this is referred to as **Close Monitoring**).  All other assays should have at least a primary control (**Regular Monitoring**).  Both compounds need to be run once per run, unless plate variability is suspected.  In that case the primary control compound needs to be run once per plate.  The purpose of the primary control is to ensure that there isn't any "assay drift", i.e. that the same compound has a stable $K_i/K_b/EC_{50}$ over time, and that the assay reproducibility (MSR) is stable over time.  The purpose of the secondary control is to examine the stability of results over a

dose-range.  If problems do develop, then it is important to examine whether the entire dose-range is equally affected (a small problem) or whether the dose-range is differentially affected (a big problem).  Also, two controls permit direct calculation of both the within-run and overall MSR's, and a check that the MSR is consistent over a range of potencies.

The activity of the primary control should be at or near the most potent compound available, and ideally should be the Lead compound.  There should also be sufficient stock of a single lot of the compound so that it can be run on a continuous basis for some period of time.  Since the control compound is supposed to be representative of the test compounds, it should receive the same sample handling as all the test compounds, and not be specifically prepared and added to the assay outside of normal test compound procedures.

For Close Monitoring, the secondary control should be >100 fold less potent then the primary control.  Otherwise it has the same requirements as the primary control.  As the SAR develops the potency traditionally improves.  So when the "best" compounds are more than 100-fold more potent than the primary control then select a new primary control.  If the assay has a secondary control then the old primary control becomes the new secondary control, and the existing secondary control is dropped.  If there is no secondary control then it is suggested to run both primary controls over the first 6 runs of the new primary control.
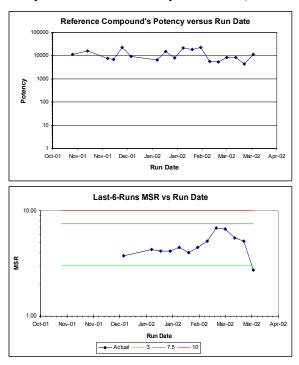
A scatter plot for control compound log-$K_i$/$K_b$/$EC_{50}$ versus run date should be updated after every run and checked for problems.  For assays with two control compounds the difference in log-$K_i$/$K_b$/$EC_{50}$ versus run date should be plotted, and for agonist and non-competitive antagonist assays the efficacy versus run date should also be plotted.  Outlier runs and trends either up or down (assay drift) should be checked visually, and problems investigated and corrected as they occur.  Outlier runs should be repeated.

After 6 runs compute the overall MSR of the assay based on the control compounds according to formula, $MSR = 10^{2\sqrt{2}s}$, where $s$ is the standard deviation of the log-$K_i$/$K_b$/$EC_{50}$ values. This MSR is the total or *overall* MSR (whereas the one computed in a test-retest study encompasses only the within-run variability), and should be less than or equal to 7.5.  This standard comes from practical experience obtained thus far with assays in the company, and not theoretical statistical considerations.  Note that this is a minimum standard that all assays should meet, and in practice chemistry requirements may indicate a smaller MSR (as low as 2-3) is required for some or all assays.  The Project/Program Team should discuss this issue with a statistician to set appropriate MSR's for their assays.

After each run, a running MSR plot should be maintained (i.e. computed from the last 6 runs) and checked to ensure the continued good reproducibility of the assay.

### *Examples*
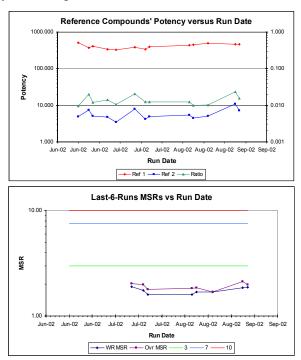
*Example 1* illustrates results for an assay with a single control.  The left panel shows the potency versus run date scatter plot, the right panel the moving MSR chart.  The MSR points are based on the last 6 runs of the assay, i.e. the first point is computed using runs 1-6, the second point uses runs 2-7, etc.  The Mean Summary section indicates the highest/lowest/last $IC_{50}$'s in the period were 22.63, 4.42 and 11.25 uM respectively (chart units are in nM).  The overall average was 10.17 uM.  The potency has no apparent temporal trends, and no unusual observations.  The right panel shows the trends in MSR over time, which appears to increase until mid Feb-2002, and then decrease.  However, the magnitude of the increase trends is quite small and well within the variation of an estimate based on a sample of size 6.  The highest/lowest/latest MSR's are 6.8, 2.7 and 2.7 respectively.  The overall MSR is 4.4, which is not the average of the 6-run MSR's but instead is a single estimate derived using the entire sample (18 data points in this case).  This is a stable assay with moderate assay variation ($3 < MSR < 5$).



| Mean Summary | | | | MSR Summary | |
|---|---|---|---|---|---|
| High | 22630.00 | | | High | 6.8 |
| Low | 4420.00 | | | Low | 2.7 |
| Overall | 10172.76 | | | Overall | 4.4 |
| Current | 11250.00 | | | Current | 2.7 |

Example 1.  Potency, MSR Chart, and Summary Statistics for an Assay with One Control
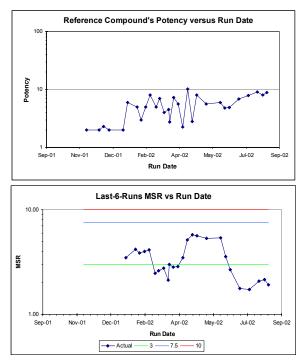
*Example 2* illustrates an assay with two controls.  In the left panel the red and blue lines represent the two compounds, and are positioned using the left axis.  The green line is the potency ratio between the two compounds and is positioned using the right axis.  The right panel shows the moving MSR values both within run and overall.  The Overall-Overall MSR is the value to be reported.  The within-run MSR's are only for comparison backwards to the test-retest study results, and for times when compounds are compared within the same run of an assay.  As with example 1, there are no apparent temporal problems, i.e. this is a stable assay with an overall MSR of 2.0.  This assay is less variable than the assay in example 1.
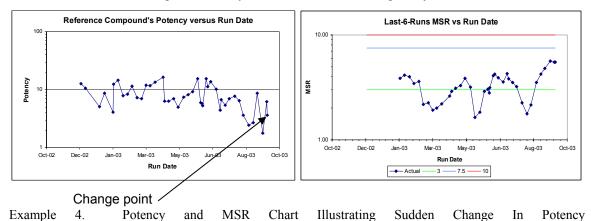




| | | Mean Summary | | | | MSR Summary | |
|---|---|---|---|---|---|---|---|
| | Ref 1 | Ref 2 | Ratio | | | WR | Overall |
| High | 505.26 | 10.84 | 0.02 | | High | 1.9 | 2.1 |
| Low | 324.19 | 3.42 | 0.01 | | Low | 1.6 | 1.7 |
| Overall | 409.51 | 5.53 | 0.01 | | Overall | 1.8 | 2.0 |
| Current | 462.99 | 7.21 | 0.02 | | Current | 1.9 | 2.0 |

Example 2.  Potency, MSR Chart, and Summary Statistics for an Assay with Two Controls

*Examples 3 and 4* illustrate problems with a shift in compound potency.  Example 3 illustrates a steady degradation in potency over time, whereas Example 4 illustrates a more sudden shift in potency at a particular point in time.  In Example 3 the assay variability appears to be shrinking, while in Example 4 it appears to be stationary.  Repetitive freeze-thaw cycles of a compound may cause a slow degradation in potency whereas a change in lot of a key assay ingredient may result in a sudden potency shift.  In both cases it is important to identify the cause and correct it as soon as possible.
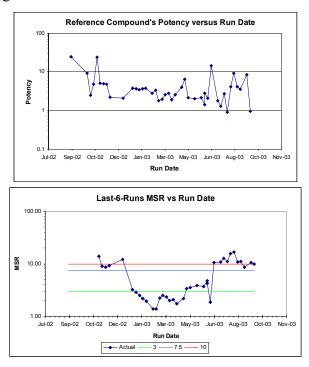


Example 3.  Potency and MSR Chart Illustrating Assay Drift



Change point

Example    4.      Potency    and    MSR    Chart    Illustrating    Sudden    Change    In    Potency

*Example 5* illustrates an assay with stable potency, but in June the assay variability increased.  The moving MSR was stable around 3, but after June increased to over 10, and remained there.  This also is most likely caused by a change in the assay process around that time.  Again it is important to identify and correct the cause as soon as possible.  Note however that a single outlier will cause the MSR chart to increase for the next 6 runs, and so it usually takes more time to correctly distinguish a change in assay variability from a single outlier result.



Example 5.  Potency and MSR chart for change in assay variability

## Retrospective Studies

During the course of project/program development numerous compounds are repeatedly evaluated and stored in archival databases.  This data can be mined to examine the reproducibility of assay results.  This work should always be done by a statistician as the repeated compounds are not a random selection of all compounds, and may be biased with respect to time of evaluation, potency, structure and "assayability" (the latter term is meant to reflect conditions such as solubility, quenching, stickiness to plastic and other practical problems).  In spite of these potential problems retrospective studies can be a very useful exercise, particularly in establishing the acceptability of older assays that have never been formally evaluated for reproducibility.  In addition, the MSR can be examined over various subsets such as potency range, structure and run date to check that the control compound MSR's are representative of the test compounds with respect to potency range, structure and run date.

## Bridging Studies

If a key aspect of an assay changes, such as an equipment change or lot of a reagent, then a test-retest study should be conducted to verify equivalence of the two protocols.  A

judgment should be made on a case-by-case basis of whether the full protocol outlined in Section II.B needs to be made, or only a single run under old and new conditions (i.e. one might do just Step 4 of the procedure, or one might do both Steps 3 and 4 depending upon the severity of the protocol change).  Also in cases of specific modifications such as replacing equipment for a particular step in the assay an experiment can be designed to validate that the replacement is equivalent to the original in the conduct of that step of the assay.

### Dimethylsulfoxide: biological compatibility and compound storage.

Dimethylsulfoxide (DMSO) is a universal solvent for all compounds tested in high, medium and low throughput screens (HTS, MTS and LTS). Compounds are initially dissolved in 100% DMSO and further diluted into water and assay buffers in subsequent dilutions for screening and $IC_{50}$ or $K_i$ determinations. It is extremely important that the DMSO compatibility of biological reagents such as enzymes, receptors, protein/peptide reagents and cells be established to ensure that the screening assays are not adversely affected. In general, the final DMSO concentrations in cell-based assays are <0.2% and are <1% in biochemical assays. It is highly recommended that the tolerable DMSO concentration be determined individually for each validated assay.

DMSO is also used as a cryoprotectant in the freezing of cell cultures at ATCC. The product is cell culture grade and has been tested to ensure cell viability. Each lot is also tested for the absence of bacteria, fungi, and endotoxin.

When solubilized compounds are stored in DMSO, it is important to understand the stability of these compounds under various storage conditions and freeze-thaw cycles. A detailed study of these effects was published recently (1).  It is believed that the degradation of DMSO solubilized compounds is mainly due to moisture absorbed from the air. This can happen during frequent freeze-thaw cycles of compounds stored frozen in DMSO, or frequent exposure to air during repeated access for biological testing (cherry-picking).

### Recommended storage conditions for DMSO solubilized compounds:

- 96- well polypropylene plates.
- Storage temperature: 10 degree C or room temperature.
- Inert gas atmosphere: argon flush.
- Minimal exposure to moist environments.

### References:

1. Cheng. X., Hochlowski J, Tan H, Hepp, D, beckner C, Kantor S, Schmitt R, Studies on Repository Compound Stability in DMSO Under Various Conditions. J Biomol Screening, 2003, 8(3), 292-304.

2. Kozikowski, BA, Burt, TM, Tirey, DA, Williams LE, . Kuzmak, BR, Stanton, DT, Morand, KL, and Nelson, SL The Effect of Room-Temperature Storage on the Stability of Compounds in DMSO J Biomol Screen 2003 8: 205-209.

# SECTION IV

# ENZYMATIC ASSAYS

# Table of Contents

## SECTION V:  ENZYMATIC ASSAYS

## A. Enzyme Assay Development Flow Chart

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**Acquire Reagents**

Enzyme target, substrate, co-factors, additives control inhibitors, enzyme inactive mutants, labeled reagents based on assay design

---

**Setup Instrumentation**

Calibration performance testing.  Establish linearity of instrument performance with appropriate product measured.

---

**Assay Concept Validation Experiments**

Establish preliminary assay parameters, reagent suitability and stability, linearity of enzyme activity, signal window, and data analysis models.  Determine initial velocity conditions, Km and Vmax with selected substrates.  Collaborate with an experienced enzymologist and statistician.

---

**Determine Optimization Requirements**

Use experimental design techniques to optimize critical assay factors and reproducibility.  Collaborate with a statistician.

---

**Validation Experiments**

Robustness verification (day-to-day, scaleup, automation, operator effects)

---

**Method Documentation**

Prepare and implement SOP

---

## B. Introduction

Enzyme inhibitors are an important class of pharmacological agents.  Often these molecules are competitive, reversible inhibitors of substrate binding.  This section describes the development and validation of assays for identification of competitive, reversible inhibitors.  In some cases other mechanisms of action may be desirable which would require a different assay design.  A separate approach should be used if seeking a non-competitive mechanism that is beyond the scope of this document and should be discussed with an enzymologist and chemist (for a reference see "Enzyme Structure and Mechanism" by Alan Fersht, WH Freeman and Co., NY, 1985, pp327-330).

## C. Concept

Enzymes are biological catalysts involved in important pathways that allow chemical reactions to occur at higher rates (velocities) than would be possible without the enzyme. Enzymes are generally globular proteins that have one or more substrate binding sites. The kinetic behavior for many enzymes can be explained with a simple model proposed during the 1900's:

$$\text{E} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{ES} \xrightarrow{k_2} \text{E} + \text{P}$$

where E is an enzyme, S is substrate and P is product(s).  ES is an enzyme-substrate complex that is formed prior to the catalytic reaction.  $k_1$ is the rate constant for enzyme-substrate complex (ES) formation and $k_{-1}$ is the dissociation rate of the ES complex.  In this model, the overall rate-limiting step in the reaction is the breakdown of the ES complex to yield product, which can proceed with rate constant $k_2$.  The reverse reaction (E + P → ES) is generally assumed to be negligible.

Assuming rapid equilibrium between reactants (enzyme and substrate) and the enzyme-substrate complex resulted in mathematical descriptions for the kinetic behavior of enzymes based on the substrate concentration (see *Enzyme Kinetics: Behavior and analysis of rapid equilibrium and steady state enzyme systems." By Irwin H.* Segel, John Wiley and Sons, NY 1975 for these mathematical derivations).  The most widely accepted equation (derived independently by Henri and subsequently by Michaelis and Menten) relates the velocity of the reaction to the substrate concentration as shown in the equation below, which is typically referred to as the Michaelis-Menten equation:

$$v = \frac{[S]\ V_{max}}{[S] + K_m}$$

where

        v = rate if reaction
        $V_{max}$ = maximal reaction rate
        [S] = substrate concentration
        $K_m$ = Michaelis-Menten constant

For an enzymatic assay to identify competitive inhibitors, it is essential to run the reaction under *initial velocity conditions* with substrate concentrations at or below the $K_m$ value for the given substrate. The substrate should either be the natural substrate or a surrogate substrate, like a peptide, that mimics the natural substrate. The optimal pH and buffer component concentrations should be determined before measuring the $K_m$ (see section on optimization experiments).

## What is initial velocity?

- Initial velocity is the initial linear portion of the enzyme reaction when less than 10% of the substrate has been depleted or less than 10% of the product has formed. Under these conditions, it is assumed that the substrate concentration does not significantly change and the reverse reaction does not contribute to the rate.

- Initial velocity depends on enzyme and substrate concentration and is the region of the cureve in which the velocity does not change with time. This is not a predetermined time and can vary depending on the reaction conditions.

## What are the consequences of not measuring the initial velocity of an enzyme reaction?

- The reaction is non-linear with respect to enzyme concentration.

- There is an unknown concentration of substrate.

- There is a greater possibility of saturation of the detection system

- The steady state or rapid equilibrium kinetic treatment is invalid

Measuring the rate of an enzyme reaction when 10% or less of the substrate has been depleted is the first requirement for steady state conditions. At low substrate depletion (i.e. initial velocity conditions) the factors listed below that contribute to non-linear progression curves for enzyme reactions, do not have a chance to influence the reaction.

- Product inhibition

- Saturation of the enzyme with substrate decreases as reaction proceeds due to a
  decrease in concentration of substrate (substrate limitation)

- Reverse reaction contributes as concentration of product increases over time

- Enzyme may be inactivated due to instability at given pH or temperature

## D. Reagents and Method Development

For any enzyme target, it is critical to ensure that the appropriate enzyme, substrate,
necessary co-factors and control inhibitors are available before beginning assay
development.  The following requirements should be addressed during the method design
phase:

1.  Identity of the enzyme target including amino acid sequence, purity, and the amount
    and source of enzyme available for development, validation and support of
    screening/SAR activities.  One should also ensure that contaminating enzyme
    activities have been eliminated.  Specific activities should be determined for all
    enzyme lots.

2.  Identify source and acquire native or surrogate substrates with appropriate sequence,
    chemical purity, and adequate available supply.

3.  Identify and acquire buffer components, co-factors and other necessary additives for
    enzyme activity measurements according to published procedures and/or exploratory
    research.

4.  Determine stability of enzyme activity under long-term storage conditions and during
    on bench experiments.  Establish lot-to-lot consistency for long-term assays.

5.  Identify and acquire enzyme inactive mutants purified under identical conditions (if
    available) for comparison with wild type enzyme.

## E. Detection System Linearity

Instrument capacity needs to be determined by detecting signal from product and plotting
it versus product concentration.  Figure 1 below demonstrates what can happen if a
detection system has a limited linear range.  In the Capacity 20 trace, the system becomes
non-linear at concentrations of product that are greater than 10% of the total product
generated.  This limited linear range would severely compromise measurements, since it
is essential that the enzyme reaction condition be within the linear portion of the
instrument capacity.  Subsequent assay analysis would be affected if the enzyme reaction

were performed outside of this linear portion.  The Capacity 100 trace represents a more ideal capability of an instrument that allows a broad range of product to be detected.

The linear range of detection for an instrument can be determined using various concentrations of product and measuring the signal.  Plotting the signal obtained (Y axis) versus the amount of product (X axis) yields a curve that can be used to identify the linear portion of detection for the instrument.

## Detection System Linearity
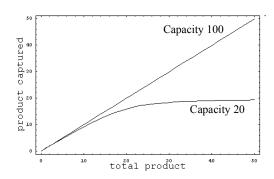


**Figure 1.**
Signal saturation can lead to false measurements of
assay parameters, such as $K_m$

## F.  Enzyme Reaction Progress Curve

A *reaction progress curve* can be obtained by mixing an enzyme and it's substrate together and measuring the subsequent product that is generated over a period of time. The initial velocity region of the enzymatic reaction needs to be determined and subsequent experiments should be conducted in this linear range, where less than 10% of the substrate has been converted to product.  If the reaction is not in the linear portion, the enzyme concentration can be modified to retain linearity during the course of the experiments.  Both of these steps (modifying the enzyme and analyzing the reaction linearity) can be conducted in the same experiment.  An example is shown below in Figure 2.

## Reaction Progress Curves
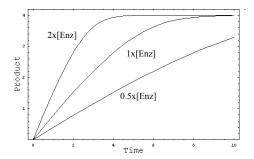


**Figure 2**.  Plateau is due to substrate depletion

In this set of data, product is measured at various times for three different concentrations of enzyme and one substrate concentration.  The curves for the 1X and 2X relative levels of enzyme reach a plateau early, due to substrate depletion.  To extend the time that the enzyme-catalyzed reaction exhibits linear kinetics, the level of enzyme can be reduced, as shown for the 0.5 X curve.  These curves are used to define the amount of enzyme, which can be used to maintain initial velocity conditions over a given period of time.  These time points should be used for subsequent experiments.

Note that all three of the reaction progress curves shown in the example above approach a similar maximum plateau value of product formation.  This is an indication that the enzyme remains stable under the conditions tested.  A similar experiment performed when enzyme activity decreases during the reaction is shown in Figure 3 below.  In this case, the maximum plateau value of product formed does not reach the same for all levels of tested enzyme, likely due to enzyme instability over time.

## Enzyme activity over time
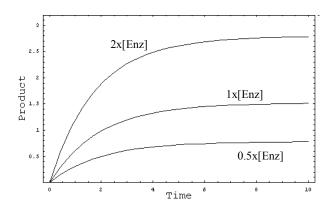


**Figure 3.**
Plateau is due to loss of enzyme activity
(note: plateaus do not converge)

## Measuring initial velocity of an enzyme reaction

- Keep temperature constant in the reaction by having all reagents equilibrated at the same temperature.

- Design an experiment so pH, ionic strength and composition of final buffer are constant.  Initially use a buffer known for the enzyme of interest either by consulting the literature or by using the buffer recommended for the enzyme.  This buffer could be further optimized in later stages of development.

- Perform the time course of reaction at three or four enzyme concentrations.

- Need to be able to measure the signal generated when 10% product is formed or to detect 10% loss of substrate.

- Need to measure signal at t=0 to correct for background (leave out enzyme or substrate).

For kinase assays, the background can be determined by leaving out the enzyme or the substrate. The condition resulting in the highest background level should be used. EDTA is **not** recommended for use as the background control during validation of a kinase assay. Once the assay has been validated, if the background measured with EDTA is the same than both the no enzyme and no substrate control, then EDTA could be used.

## G. Measurement of $K_m$ and $V_{max}$

Once the initial velocity conditions have been established, the substrate concentration should be varied to generate a saturation curve for the determination of $K_m$ and $V_{max}$ values. **Initial velocity conditions must be used**. The Michaelis-Menten kinetic model shows that the $K_m = [S]$ at $V_{max}/2$. In order for competitive inhibitors to be identified in a competition experiment that measures $IC_{50}$ values, a substrate concentration around or below the $K_m$ must be used. Using substrate concentrations higher than the $K_m$ will make the identification of competitive inhibitors (a common goal of SAR) more difficult.

For kinase assays, the $K_m$ for ATP should be determined using saturating concentrations of the substrate undergoing phosphorylation. Subsequent reactions need to be conducted with optimum ATP concentration, around or below the $K_m$ value using initial velocity conditions. However, it would be best to determine $K_m$ for ATP and specific substrate simultaneously. This would allow maximum information to be gathered during the experiment as well as address any potential cooperativity between substrate and ATP.

A requirement for steady state conditions to be met means that a large excess of substrate over enzyme is used in the experiment. Typical ratios of substrate to enzyme are greater than 100 but can approach one million.

### What does the $K_m$ mean

- If $K_m >>> [S]$, then the velocity is very sensitive to changes in substrate concentrations. If $[S] >>> K_m$, then the velocity is insensitive to changes in substrate concentration. A substrate concentration around or below the $K_m$ is ideal for determination of competitive inhibitor activity.

- $K_m$ is constant for a given enzyme and substrate, and can be used to compare enzymes from different sources.

- If $K_m$ seems "unphysiologically" high then there may be activators missing from the reaction that would normally lower the $K_m$ *in vivo*, or that the enzyme conditions are not optimum.

## How to measure $K_m$

- Measure the initial velocity of the reaction at substrate concentrations between 0.2-5.0 $K_m$. If available, uses the $K_m$ reported in the literature as a determinant of the range of concentration to be used in this experiment. Use 8 or more substrate concentrations.

- Measuring $K_m$ is an iterative process. For the first iteration, use six substrate concentrations that cover a wide range of substrate concentrations, to get an initial estimate. For subsequent iterations, use eight or more substrate concentrations between 0.2-5.0 $K_m$. Make sure there are multiple points above and below the Km
- For enzymes with more than one substrate, measure the $K_m$ of the substrate of interest with the other substrate at saturating concentrations. This is also an iterative process. Once the second Km is measured, it is necessary to check that the first Km was measured under saturating 2$^{nd}$ substrate concentrations.

- Fit the data to a rectangular hyperbola function using non-linear regression analysis. Traditional linearized methods to measure Km s should not be used.

Figures 4 and 5 demonstrate a typical procedure to determine the $K_m$ for a substrate. In Figure 4, reaction product is measured at various times for 8 different levels of substrate. The product generated (Y axis) is plotted against the reaction time (X axis). Each curve represents a different concentration of substrate. Note that all the curves are linear, indicating that initial velocity conditions (<10% of substrate conversion) have been met.
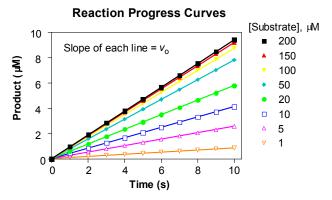


**Figure 4.** Reaction progress curves at 8 substrate concentrations

The initial velocity ($v_o$) for each reaction progress curve is equivalent to the slope of the line, which is defined as the change in the product formed divided by the change in time. This is expressed by the equation below and can be calculated using linear regression or other standard linear method:

$$\frac{\Delta Y}{\Delta X} = \text{Slope} = v_o$$

The resulting slopes (initial velocity, $v_o$) for each of the reaction progress curves are plotted on the Y-axis versus the concentration of substrate (X axis) and a nonlinear regression analysis using a rectangular hyperbola model is performed as shown in Figure 5 below.

**Plot of $v_o$ vs. [S]**
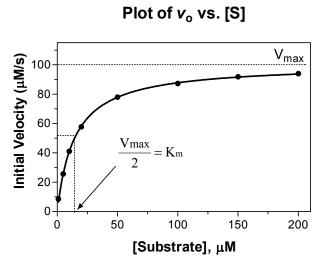


**Figure 5.** Initial velocity versus substrate concentration

The $V_{max}$ and $K_m$ for the system is calculated from the nonlinear regression analysis. The meaning of each term is shown in Figure 5. The $K_m$ is the substrate concentration which results in an initial reaction velocity that is one-half the maximum velocity determined under saturating substrate concentrations.

Linear transformations, such as a double reciprocal Lineweaver-Burke plot of the initial velocity/substrate concentration data (i.e. $1/v_o$ vs. $1/[S]$, should not be used for calculating the $K_m$ and $V_{max}$ from saturation type experiments such as those described above. These linear transformations tend to distort the error involved with the measurement and were used before programs that can perform nonlinear regression analysis were widely available.
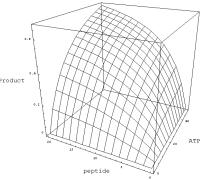
An additional parameter, often seen in the literature, which can sometimes be useful to describe the efficiency of an enzyme, is the catalytic constant (or turnover number) that is termed $k_{cat}$. The $k_{cat}$ value can be determined from saturation data (Figure 5) from the following equation:

$$kcat = \frac{Vmax}{[E]i}$$

where $[E]_i$ is the initial enzyme concentration and $V_{max}$ is the maximum velocity determined from the saturation hyperbola.

For kinase reactions where the $K_m$ for ATP and substrate need to be determined, it is best if a multi-dimensional analysis is used to measure both $K_m$'s simultaneously. An example is shown in Figure 6 below.

Determine Km's for ATP and peptide/protein



•Check time dependence of four corners
•Watch for cooperativity
•Global fit at least a 4x4 matrix

**Figure 6.** Simultaneous determination of $K_m$ for ATP and specific substrate

If this method is used, it is important to demonstrate that in the extreme conditions (particularly low substrate, high ATP concentrations) the linearity of the instrument is maintained. In addition, it is important that linearity of the reaction is maintained at all conditions. Proper background controls must be used. The best condition would be a combination of the best signal to noise ratio while maintaining the substrate and ATP concentration as low as possible. Consult with a biochemist and statistician experienced in these techniques to ensure appropriate data analysis methods are utilized.

## H. Determination of IC$_{50}$ for Inhibitors

Concentration-response plots are used to determine the effects of an inhibitor on an enzymatic reaction. These experiments are performed at constant enzyme and substrate concentrations and are the primary type of analysis performed for structure-activity relationship (SAR) measurements for compounds of interest.

A typical concentration-response plot is shown in Figure 7. Fractional activity (Y axis) is plotted as a function of inhibitor concentration (X axis). The data are fit using a standard four-parameter logistic nonlinear regression analysis.
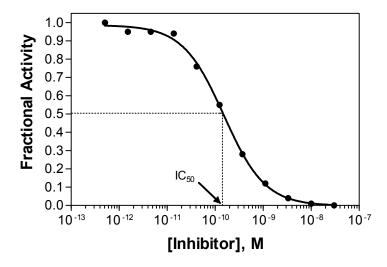


**Figure 7.** Concentration-Response plot for an enzyme inhibitor

The concentration of compound that results in 50% inhibition of maximal activity is termed the IC$_{50}$ (inhibitor concentration yielding 50% inhibition). It is important to use enough inhibitor concentrations to provide well-defined top and bottom plateau values. These parameters are critical for the mathematical models used to fit the data. Other criteria for successful concentration-response curves are listed in the discussion below.

## I. IC$_{50}$ Determination for SAR

- Use a minimum of 10 inhibitor concentrations for an accurate IC$_{50}$ determination. Equally spaced concentration ranges (i.e. 3-fold or half-log dilutions) provide the best data sets for analysis.

- Ideally, half the data points on the IC$_{50}$ curve are above the IC$_{50}$ value and half are below the IC$_{50}$ value, including a minimum and maximum signal.

- The lower limit for determining an $IC_{50}$ is ½ the enzyme concentration (Tight binding inhibitors, Chapter 9, Copeland, R.A., 2$^{nd}$ Edition, 2000).

- Screening strategies for defining an initial SAR include: determination of the % inhibition at a single concentration; determination of the % inhibition at a high and a low concentration of inhibitor; and finally, determination of an apparent $IC_{50}$ using fewer concentrations.

## Criteria for reporting IC50's

- The maximum % inhibition should be greater than 50%.

- Top and bottom values should be within 15% of theory

- The 95% confidence limits for the $IC_{50}$ should be within a 2-5 fold range.

Since the $IC_{50}$ value is the most common result reported for enzymatic assays, it is important to understand how experimental conditions affect $IC_{50}$ determinations. Generally the concentrations of substrate relative to the Km and the amount of product produced have the greatest effect on the measured $IC_{50}$. The figure below demonstrates the effect of both substrate concentration and percent conversion on measured $IC_{50}$ values for a competitive inhibitor.
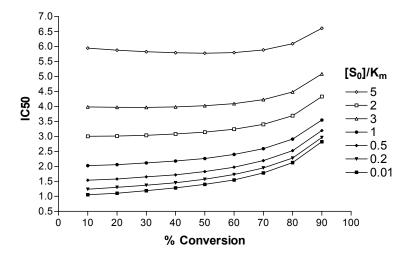


**Figure 7.** Effect of substrate concentration and % conversion on the $IC_{50}$ for an inhibitor

Figure 7 shows the effect of both substrate concentration and % conversion on measured $IC_{50}$ values. Increased substrate conversion as well as increased substrate concentrations will increase the resulting $IC_{50}$ value for a given inhibitor. The data were modeled assuming $K_i = 1.0$ for a competitive inhibitor with no product inhibition.

## J.  Optimization Experiments

Buffer composition can have significant effects on enzymatic activities.  Some buffer components can also affect compound inhibitory activities.  Various components in the buffer can be used as factors to modify in a statistical optimization experiment. Published literature information should be used in selecting these factors.  For example a factorial design experiment could be conducted while varying:

- Divalent cations, for example $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$

- Salts, for example NaCl, KCl

- EDTA

- Reducing agents such as βME, DTT, glutathione

- Bovine serum albumin

- Detergents such as Triton, CHAPS

- DMSO

- Buffer source, for example HEPES *vs.* acetate

- pH

In addition to assay conditions, enzyme stability may be affected if appropriate measures are not taken during long-term storage.  Many enzymes need to be stored at $-70^{o}C$ to maintain activity, but freeze-thaw cycles are not recommended.  Other enzymes can be stored for long periods of time at $-20^{o}C$ using an additive in the storage buffer such as 50% glycerol.

The presence of carrier proteins in the buffer (bovine serum albumin, ovalbumin, others…) as well as use of polypropylene plates (or non-binding polystyrene plates) may be essential to retain proper enzyme activity.

Enzyme instability can also occur during an assay, as demonstrated previously in Figure 3.  This type of instability can occur if the active conformation of the enzyme is not stable in the chosen assay conditions of pH, temperature, ionic strength, etc.  In addition, for enzymes that are dimerized, a large dilution into assay buffer may result in inactivation.

## K. Assay Validation

Parameters such as substrate $K_m$ and control inhibitor $IC_{50}$'s need to be determined in 3 separate experiments to assess variability.  Refer to Section IIB to assess variability the assay.

## L. References

Copeland, Robert A. *Enzymes: A practical introduction to structure, mechanism and data analysis.* Wiley-VCH, NY, 2nd Edition, 2000.

Segel, Irwin H. *Enzyme Kinetics: Behavior and analysis of rapid equilibrium and steady state enzyme systems.* John Wiley and Sons, NY 1975.

Dixon, M. and Webb, E.C. *Enzymes,* 3rd Edition. Academic Press, NY 1979.

Lai C-J-,Wu JC A Simple Kinetic Method for Rapid Mechanistic Analysis of Reversible Enzyme Inhibitors. Assays and Drug Dev. Technologies. 2003;1(4):527-535.

**General Enzyme Kinetics references on the Internet**:

http://web.indstate.edu/thcme/mwking/enzyme-kinetics.html#michaelis

http://www.ultranet.com/~jkimball/BiologyPages/E/EnzymeKinetics.html

**Enzyme kinetics simulations:**

http://www.rpi.edu/dept/chem-eng/Biotech-Environ/Canada/enzkin.html

http://interactive-mathvision.com/PaisPortfolio/Ckm/EnzymeKinetics/EKJava.html

**Software examples for fitting enzyme kinetics data**:

Graphpad Prism (http://www.graphpad.com/prism/Prism.htm)

Sigma Plot (http://www.spss.com/SPSSBI/Sigmaplot/)

GraFit (http://www.erithacus.com/grafit/)

# SECTION V

# RECEPTOR BINDING ASSAYS

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## SECTION V:  RECEPTOR BINDING ASSAYS

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↓

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⟷

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Test known agonists/antagonists, $K_i$

↓

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**Assay Buffer**
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**Assay Conditions**
Incubation time
Receptor concentration
Solvent tolerance

**Binding Parameters**
Determine $K_d$
Test known agonists/antagonists, $K_i$

**Variability Assessment**

## C. INTRODUCTION

There are two typical assay formats used for analysis of receptor-ligand interactions in screening applications, filtration and scintillation proximity assay (SPA). Both formats utilize a radiolabeled ligand and a source of receptor (membranes, soluble/purified). Receptor binding assays using non-radioactive formats (fluorescence polarization, time-resolved fluorescence, etc.) which are continually being investigated for feasibility, would have similar assay development schemes to those presented in this document.

Selection of the detection method to be used (SPA, filtration, non-radioactive) is the first step to receptor binding assay development. In some cases, investigation into more than one format may be required to meet the following desired receptor binding criteria:

- Low nonspecific binding (NSB)
- > 80% specific binding at the $K_d$ concentration of radioligand
- Less than 10% of the added radioligand should be bound (Zone A)
- Steady state obtained and stability of signal maintained
- For competition assays, the radioligand concentration should be at or below the $K_d$
- No dose response in the absence of added receptor
- Reproducible
- Appropriate signal window (i.e. Z-factor > 0.4, SD window > 2 SD units)

While developing receptor binding assays, some of the experiments may need to be performed in an iterative manner to achieve full optimization. In addition preliminary experiments may be required to assess the system.

In many instances, a multi-variable experimental design can be set up to investigate the impact of several parameters simultaneously, or to determine the optimum level of a factor. It is strongly recommended that full assay optimization be performed in collaboration with an individual trained in experimental design.

Experimental design and assay variability is addressed in detail in other sections of this handbook.

The following pages should be used as a general developmental guide to receptor binding assays using SPA or filtration formats.

## D. REAGENTS

Quality reagents are one of the most important factors involved in assay development. Validated reagents of sufficient quantity are critical for successful screen efforts over a long period of time. The primary reagents required for a radioactive receptor binding assay which are discussed on the following pages are:
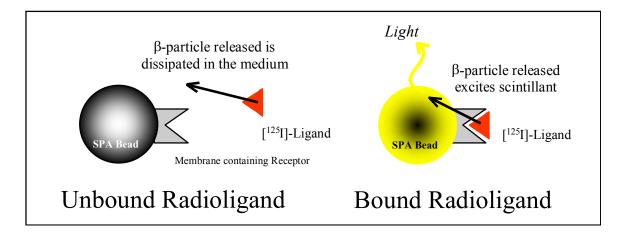
   Receptor (membranes or purified)
   Radioligand

A section on methods of generating reagents for membrane binding assays can be found in the *Appendix for the Receptor Binding Assays* section of this handbook.

## E. SCINTILLATION PROXIMITY ASSAYS (SPA)

### Concept

SPA assays do not require a separation of free and bound radioligand and therefore are amenable to screening applications. A diagram for a standard receptor binding SPA is shown below for a $^{125}$I radioligand.



General Steps for an SPA assay:

1)  Add and incubate test compound, radioligand, receptor and SPA beads in a plate (in some cases, the SPA beads are added at a later time point).

2)  Count plates in microplate scintillation counter. The appropriate settling time needs to be determined experimentally.

| Advantages | Disadvantages |
|---|---|
| Non-separation method | More expensive - requires license |
| No scintillation cocktail required | Lower counting efficiency |
| Reduced liquid radioactive waste | Primarily for $^3$H and $^{125}$I ($^{33}$P, $^{35}$S possible) |
| Reduced handling steps (add, incubate, read) | Non-proximity effects |
| Multiple bead types (WGA, PEI-coated, etc.) | Quenching by colored compounds |
|  | Difficult to perform kinetic experiments |
|  | Bead settling effects |

Many of the advantages and disadvantages are addressed in the following sections.


## F.  SPA ASSAY FORMAT


### Bead Type

The SPA bead surface-coupling molecule selected for use in a receptor binding assay must be able to capture the receptor of interest with minimal interaction to the radioligand itself.  The table below lists the available SPA bead capture mechanisms that can be used with various receptor sources.

| Receptor Source | SPA Bead | Capture Mechanism |
|---|---|---|
|  |  |  |
| Membranes | WGA[1] | Glycosylation sites |
|  | Poly-L-lysine | Negative charges |
|  |  |  |
| Soluble/Purified | WGA | Glycosylation sites |
|  | Streptavidin | Biotinylated site |
|  | Antibody[2] | Specific antibody |
|  | Copper | His-Tag |
|  | Glutathione | GST-fusion |

[1]Wheat germ agglutinin (WGA) SPA beads are available in standard untreated format and two different versions that have been treated with polyethyleneimine (PEI).
In addition to the SPA bead types listed above, FlashBlue GPCR beads are available from Perkin Elmer Life and Analytical Sciences.
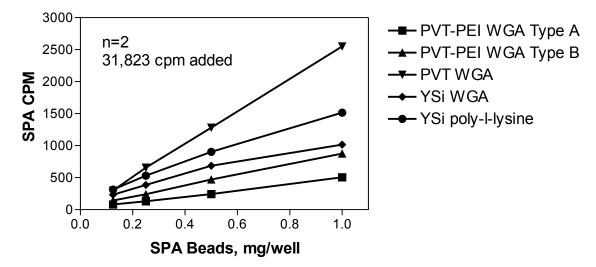
[2]Secondary antibody SPA beads are available to capture specific antibodies from the following species:  Rabbit, Sheep/Goat, Guinea pig, mouse.  Protein A SPA beads are also available for antibody capture.

In addition to the capture mechanism, two types of SPA beads are available:

> Plastic SPA beads, made of polyvinyltoluene (**PVT**), act as a solid solvent for diphenylanthracine (DPA) scintillant incorporated into the bead

> A Glass SPA bead, or Yttrium silicate (YSi), uses cerium ions within a crystal lattice for the scintillation process.  In general, YSi is a more efficient scintillator than PVT is, but YSi SPA beads are requires continuous mixing even during dispensing.

Typical experiments to investigate nonspecific binding of radioligand to SPA beads include varying the amount of radioligand (above and below the predicated $K_d$ value) and the amount of SPA beads (0.1 mg to 1 mg) in the absence of added membrane protein. Results from this experiment can identify the proper type of SPA beads to use in future experiments, as well as the baseline background due to non-proximity effects.  An example experiment using a kit from GE Healthcare (formerly Amersham Biosciences) that contains several different SPA bead types (Select-a-Bead kit, #RPNQ0250) is shown below.



For this example, which was performed in the absence of added membrane receptor, the PVT-PEI WGA Type A SPA beads yields the lowest interaction with the radioligand and was used for further assay development.  An increase in signal with an increasing amount of added SPA beads is normal.  Additives may be useful in decreasing high levels of nonspecific binding of radioligand to the SPA beads (see table of Agents which Reduce NSB in the *Assay Buffer* section).

## Plate Type

The type of plate that is used for SPA receptor binding assays may be influenced by the following factors:

        Counting instrument used (Trilux, TopCount, CLIPR, LeadSeeker)
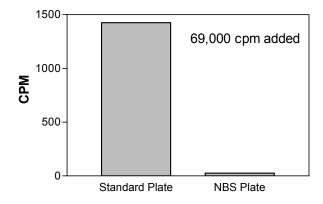        Miniaturization (96-well, 384-well)
        Binding of radioligand to plastics
        Liquid dispensing/automation equipment

The table below lists typical choices for SPA assays:

| Plate Type | Instrument | # of Wells | Comments |
|------------|-----------|-----------|----------|
| Costar #3632 | Trilux | 96 | White/Clear-bottom, 96-well |
| Costar #3604 | Trilux | 96 | White/Clear-bottom, 96-well, non-binding surface (NBS), may be useful when ligands are sticky |
| PE LAS 401 | Trilux | 96 | Clear/flexible, not amenable to automation or liquid dispensing instrumentation |
| Costar#3706 | Trilux | 384 | White/Clear-bottom, 384-well |
| PE LAS Optiplate | TopCount | 96 | White/solid bottom, 96-well |

The data shown below demonstrates an advantage of the NBS plates when using a radioligand, which binds nonspecifically to plate plastic.



69,000 CPM of $^{125}$I-labeled ligand added to the well, incubated for 60 min. Radioactivity removed and wells washed. SPA beads then added. Data demonstrates that a radioligand sticking to the plate surface can elicit an SPA signal. NBS plate yields significantly less nonspecific binding of radioligand.

## Order of Addition

The order of addition for reagents may affect assay performance as well as ease of automation. Three basic formats have been used:

| Method | Advantage |
|--------|-----------|
| Membrane pre-coupled to SPA bead | May aid in lowering NSB |
| Time zero (T=0) addition of SPA beads | Easily automated |
| Delayed addition of SPA beads | Optimum ligand/receptor interaction possible |

Time zero or delayed additions are the most commonly used formats in HTS, with time zero addition requiring fewer manipulation steps.  Experiments may be required to determine the optimum method to be used for a particular receptor to maximize signal to background levels.

In addition, the effect of DMSO on intermediate reactants should be investigated.  If compounds in DMSO are added into the wells first (most common method for screening efforts), other reagents added (i.e. radioligand, membranes, beads, etc.) may be affected by the concentration of DMSO, or if the time before reaching the final reaction mixture becomes significant.

**Non-Specific Binding (NSB)/Non-proximity Effects (NPE)**
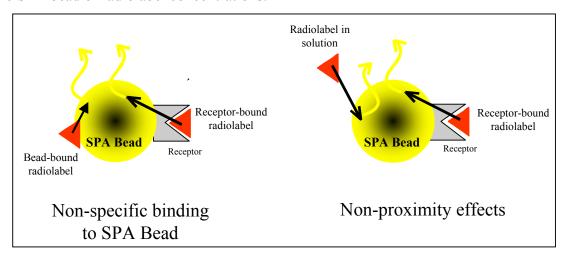
In order to obtain the maximum signal to noise ratio possible for SPA receptor binding assays, it is important to understand the two different types of signals associated with the radioligand and SPA beads, which may contribute to the total assay background levels.

**Non-Specific Binding (NSB) to SPA Beads**

This signal is attributed to radiolabel which may adhere to the SPA beads themselves and not through a specific interaction with the receptor attached to the SPA bead (Left panel, below). This component of background signal can be determined in the presence of an excess concentration of competitor in the absence of the membrane receptor. Reduction of this factor can be accomplished through the careful use of buffering systems and the appropriate bead type. Determination of NSB to the SPA beads is separate from the NSB associated with membrane receptor preparations. A competition experiment using an unlabeled compound in the absence or presence of added receptor may assist in identifying nonspecific binding problems.

**Non-Proximity Effects (NPE)**
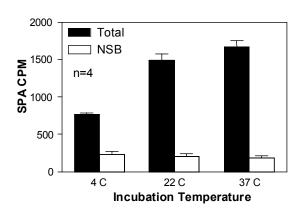
NPE occurs when either the concentration of the radioligand or the concentration of SPA beads is sufficiently high enough to elicit a signal from the emitted β-particles. This can occur even though the labeled ligand is not attached directly to the SPA bead through the interaction with the receptor or the nonspecific interaction with the bead (Right panel, below). In general, this signal is a linear function, directly proportional to the concentrations of each of these reagents. Therefore, a careful balance between radiolabel and SPA beads is crucial to maximize signal and sensitivity while minimizing NPE and ultimately cost. The only technique available to minimize NPE is adjustment of the SPA bead or radiolabel concentrations.



Non-specific binding to SPA Bead

Non-proximity effects

For routine SPA binding assays, nonspecific binding may be a combination of nonspecific binding to SPA beads as well as nonspecific binding to the receptor, and are expressed as one. Total nonspecific binding is measured in the presence of an excess concentration of unlabeled competitor.

### Temperature

Typically, receptor binding assays used in screening efforts are performed at room temperature. Comparison experiments may be required if other temperatures are considered. A kinetic analysis may be necessary as well. The data below depicts an SPA receptor binding assay performed at three temperatures.



**Note:** Since in nearly all cases, the microplate scintillation counter is at room temperature, and a 96-well plate requires approximately 16 minutes to read, it is difficult to perform SPA assays at temperatures other than room temperature. Data shown at the left was generated by incubation of a limited number of wells (n=4, different plates) at the indicated temperatures and counting them rapidly in the instrument. The information is useful in areas where there are significant variations in day-to-day laboratory temperatures

## G. ASSAY BUFFER

Identify appropriate starting buffer from literature sources or based on experience with similar receptors. Binding assays may require $CaCl_2$, $MgCl_2$, NaCl or other agents added to fully activate the receptor. pH is generally between 7.0 to 7.5. Commonly used buffers include TRIS or HEPES at 25 mM to 100 mM. Protease inhibitors may be required to prevent membrane degradation.

The following are possible factors that can be investigated in a statistically designed experiment to improve radioligand binding to membrane receptors, or reduce radioligand binding to SPA beads. The optimization of the assay buffer may be an iterative process in conjunction with the optimization of the assay conditions to achieve acceptable assay performance. Typical concentrations or concentration ranges for some reagents are listed in the tables below. Other reagents may be required depending on the individual receptor/ligand system.

Note that for most instances, the highest purity reagents should be tested. In some cases, such as with BSA, several forms (fatty acid free, fatty acid containing) may need to be investigated.

| Agents which Reduce NSB | |
|---|---|
| BSA | 0.05 % - 0.3 % |
| Ovalbumin | 0.05 % - 0.3 % |
| NP-40 | 0.05 % - 0.3 % |
| Triton X-100 | 0.05 % - 0.1 % |
| Gelatin | 0.05 % - 0.3 % |
| Polyethylenimine | 0.01% - 0.1 % |
| CHAPS | 0.5 % |
| Tween-20 | 0.05 % - 0.1 % |
| Fetal bovine serum (FBS) | up to 10% |

| Antioxidants/Reducing Agents | |
|---|---|
| Ascorbic Acid | 0.1 % |
| Pargyline | 10 $\mu$M |
| DTT | 1 mM |

| Reduce SPA Bead Settling Effects | |
|---|---|
| Glycerol | 10 - 20 % |
| Glucose | 10 mM |
| Polyethylene glycol (PEG) | 5 % - 10 % |

| Divalent Cations | |
|---|---|
| Magnesium ($Mg^{2+}$) | 1 mM 10 mM |
| Sodium Acetate | 10 mM - 50 mM |
| Calcium ($Ca^{2+}$) | 1 mM - 10 mM |
| Zinc ($Zn^{2+}$) | 10 $\mu$M - 50 $\mu$M |

| Other Buffer Additives | |
|---|---|
| NaCl | 100 mM - 150 mM |
| KCl | 5 mM - 80 mM |
| TRIS | 10 mM - 50 mM |
| HEPES | 5 mM - 100 mM |
| Phosphate Buffer | 20 mM |
| pH | 7.0 - 8.0 |
| Aprotinin | 500 units/ml |
| EDTA | 0.5 mM - 5 mM |

In addition to Aprotinin and EDTA, other protease inhibitors may be required for receptor stability.  As a starting point, Complete™ tablets from Roche Molecular Biochemicals are commonly used.

## H. ASSAY CONDITIONS
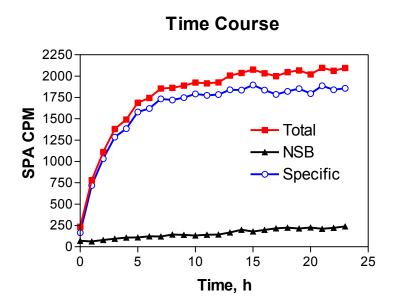
### Incubation Time - Signal Stability

*Setup:*    Measure total binding (receptor + radioligand + SPA beads) and nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) at various times using repetitive counting on the microplate scintillation counter.

*Results Analysis:*    Plot total, NSB and specific binding (total binding - NSB) versus time

Since steady state will require a longer time to reach at lower concentrations of radioligand, these experiments are usually performed at radioligand concentrations below the $K_d$ (i.e. 1/10 $K_d$) if signal strength permits.  In addition, the total concentration of radioligand bound should be equal to less than 10% of the concentration added to avoid ligand depletion.  The receptor concentration added must be lowered if this condition is not met.

This experiment is used to determine when a stable signal is achieved and how long a stable signal can be maintained. The signal is a combination of receptor/ligand reaching steady state and bead settling conditions. As SPA beads become packed at the bottom of the well, the efficiency of counting (particularly with $^{125}I$) increases. Therefore, it is important to determine when a uniform signal is obtained and adopt this time window as standard practice. In many assays 8-16 hours are required for stable signal counting. Use approximately 0.125-0.5 mg SPA beads depending on results from preliminary experiments.

An example of an incubation time course is shown below. A minimum of 10 hours incubation time was chosen in this example and the interaction was stable for at least 24 hours.
Failure to operate a receptor/ligand binding assay at steady state conditions may result in erroneous calculations for binding constants ($K_d$ or $K_i$).
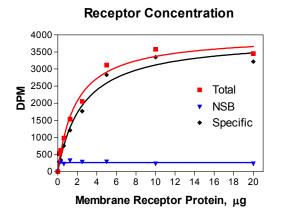


**Time Course**

**Receptor Concentration - Zone A**

*Setup:*    Measure total binding (receptor + radioligand + SPA beads) and nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) at various levels of added receptor (typical µg amounts vary depending on the source and purity of receptor).
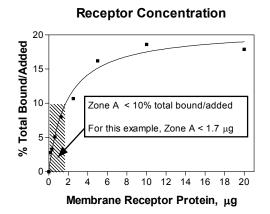
*Results Analysis:*  Plot total, NSB and specific binding (total - NSB) versus receptor amount. Plot total bound/total added expressed as a percent versus receptor concentration. Determine the level of receptor that yields <10% total binding/total added (Zone A).

It is ideal to keep the total amount of radioligand bound at less than 10% of the total amount added to avoid ligand depletion. This is considered the acceptable limit and is referred to as "Zone A". Saturation experiments must be performed at <10% total ligand binding at all concentrations tested

($0.1 \times K_d$ to $10 \times K_d$), so an initial protein variation experiment at a radioligand concentration that is $0.1 \times K_d$ is typically performed.

The example shown below uses radioligand at $< 0.1 \ K_d$ and an increasing amount of membrane receptor protein.  Two plots are shown:  (Left) raw SPA data for total, NSB and specific; (Right) total bound/total added expressed as a percent.  In this example, receptor levels less than 1.7 μg/well would meet Zone A requirements.
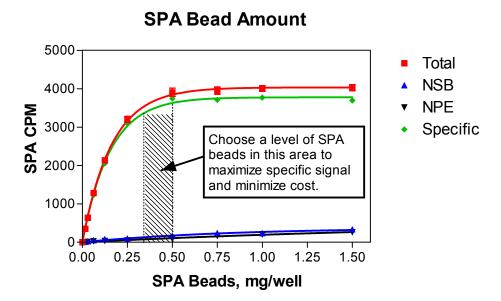
**Receptor Concentration**



**Receptor Concentration**



Zone A  < 10% total bound/added

For this example, Zone A < 1.7 μg

Total counts added (using liquid scintillation counting) are determined differently than the bound counts (SPA), therefore, in order to plot the % Total Bound/Added, the efficiency for each method must be taken into account, and any CPM data converted to DPM (described in APPENDIX).  You cannot compare the CPM data from one instrument/scintillation method to that of another.  The Section entitled "DPM Mode for SPA" demonstrates a representative method for determining efficiency for SPA bead counting.  DPM for liquid scintillation counting can be obtained from the instrument directly.  The stable signal count time must be determined prior to these experiments.  If the signal dips after a high concentration of receptor, then the SPA beads may be in limited amounts.

## SPA Bead Amount

*Setup:*    Measure total binding (receptor + radioligand + SPA beads) nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) and non-proximity effects (radioligand + SPA beads) at various SPA bead levels (typically 0.125 mg to 1.5 mg) using the determined optimum incubation time and optimum receptor concentration.

*Results Analysis:*  Plot total, NSB, NPE, and specific binding (total - NSB) versus SPA bead amount.  Choose a bead concentration beyond the linear range, at or near the initial saturation level on the specific binding curve.

## SPA Bead Amount



Non-proximity effects (NPE) can be determined in the absence of added receptor.  Ideally, the NPE signal would be identical to the nonspecific signal in the presence of unlabeled competitor.  A level of SPA beads at 0.35 mg - 0.5 mg would provide the best economical signal for this example.

**Solvent Interference**

*Setup:*   Measure total binding (receptor + radioligand + SPA beads) and nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) at various concentrations of DMSO (or other solvent) using the determined optimum incubation time, optimum receptor concentration and optimum SPA bead amount.

*Results Analysis:*   Plot total and NSB versus final assay concentration of DMSO

If the developed SPA receptor binding assay will be used to test organic compounds, interference with DMSO will need to be determined.  As shown in the example data below, there can be significant signal reduction if the DMSO concentration becomes too high.

### DMSO Interference



The level of DMSO in a SPA binding assay is determined by data in experiments such as the one in the example above and by the requirement set to maintain compound solubility.

Additional solvents (methanol, ethanol, etc.) or other agents (i.e. β-cyclohexadextrin) may need to be tested if compounds will be received in these other diluents.

Once determined, the solvent should be included in any further assay development or compound testing, including controls.

As an additional verification of minimal solvent interference, test competitive binding with a known competitor in the absence or presence of solvent at the determined level to be used in assays. Ideally, the test compound will have high affinity for the receptor and be freely soluble in aqueous buffer.  The $IC_{50}$ should not change in the absence or presence of the solvent.

# I. BINDING PARAMETERS

The determination of the equilibrium dissociation constant for the radioligand ($K_d$) or equilibrium dissociation constants for unlabeled compounds ($K_i$) should be performed after the SPA receptor binding assay has been fully optimized for the conditions outlined in the prior sections.

Three methods are described for the determination of the receptor affinity for the radioligand, $K_d$:
Saturation analysis
Homologous competition
Association rate at various radioligand concentrations

A heterologous competition binding assay is used to determine the affinity of the receptor for an unlabeled compound, $K_i$.

**<u>Saturation Binding</u>**

An equilibrium saturation binding experiment measures total and nonspecific binding at various radioligand concentrations. The equilibrium dissociation constant or affinity for the radioligand, $K_d$, and the maximal number of receptor binding sites, $B_{max}$, can be calculated from specific binding (total - NSB) using non-linear regression analysis.

Requirements:
* Steady state for low concentrations of radioligand (i.e. 1/10 estimated $K_d$) has been reached - perform association experiment to verify if necessary.
* Ensure that <10% of the added radioligand is bound (at all radioligand concentrations tested) to prevent ligand depletion - if this is not met, lower the receptor concentration

The range of radioligand concentrations tested in a saturation binding experiment is typically from 1/10 $K_d$ to 10x $K_d$ to yield an appropriate curve for nonlinear regression analysis methods. Radioligand specific activity, concentration or expense may prevent a wide range of concentrations from being used.

A high concentration of unlabeled compound (1000 x $K_i$ or $K_d$ value) is used to determine nonspecific binding. Ideally, the unlabeled compound should be structurally different than the radioligand. Nonspecific binding should be less than 50% of the total binding at the highest concentration of [L] tested.

Calculate $K_d$ for specific binding using a one-site binding hyperbola nonlinear regression analysis (i.e. GraphPad Prism) as shown in the equation below:
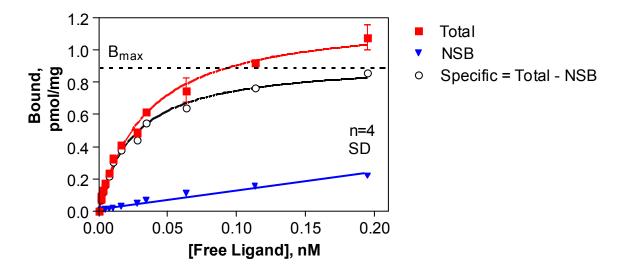
$$Bound = \frac{B_{max} \times [L]}{[L] + K_d}$$

$B_{max}$ is the maximal number of binding sites (pmol/mg), and $K_d$ (nM, pM, etc.) is the concentration of radioligand required to reach half-maximal binding.

***Setup:***   Measure total binding (receptor + radioligand + SPA beads) and nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) at various concentrations of radioligand using the determined optimum incubation time, optimum receptor concentration and optimum SPA bead amount.  Include the expected concentration of DMSO or other solvent for compound testing.  To assess non-proximity effects (NPE), a condition without receptor can be included (radioligand + SPA beads).

***Results Analysis:***   Plot total, NSB and specific binding (total - NSB) versus free concentration of radioligand.  Plot NPE if no receptor condition was performed.

A representative saturation binding experiment is shown below.  Y-axis data has been expressed in pmol/mg, using conversion methods shown in the Appendix.



A listing of the calculations required for analysis of saturation binding data is shown below.  Details for each of these calculations are shown in the Appendix.

a)  Determine total radioactivity added per well by counting an aliquot of each radioligand mix in a gamma counter or a liquid scintillation counter.  Convert to DPM if necessary using the equation below:

$$DPM = CPM/Efficiency$$

b)  Convert binding data (total bound, NSB) from CPM to DPM data using above equation.

c)  Calculate specific binding in DPM:  Specific bound = Total Bound - NSB

d)  Calculate unbound (free) DPM:  Free = Total Added - Total Bound

e)  Convert free DPM to concentration units (i.e. nM) using the radioligand specific activity (expressed as DPM/fmol) and the volume of sample used.

f) Convert Total bound, NSB and Specific bound DPM to pmol/mg units using specific activity expressed as DPM/fmol and the amount of receptor added per assay well in mg units.

g) Plot Bound (in pmol/mg) on Y-axis versus Free concentration of radioligand (in nM) on X-axis.

h) Determine $K_d$ and $B_{max}$ using a non-linear regression analysis for a single site binding (hyperbola).


## Considerations/Assumptions for Saturation Binding Experiments

The binding reaction must be at equilibrium for all concentrations of radioligand. Lower concentrations of radioligand require longer times to reach equilibrium.

Less than 10% of the total added radioligand should be bound at all concentrations of radioligand tested. At lower concentrations of radioligand, it is more likely that greater than 10% of the added radioligand will be bound (if this is the case, receptor concentration should be lowered).

If reagents and the assay system allow, radioactive concentrations of at least 10 times the $K_d$ should be tested to provide suitable data for a nonlinear regression analysis and accurate determination of the binding parameters. The $K_d$ and $B_{max}$ values can be calculated from less than complete data sets, but the statistical reliability of the returned values may be lower.

Ideally, nonspecific binding should be less than 50% of the total binding.

No positive or negative binding cooperativity

Binding is reversible and obeys the Law of Mass Action:


## Scatchard Plots

In the past, nonlinear saturation binding data was transformed into linear data followed by analysis using linear regression, resulting in a Scatchard (or Rosenthal) plot. Although perhaps useful for the display of data, the Scatchard plot is not used anymore for the determination of $K_d$ or $B_{max}$ values. These values are determined using nonlinear regression analysis as described above. Scatchard plots distort the experimental error (X value is used to calculate Y), hence the assumptions of linear regression are violated and the resulting values are not accurate.

**It is inappropriate to analyze transformed data for the determination of $K_d$ and $B_{max}$.**

**Homologous Competition**

A homologous competition is a concentration response curve with an unlabeled compound that is identical to the radioligand being used. Radioligand concentration is constant in the experiment. Homologous competition experiments can be used as an alternative to saturation experiments to determine receptor affinity ($K_d$) and density ($B_{max}$), provided the criteria shown below are met. When using [$^{125}$I]-ligands, a non-radioactive iodo-ligand should be used if possible.

Assumptions:
1) The receptor has identical affinity for the radioligand and unlabeled ligand.
2) There is no cooperativity.
3) No ligand depletion (<10% of the total added radioligand is bound)
4) Nonspecific binding is proportional to the concentration of labeled ligand.

The concentration-response curve should ideally descend from 90% specific binding to 10% specific binding over an 81-fold (or approximately 2 log scales) increase in concentration of the unlabeled ligand.

A homologous competition experiment has been designed correctly if the $IC_{50}$ is between 2 and 10 times the concentration of radioligand.

Two methods can be used to analyze data from a homologous competition experiment and determine the $K_d$ and $B_{max}$. They are described below as *Results Analysis 1* and *Results Analysis 2*. The experimental setup is identical for both types of analysis.

*Setup:*   Measure binding (receptor + radioligand + SPA beads) at various concentrations of unlabeled competitor using a single concentration of radioligand ($\leq K_d$) and the determined optimum incubation time, optimum receptor concentration and optimum SPA bead amount. In some cases ($^3$H-label with low specific activity), concentrations above the Kd may be required. Total binding is determined in the absence of any added competitor. Nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) is included for calculation of specific binding.
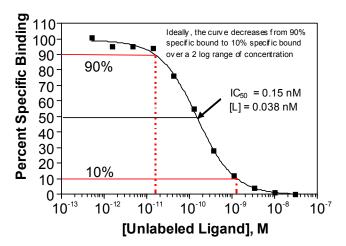
*Results Analysis 1:*   Plot specific bound (Bound - NSB) at each concentration of unlabeled competitor. Conversion to percent specific bound is performed using the following equation:

$$\% \text{ Specific Bound} = \frac{\text{Bound - NSB}}{\text{Total Bound - NSB}}$$

Step 1. Determine the $IC_{50}$ using a sigmoidal dose-response (variable slope), which is also known as a four-parameter logistic nonlinear regression analysis (i.e. using GraphPad Prism) as shown in the equation below (use log concentration values for proper analysis):

$$Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(Log\ EC_{50} - X) * Hill\ Slope)})}$$

Representative data for a homologous competition:



Step 2: Determine the $K_d$ and $B_{max}$ using nonlinear regression analysis (i.e. GraphPad Prism) for the following equation:

$$Y = \frac{B_{max}\ x\ [Hot]}{([Hot] + [Cold] + K_d)} + NSB$$

> [Hot] is the concentration of radioligand used in the assay (in nM)
> [Cold] is the concentration of competitor, which varies, (in nM)
>
> In GraphPad Prism, enter Y in CPM or DPM and X in log concentration of competitor.
>
> Calculate $K_d$ and $B_{max}$ with above curve fit analysis. Use instrument counting efficiency and specific activity of radioligand to convert the calculated maximum signal units (CPM or DPM) to pmol/mg units.

The calculated $IC_{50}$ for this homologous competition experiment is $1.5 \times 10^{-10}$ M.
The concentration of [L] used in this homologous competition is $3.8 \times 10^{-11}$ M.

The calculated $IC_{50}$ is between 2 and 10 times the concentration of added [L].

The concentration-response curve descends from 90% specific binding to 10% specific binding over an 86-fold increase in concentration of the unlabeled ligand.

Inputting X (log concentration) and Y (Total DPM) into the homologous binding analysis equation above yields (in parentheses are the values obtained from saturation binding analysis):

Log $K_d$ = -9.962 $\longrightarrow$ Antilog of -9.962 = $1.09 \times 10^{-10}$ M = 109 pM (79 pM)
$B_{max}$ = 27773 DPM $\longrightarrow$ Convert using specific activity, $B_{max}$ = 12.5 pmol/mg (5 pmol/mg)

*Results Analysis 2:*

**Alternatively**, convert specific DPM bound to molar units (i.e. nM) bound.

1) The molar concentration of labeled ligand ([L]) is calculated using the DPM added per well, the specific activity and the conversion factor, $1\ \mu Ci = 2.2\ x\ 10^6$ DPM.

   The formula is [L] nM = (specific counts) * (1/2200000) * (1 / Specific Activity) * 10000

2) This concentration is added to all concentrations of unlabeled ligand to determine the final added ligand concentration.

3) As the added ligand concentration increases (due to increase added unlabeled ligand), the specific activity of the labeled ligand is decreased.

4) For each specific DPM bound determine the specific molar units bound by using the corresponding specific activity in that condition.

   The formula is [RL] = (added ligand) * (specific DPM) / (DPM added per well).

5) Use a one-site binding (hyperbola) similar to the saturation binding data to calculate $K_d$ and Bmax.
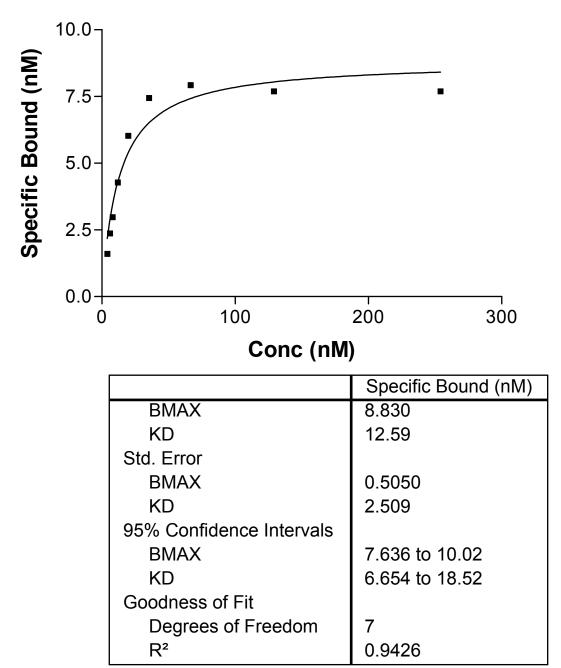
**Example:**
Labeled ligand specific activity is 90 Ci/mmol and 66398 dpm are added per well (100 μl final volume). The concentration of labeled ligand in all wells is 3.3 nM.
At unlabeled ligand concentration of 125 nM, the final added ligand (unlabeled + labeled) is 128.3 nM.

If the specific binding at 125 nM unlabeled ligand condition is 3283 DPM, then the specific molar unit bound would be (3283 x128.3)/66398 = 6.34 nM.

Representative results for homologous competition analyzed using the Results Analysis 2 method is shown on the following page.

**Homologous Competition for Determination of K$_d$**



| | Specific Bound (nM) |
|---|---|
| BMAX | 8.830 |
| KD | 12.59 |
| Std. Error | |
| BMAX | 0.5050 |
| KD | 2.509 |
| 95% Confidence Intervals | |
| BMAX | 7.636 to 10.02 |
| KD | 6.654 to 18.52 |
| Goodness of Fit | |
| Degrees of Freedom | 7 |
| R² | 0.9426 |

In this example data, the K$_d$ determined from a homologous competition experiment is 12.6 nM.

## Association Rate at Various Radioligand Concentrations (Optional)

An optional method, which can be used early in development for both determination of optimum incubation time and provide an estimate for the $K_d$, is to perform an association rate experiment at various radioligand concentrations.

***Setup:***   Measure total binding (receptor + radioligand + SPA beads) and nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) at various times and at various concentrations of added radioligand.

***Results Analysis:***   Plot specific binding (total binding - NSB) versus time at each radioligand concentration tested.

Calculate the observed association rate constant ($k_{obs}$) by fitting the signal versus time data to a one-phase exponential association nonlinear regression analysis for each concentration of radioligand tested.  The $k_{obs}$ value is returned as one of the resulting curve fit parameters.  There will be different $k_{obs}$ for each radioligand concentration.
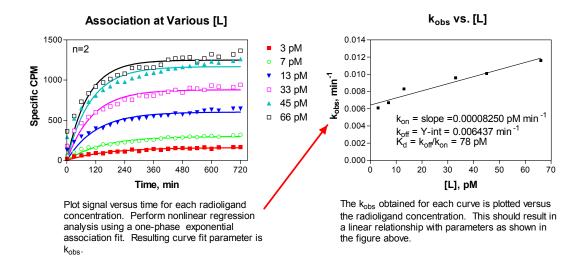
Plot the observed association rate constant ($k_{obs}$) versus concentration of [L].

This should result in a linear function with a slope equal to the association <u>rate</u> constant ($k_{on}$) and the Y-intercept equal to the dissociation <u>rate</u> constant ($k_{off}$).  An estimate for the equilibrium dissociation constant ($K_d$) can be calculated using the equation below with the kinetically determined rate constants:

$$K_d = k_{off}/k_{on}$$

***Example Data:***
***SPA Method: Reaction mix was read at different time points***



Plot signal versus time for each radioligand concentration.  Perform nonlinear regression analysis using a one-phase  exponential association fit.  Resulting curve fit parameter is $k_{obs}$.

The $k_{obs}$ obtained for each curve is plotted versus the radioligand concentration.  This should result in a linear relationship with parameters as shown in the figure above.

The $K_d$ calculated from **<u>saturation binding</u>** for this receptor was 35 pM.

**Heterologous Competition**

Experimentally, a heterologous competition is identical to a homologous competition. Heterologous competition assays measure concentration-response binding with unlabeled ligands that are structurally different than the radioligand. The $IC_{50}$ for the unlabeled compound is determined from the experimental data and the equilibrium dissociation constant, $K_i$, can be calculated using a mathematical formula (Cheng-Prusoff equation).

*Setup:*    Measure binding (receptor + radioligand + SPA beads) at various concentrations of unlabeled competitor using a single concentration of radioligand ($\leq K_d$) and the determined optimum incubation time, optimum receptor concentration and optimum SPA bead amount. Total binding is determined in the absence of any added competitor. Nonspecific binding (receptor + radioligand + excess unlabeled competitor + SPA beads) is included for calculation of specific binding.

*Results Analysis:*    Plot specific bound (Bound - NSB) at each concentration of unlabeled competitor. Conversion to percent specific bound is performed using the

following equation:    $\% \text{ Specific Bound} = \dfrac{\text{Bound - NSB}}{\text{Total Bound - NSB}} \times 100$

Determine $IC_{50}$ using a sigmoidal dose-response (variable slope), which is also known as a four-parameter logistic nonlinear regression analysis (i.e. using GraphPad Prism) as shown in the equation below:

$$Y = \text{Bottom} + \frac{(\text{Top - Bottom})}{(1 + 10^{(\text{Log EC50 - X}) * \text{Hill Slope}})}$$

where Y is the specific binding and X is the log concentration of competitor.

Calculate the equilibrium dissociation constant for the unlabeled compound ($K_i$) using the Cheng-Prusoff equation (valid when Hill Slope is near unity):

$K_i = IC_{50}/[1 + ([L]/K_d)]$

where
$K_i$ is the equilibrium dissociation constant for the unlabeled compound
$IC_{50}$ is the concentration causing 50% inhibition of binding
[L] is the concentration of radioligand
$K_d$ is the equilibrium dissociation constant for the radioligand
Further calculation details on the Cheng-Prusoff equation can be found in the Appendix.

A representative heterologous competition curve is similar to the one shown in the homologous competition section.

Several assumptions, based on specific criteria, are made to allow calculations using the Cheng-Prusoff equation to be reliable:
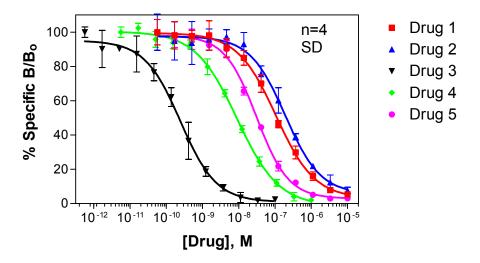
1) Law of Mass Action applied (10-90% of displacement occurs over 81-fold concentration range)
2) A single class of receptor binding sites
3) No ligand depletion
4) Receptor concentration $< K_d$
5) Assay is at equilibrium or steady state
6) The concentration of the added inhibitor is equal to the free concentration of the inhibitor

For special cases associated with high affinity compounds, where ligand depletion must be accounted for, see page 37 of this section.

## Pharmacological Profile

A pharmacological profile is a heterologous competition testing several unlabeled compounds simultaneously. The $K_i$ for each compound can be computed and compared to each other. A rank affinity can also be calculated. The data below demonstrates a typical pharmacological profile with representative $IC_{50}$, $K_i$ and rank affinity data shown in the table.

Notice that different concentration ranges may be required for each drug to fully define top and bottom portions of the curves

The $IC_{50}$ is determined from experimental data, the $K_i$ is calculated using the Cheng-Prusoff equation and the Relative Affinity is relative to a particular compound of interest (Drug 1 in this example.

| **Drug** | **$IC_{50}$, nM** | **$K_i$, nM** | **Relative Affinity** |
|---|---|---|---|
| 1 | 106 | 53 | 1.00 |
| 2 | 190 | 95 | 0.56 |
| 3 | 0.25 | 0.13 | 424 |
| 4 | 8.9 | 4.5 | 11.9 |
| 5 | 30.9 | 15.5 | 3.4 |

[L] = 0.025
$K_d$ = 0.025

Relative Affinity = IC50 for Drug 1/IC50 for Drug

A typical plate setup for competitive binding is shown in the ***Plate Layout*** section of the **Appendix**. A control compound is tested on each plate and can be used for determination of the relative affinity. This process aids in analyzing the statistical significance of differences between the individual compounds.

**Guidelines for Nonlinear Regression Curve Fitting**
See section XI for further information regarding guidelines.

Nonlinear regression analysis should be either:
        4 parameter logistic fit
        3 parameter logistic fit (Top constant = 100)
        3 parameter logistic fit (Bottom constant = 0)
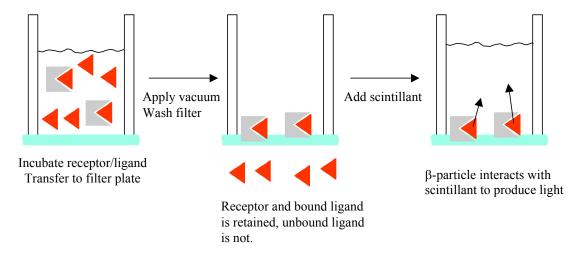
The slope parameter should be fixed nor should both top and bottom be fixed at the same time. Consult with a statistician for questions.

## J.  FILTRATION ASSAYS

**Concept**

Filter assays differ from SPA because a separation of free radioligand and radioligand bound to the receptor is required for measurement.  However, many of the assay development and optimization steps are the same.  Specific information to the filter assay format is included in this section, and reference back to the text under the SPA section is made when there is no significant difference between the two formats.  A diagram for a standard filtration assay is shown below.



General Steps for a filtration assay:

1.  Add and incubate test compound, radioligand and receptor in a plate (this can be a separate plate or if validated, the filtration plate directly)

2.  Apply vacuum to "trap" receptor and bound radioligand onto filter and remove unbound radioligand.  Wash several times with an appropriate buffer to minimize nonspecific binding.

3.  Allow filters to dry.  Add liquid scintillation cocktail or other scintillant (i.e. solid Meltilux).

4.  Count filters in microplate scintillation counter.  Some time between adding the scintillant and counting may be required.

| Advantages | Disadvantages |
|---|---|
| Less color quenching | Separation method (dissociation of ligand) |
| Traditional, trusted method | Generates large volumes of liquid waste |
| Higher efficiency than SPA | Variable vacuum across plate |
| Kinetic experiments easier | Nonspecific binding to filters |
| Association/Dissociation | Accumulation of radioactivity on unit |
| | Requires more handling steps |

## K. FILTER ASSAY FORMAT

**Filter Type**

The most commonly used filters for receptor binding are listed below:

**GF/B** - glass fiber filters with 1.0 µM pore size

**GF/C** - glass fiber filters with 1.2 µM pore size

**Durapore** - PVDF filters with various pore sizes such as 0.22, 0.65, 1.0 µM.

Depending on the radioligand, receptor and other assay factors, it may be necessary to perform experiments with more than one type of filter to determine the best one for the system under investigation.

Solid white, opaque plates are used to minimize cross-talk in the counting instrument.

The plate type being used should match the filtering apparatus:

MAP Titertek:  Millipore Multiscreen filters
Millipore vacuum manifold:  Millipore Multiscreen filters
Brandel M96 Harvester:  Several harvester-type plates acceptable
Packard Filtermate 196:  Unifilter type plates
TomTec Harvester:  Filter mats

| Plate Type | Harvester Instrument | Counting Instrument | Comments |
|---|---|---|---|
| Unifilter GF/C or GF/B | Packard or Brandel | Trilux or TopCount | Filter from an assay plate to the filter plate with washing of the assay plate possible |
| Multiscreen-FC or Multiscreen-FB | MAP or individual manifold | Trilux or TopCount | Removable bottom plastic piece.  Requires solid white adapter for TopCount or clear plastic liner and cassette for Trilux |
| Multiscreen-GV | MAP or individual manifold | Trilux or TopCount | 0.22 mM Durapore membrane.  Removable bottom plastic piece.  Requires solid white adapter for TopCount or clear plastic liner and cassette for Trilux |

The speed of separation is important, particularly for lower affinity interactions (<1 nM), and can be influenced by the filter plate type.  Dissociation of bound radioligand from a receptor interaction with an affinity of 1 nM can occur in as little as 1.7 min.  Lower affinity interactions can dissociate even quicker, when the separation process disrupts equilibrium.

## Order of Addition

The order of addition for reagents may affect assay performance as well as ease of automation.  A standard format for order of reagent addition in a filtration method is as follows:

1. Test compound
2. Radioligand
3. Receptor

Experiments may be required to determine the optimum order of addition and if there is any effect by locally high concentrations of DMSO present during the initial additions into the wells.

## Non-Specific Binding

Radioligands may bind nonspecifically to components of the assay system such as tubes, pipette tips, assay plates or filters.  This may lead to ligand depletion and certain binding assumptions may not be met.  To test for nonspecific binding, perform an experiment in the absence of membranes.  The amount of activity added can be tracked at each step of the assay to determine where any losses or nonspecific binding is occurring.

Some potential solutions to minimize nonspecific binding include the following:

- Pretreatment of tubes (siliconization)
- Additions to assay buffers (See table of Agents which Reduce NSB in the *Assay Buffer* section)
- Different filter plate manufacturers (Packard, Millipore, Brandel, Polyfiltronics, etc.)
- Different filter plate types (GF/C, GF/B, Durapore, etc.)

Since there may be non-receptor binding (to system components as described above), the use of an unlabeled ligand at a 100-fold excess may not be adequate to fully define all of the nonspecific binding.

## Temperature

See SPA section on *Temperature*.

The filtration format can accommodate temperatures other than room temperature easier than the SPA format.  The receptor/ligand/compound can be incubated at the desired temperature and then filtered to capture bound radioactivity.  Since the filtration process is rapid, there is not a significant temperature drop during that time.  Once the scintillant is added, the filter plates can be counted in the microplate scintillation counter at room temperature.

**Plate Treatment Conditions**

Filter plates are usually pre-wetted to ensure even distribution of the receptor/ligand reagents. If there is no ligand sticking problems, the pre-wet can be accomplished with Wash Buffer.

Pretreatment of filters with polyethylinimine (PEI) is a common practice to minimize ligand binding to filters:
1. Presoak 30 to 60 min in 0.1% to 0.5% PEI (in water)
2. Treat at $4^{o}$C to minimize filter degradation
3. Filter away PEI, then wash with ice-cold buffer prior to filtration of receptor sample

Pretreatment with carrier proteins, serum, or detergents has also been used to minimize binding of ligands to filter plates.

**Note of caution**: Millipore Multiscreen glass fiber filter plates have a 0.65 mm Durapore support membrane under the GF filter. Some treatments (including PEI) may change or compromise the stability of this support membrane. Appropriate experiments should be designed to test for stability when using these types of plates.


**Vacuum Pressure**

The vacuum pressure used for filtration binding assays is a balance between having enough pressure to filter the samples rapidly and prevent ligand dissociation and having too much pressure which can affect filter integrity or the level of membranes retained on the filter. The pressure to be used should be determined experimentally, with a starting guideline of 5 to 10 mm Hg. If necessary, install an appropriate regulator to control consistent vacuum pressure.


**Wash Buffer**

Several washes of the filters are required to remove as much unbound radioligand as possible and to maximize specific binding. Generally, an ice-cold buffer is used to prevent or reduce dissociation of bound radioligand from the receptor.


**Filter Plate Drying Time**

If filters are not completely dry prior to the addition of liquid scintillant, the residual water present in the filters can interact with the scintillant to reduce counting efficiency. Dry filters require less liquid scintillant to achieve maximum signal than wetted filters. Drying filters completely may not be practical for medium or high throughput screening applications.

## Type and Volume of Scintillant

The type of microplate scintillation counter being used may dictate the type of scintillant required for proper counting conditions.

TopCount - must use a "slow" scintillator such as Microscint-20 or Microscint-40

Trilux - can use virtually any scintillant designed for microplate scintillation counting (Microscint-20/Microscint-40, Optiphase Supermix, Meltilux)

Regular liquid scintillation cocktail such as Ready Pro should not be used, as a rule, for microplate scintillation counting as their load capacities may not be adequate and they may not be compatible with microplate plastics.

General volumes of liquid scintillant used are in the range of 40 to 150 μl.  As mentioned above, the volume of scintillant used may depend on the dryness of the filters.

## Exposure Time to Scintillant

With filter plates, some of the radioligand may be embedded within the filter and require some time to become accessible to the liquid scintillant for photon generation and signal detection.  Therefore, an incubation time may be required following the addition of liquid scintillant and prior to counting. In addition to increasing the maximum signal, the variability of the signal may be reduced following an incubation time as demonstrated in the figure on the following page.

When processing large numbers of plates, it is important that a stable counting signal has been reached, so that all plates from the first counted to the last counted, are comparable.

Over time, more radioactive particles will be removed from the filter and make contact with the liquid scintillant in the well.  As the data to the left shows, this can improve signal strength and decrease variability.

**Exposure Time to Scintillant**

## L.  ASSAY BUFFER

See the *Assay Buffer* section in the SPA part of this document.

Many of the buffer additives and reagents described for the SPA format are relevant for receptor binding assays in a filtration format.

## M. ASSAY CONDITIONS

### Incubation Time - Signal Stability

*Setup:*        Measure total binding (receptor + radioligand) and nonspecific binding (receptor + radioligand + excess unlabeled competitor) at various times.
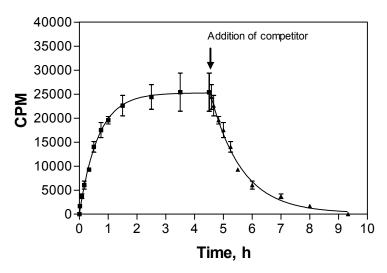
At least two methods could be used to obtain the association/dissociation data.  Both methods should yield the same result.  If not, there may be a problem with receptor stability.

Method 1:  Add and mix together enough receptor and radioligand for all time points in the experiment.  At various times, filter an aliquot of the receptor/radioligand mixture and wash the filters with Wash Buffer.  The last aliquots to be filtered will be the longest incubation time points.

Method 2:  Prepare receptor and radioligand separately.  At various time points, combine the two in the microplate.  After all points have been added, filter the reactions at the same time. The last wells to be mixed will be the shortest incubation times.

Dissociation, which can be measured more conveniently using the filtration format than SPA, is performed by adding an excess amount of unlabeled competitor after a receptor/radioligand mixture has reached steady state (plateau on the association curve).  The figure below demonstrates an association/dissociation experiment (total binding only shown).

## Association/Dissociation



**Results Analysis:**    Plot specific binding (total binding - nonspecific binding) versus time. Fit the association data to a one-phase exponential association curve and the dissociation data to a one-phase exponential decay curve. In the example above, a minimum reaction time of 2.5 hours would be adequate.

In addition to determination of the appropriate primary incubation time for steady state, a kinetic estimate for the equilibrium dissociation constant, $K_d$, can be made from the results of an association/dissociation experiment.

Association Experiment:
    Obtain $k_{obs}$, expressed in min$^{-1}$, from the nonlinear regression analysis of data

Dissociation Experiment:
    Obtain $k_{off}$, expressed in min$^{-1}$, from the nonlinear regression analysis of data

Calculate association rate constant, $k_{on}$ (in Molar$^{-1}$ min$^{-1}$)

$$kon = \frac{kobs - koff}{[radioligand]}$$

Calculate equilibrium dissociation constant, $K_d$ (in Molar):

$$K_d = k_{off}/k_{on}$$

## Receptor Concentration - Zone A

*Setup:*   Measure total binding (receptor + radioligand) and nonspecific binding (receptor + radioligand + excess unlabeled competitor) at various levels of added receptor.

*Results Analysis:*  Plot total, NSB and specific binding (total - NSB) versus receptor amount.  Plot total bound/total added expressed as a percent versus receptor concentration.  Determine the level of receptor that yields <10% total binding/total added (Zone A).

See the *Receptor Concentration - Zone A* section in the SPA part of this document for further details and an example.

## Solvent Tolerance

*Setup:*   Measure total binding (receptor + radioligand) and nonspecific binding (receptor + radioligand + excess unlabeled competitor) at various concentrations of DMSO (or other solvent) using the determined optimum incubation time and optimum receptor concentration.

*Results Analysis:* Plot total and NSB versus final concentration of solvent

See the *Solvent Tolerance* section in the SPA part of this document for further details and an example.

# N. BINDING PARAMETERS

## Saturation Binding

See the *Saturation Binding* section in the SPA part of this document for further details and an example.

## Homologous Competition

See the *Homologous Competition* section in the SPA part of this document for further details and an example.

**Association Rate at Various Radioligand Concentrations (Optional)**

See the *Association Rate at Various Radioligand Concentrations (Optional)* section in the SPA part of this document for further details and an example.

**Heterologous Competition**

See the *Heterologous Competition* section in the SPA part of this document for further details and an example.

**Pharmacological Profile**

See the *Pharmacological Profile* section in the SPA part of this document for further details and an example.

# O. SPECIAL CIRCUMSTANCES

**High Affinity Competitors**

For high affinity competitors, the assumption related to inhibitor depletion may not be met and an alternative analysis method can be used.

When the assay is designed properly, ligand depletion should not be a problem. However, once competitors reach an activity 2 to 3 fold lower than the ligand, inhibitor depletion can be an issue. Assuming that the hill slope for these compounds is near 1, the $K_i$ computed using the Cheng-Prusoff equation could be compared to the $K_i$ found by fitting the tightly bound inhibitor model below.

$$a = K_d\left(1 + \frac{K_d}{[L]}\right)$$
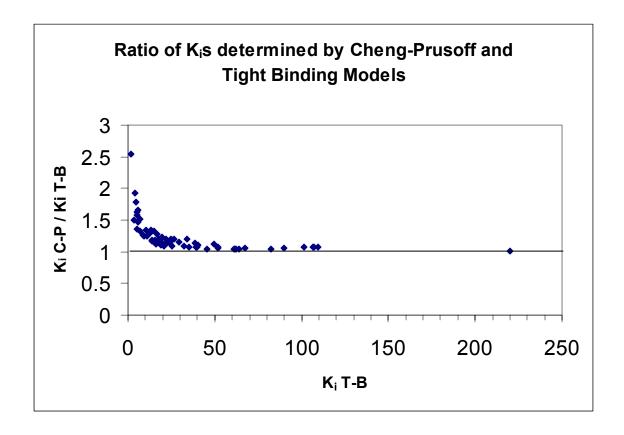
$$b = \left([I]_t K_d + K_i[L]\left(1 + \frac{K_d}{[L]}\right) - K_d[R]_t\right)$$

$$c = -[R]_t[L]K_i$$

$$[RL] = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

The ligand concentration [L] and the $K_d$ are exactly those that would be used in the Cheng-Prusoff equation.  The inhibitor concentration, $[I]_t$, is the concentration tested.  The $K_i$ and receptor concentration $[R]_t$ are obtained by fitting the model.  In order to use this model, the response determined by the plate reader, which measure the amount of receptor ligand complex [RL], must be converted to the same concentration units that are used for the ligand [L] and inhibitor $[I]_t$.  This requires the specific activity of the label and a plate reader that is calibrated well.

Even though the T-B model looks much more complex than the sigmoid curve model or the one site competition model in GraphPad Prism, both the fitted curve and the $K_i$ are virtually identical unless a substantial portion of the inhibitor is bound.  This can be seen in the graph of the radioligand binding results from an assay with $K_d \approx 100$ and ligand concentration of 4 nM that is shown below.  The ratio of the $K_i$ determined by Cheng-Prusoff to the $K_i$ determined using the T-B model is plotted against the $K_i$ determined by the T-B model.  Inhibitor depletion will always result in understating the true potency of the molecule.  Hence, the ratios are always greater than one.  Also, the $K_i$ values are virtually identical unless the $K_i$ is much lower than the $K_d$.

**Hill Slope Deviations**

A standard competitive binding curve that follows the law of mass action will descend from 90% specific binding to 10% specific binding over an 81-fold range of unlabeled drug concentrations. The steepness of the competition curve is given by a slope factor, called the Hill Slope. This parameter is determined from a nonlinear regression analysis of the competition data when using a four-parameter logistic equation. A standard competition curve that meets all assumptions would have a Hill Slope of -1.0. If the slope factor deviates from 1.0 significantly, then the binding may not follow the law of mass action and you may be dealing with a receptor with more than a single class of binding sites, solubility issues or an assay artifact.
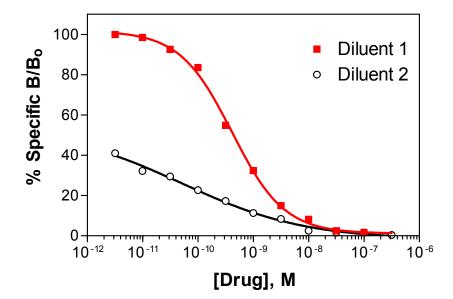
There is no adequate way to interpret the absolute value of the Hill Slope. However, there are several possible explanations when a competition curve has a calculated Hill Slope that is significantly less than 1 (shallow curve):

1. Experimental problems such as improper serial dilution of the compound

2. Curve fitting problems due to undefined top and bottom plateaus or too few data points

3. Negative cooperativity - binding on one ligand molecule reduces affinity of other binding sites

4. Heterogeneous receptors - different populations of receptors with different affinities

5. Assay variability

Although the Hill Slope for a compound may not be -1.0, repetitive determinations for the same compound should yield similar Hill Slopes each time. If this is not the case, further optimization of the receptor binding assay may be required.

Some compounds being tested may not be soluble in the standard solvent, DMSO. In addition, compounds at high concentrations may not be soluble. Both of these cases can affect the shape of competition curves (i.e. Hill Slope, top or bottom plateau, etc.) and the calculated parameters. Therefore, it is important to review each competition curve for the following features:
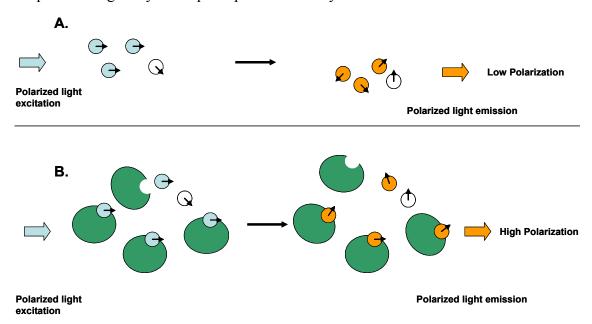
Specific binding descends from 90% to 10% over an 81-fold concentration range
The Hill Slope is at or near -1.00
Top and bottom plateaus have been appropriately defined
Data points are evenly spaced along the entire range of concentrations tested

The example below demonstrates a compound tested in Diluent 1 and Diluent 2. In Diluent 2, the compound appears to have limited solubility and exhibits a very shallow Hill Slope and poorly defined top and bottom plateaus. In Diluent 1, the compound competes with the radioligand in the expected manner.
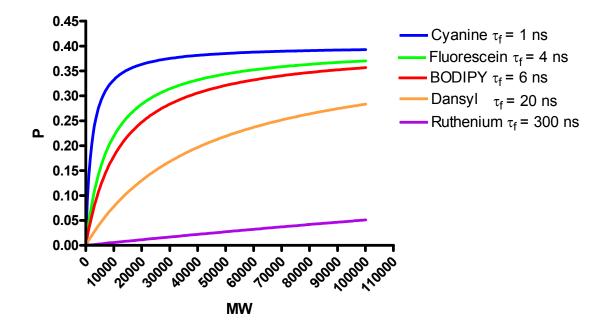
## P.  PRACTICAL USE OF FLUORESCENCE POLARIZATION IN COMPETITIVE RECEPTOR BINDING ASSAYS

### Principles of Fluorescence Polarization

Fluorescence polarization (FP) measurements have become a popular assay format for receptor binding assays. The principle of this assay is illustrated below.

**A.**

Polarized light excitation

Low Polarization

Polarized light emission

**B.**

Polarized light excitation

High Polarization

Polarized light emission

A fluorophore whose absorption vector is aligned with polarized excitation light is selectively excited. If the fluorophore tumbles rapidly relative to its fluorescent lifetime then it will be randomly orientated prior to light emission and therefore will show a low polarization value (situation A above). However, if this fluorophore's rotation is slowed down so that it tumbles slowly with respect to the fluorescent lifetime (*e.g.* by binding to a large receptor as shown in B above) it will not rotate much before light emission and will show a high polarization value. The dependence of polarization on fluorescent life-time is shown below.

[The graph above contains simulated data using the Perrin equation (Cantor and Schimmel, 1980) and taking the limiting polarization as 0.5 using T = 293 K and assuming a spherical protein in water with the fluorescence probe rigidly attached]

Typical fluorophores include fluorescein- or BODIPY-labels that have fluorescence lifetimes allowing FP measurements to be made between a small labeled-ligand (<1500 Da) and a large receptor (*e.g.* > 10,000 Da).

The increase in polarization can be measured with several microplate readers where the fluorescence is measured using polarized excitation and emission filters. Two measurements are performed on every well. Data is obtained for the fluorescence perpendicular to the excitation plane (the "P-channel") and fluorescence that is parallel to the excitation plane (the "S-channel"). For screening applications, the millipolarization units (mP) are often calculated using:

$$mP = \left[ \frac{(S - P*G)}{(S + P*G)} \right] x \ 1000$$

The proper use of S and P channel data requires two corrections. First, accurate calculation of polarization using fluorescent readers requires calculation of the instrument "G-factor". This factor corrects for any bias toward the P channel. For microplate readers, a 1
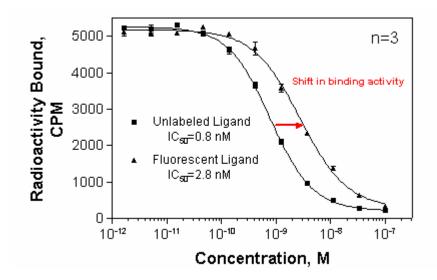
nM fluorescein solution is typically used and the G-factor that yields a value of 27 mP is entered (27 mP is the known value for a 1 nM fluorescein solution at R.T). Secondly, the S and P values should have the background fluorescence subtracted (determined using assay buffer without labeled-ligand in the well).

## Fluorescence Polarization and Receptor Binding

Receptor-binding FP assays use a small molecule labeled ligand (so called tracer) and a large unlabeled receptor. An example is a fluorescently labeled-steroidal ligand binding to a nuclear receptor-ligand binding domain (kits of this type are sold by Invitrogen/PanVera). This type of assay typically yields a minimum signal of approximately 50 mP for the unbound tracer and a maximum signal of approximately 300 mP when the tracer is fully bound to the receptor.
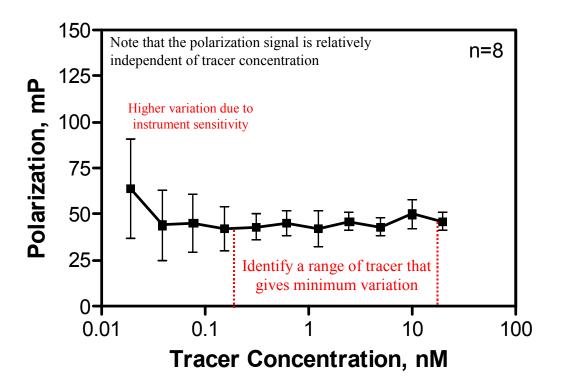
## Validate Activity of Fluorescent Tracer

The receptor binding activity of a fluorescent-labeled tracer can be determined in a competition assay using a radiolabeled ligand and traditional methods of receptor binding (filtration, SPA, charcoal precipitation, etc.). As shown in the figure below, some loss of receptor binding activity may occur following fluorescent tagging. It is important to identify lower binding activity prior to further experiments with the fluorescent tracer. Functional receptor assays, such as cAMP measurement, calcium mobilization or GTP$\gamma$S binding, can also be performed to determine if there has been a loss in biological activity as a result of the labeling process.
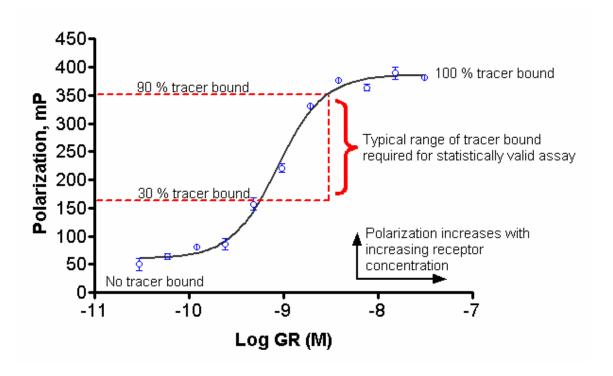
**Choosing Tracer and Receptor Concentrations**

The $K_d$ of the tracer and the amount of tracer bound under the chosen assay conditions will be required for analysis of competitive binding parameters. Typically, the $K_d$ can be estimated using radioligand-binding techniques (SPA, filtration) discussed in previous sections, provided there is not significant deviation in the potency of the tracer and the unlabeled molecule (see figure above). It may be useful to perform a tracer calibration curve by varying the amount of tracer and ensuring that the polarization signal is constant over a reasonable concentration range, inclusive of the estimated $K_d$. By definition, the polarization signal is independent of the intensity of the tracer. This also identifies the variability at the tracer concentration to be used. The polarization signal as a function of tracer concentration is shown for a representative tracer in the figure below. Note that as the signal nears the limits of sensitivity for the detector, the variation increases.

The amount of bound tracer can be measured in an experiment where the tracer is held at a constant concentration near its $K_d$ and the receptor concentration is then varied. An example of this type of experiment using the glucocorticoid receptor (GR) included in the FP kit available from Invitrogen/PanVera is shown below. Here the ligand-binding domain of GR is varied using a constant $K_d$ concentration of a labeled-steroidal ligand (Fluormone™, Invitrogen/Panvera kits; Data provided by Pharmacopeia).
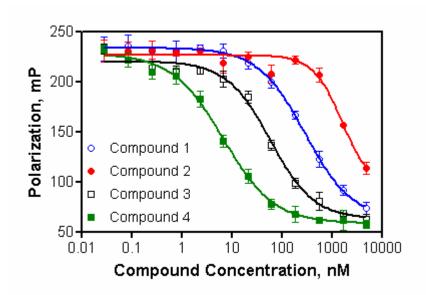


In these types of FP experiments no correction for nonspecific binding (NSB) is performed as was shown in earlier sections for radioligand-binding experiments. This is because the tracer (what is the radioactive ligand concentration in traditional assays) is held constant at a concentration usually near the $K_d$ and the protein receptor concentration is then varied over several orders of magnitude. However, this assumption should be checked by observing the polarization of the ligand in the absence of receptor. (Caution: it is possible to observe increasing FP signals when membrane receptors are used due to light scattering. In those cases, a correction may need to be made by measuring the signal in the presence and absence of the fluorescent tracer). If binding to non-specific buffer components or microtiter plates surfaces is observed then this tracer should be avoided. An analytical treatment of FP

competitive-binding data has recently been presented by Roehrl *et al*. (2004) that allows one to quantify the effect of non-specific binding on FP titration curves.

Examination of the curve above allows one to choose a receptor concentration that yields an acceptable assay window (typically a ΔmP of between 150 mP and 300 mP).

**Pharmacological Profile**

Sensitivity to known competitors should be checked at this stage to ensure that the developed FP assay is adequate for the intended purpose. An example pharmacological profile using fluorescence polarization is shown below.



**Ligand Depletion**

The FP assay format is homogenous in nature and therefore lends itself to simple "mix and read" protocols. However, to obtain an acceptable signal, the assay must be set-up with a large fraction of the tracer bound to the receptor (typically >80 %). The high amount of bound tracer requires a specific set of equations to be used when interpreting FP derived competition binding results.

In these cases, where a large amount of bound tracer exist, the Cheng-Prusoff equation as mentioned in the discussion of heterologous competition-receptor binding (see p.

27) will always lead to an overestimation of the Ki from the $IC_{50}$. This is because the Cheng-Pursoff equation is strictly given as:

$$K_i = \frac{IC_{50}}{1 + L_f/K_d} \qquad \{Eq.\ 1\}$$

In the case of FP displacement-binding, the free ligand term $[L_f]$ can not be substituted for the total ligand concentration $[L]$ because there is little free ligand available. This differs from the typical saturation-binding experiments mentioned in previous sections.

Three equations have been presented in the literature to provide a solution to this situation for simple competitive-binding. Munson and Rodbard (Munson and Rodbard, 1988) provide a correction that takes into account the amount of bound tracer. This takes the form of:

$$K_i = \frac{IC_{50}}{1 + L_o(y_o + 2)/[2K_d(y_o + 1)] + y_o} - K_d[y_o/(y_o + 2)] \qquad \{Eq.\ 2\}$$

Where $y_o$ is the bound/free ratio of tracer and Lo is the total tracer concentration.

Huang provides an alternative form of this correction in terms of the fraction of bound tracer (Huang, 2003). Rearrangement of Equation 15 given in Huang to solve for Ki yields:

$$K_i = \frac{IC_{50}}{1/(1-F_o) + L_o(2-F_o)/2K_d} - K_d[F_o/(2-F_o)] \qquad \{Eq.\ 3\}$$

Where $F_o$ is the fraction of tracer bound and $L_o$ is the total tracer concentration. Huang's result is redundant with the earlier Munson and Rodbard equation except for expressing the equation in terms of the fraction of tracer bound.  Therefore, Eq. 2 and Eq 3 yield the same correction (see below).

The final correction often used in this situation is the one derived by Kenakin (1993). Here the equation is expressed in terms of total receptor concentration ($R_o$), the total tracer concentration ($L_o$ as above) and the bound tracer concentration ($L_b$).

$$K_i = \frac{(L_b)\,(IC_{50})(K_d)}{(L_o)(R_o) + L_b(-R_o - L_o + L_b - K_d)} \qquad \{Eq.\ 4\}$$

These equations should be used instead of Cheng-Pursoff when > 10% of the tracer is bound to the receptor in the assay.

Application of Ligand Depletion Equations

Once a suitable choice of receptor and tracer concentrations have been made and the resulting assay has been shown to be useful for competitive binding analysis, one can calculate the amount of bound tracer under the assay conditions taking the lower and upper asymptotes as values for free and bound tracer respectively.

Some example competition-binding data (Fluormone™ kit, Invitrogen/Panvera) are shown in Table I to illustrate the differences between using the Cheng-Pursoff equation without correction for the amount of bound tracer or each of the above equations which correct for tracer depletion. For these competition-binding experimental results the conditions were:

Equilibrium dissociation constant, $K_d$ = 0.6 nM (Fluormone™ ligand), determined using saturation binding analysis.

Bound Tracer Concentration, $L_b$ = 0.9 nM, determined from receptor concentration experiment at constant tracer ($L_o$), by reading the mP signal and determining the % of maximum

Total Tracer Concentration, $L_o$ = 1 nM, concentration set near the $K_d$ value

Total Receptor Concentration, $R_o$ = 4 nM (GR ligand-binding domain), determined from receptor concentration experiment at constant tracer – yields statistically valid assay with robust signal

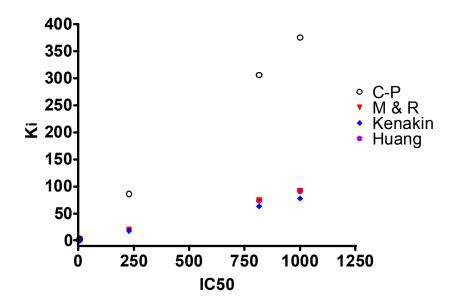These concentrations yield the following terms required for Equations 2-4:

Bound/Free ratio of Tracer, $y_o$ = $L_b/(L_o - L_b)$ = 0.9/(1-0.9) = 9

Fraction of Tracer Bound, $F_o$ = $L_b/L_o$ = 0.9/1 = 0.9

**Table I**.  **Comparison of $K_i$ values determined from ligand depletion correction formulas**.  Values are in nM.  $IC_{50}$ shown is the measured $IC_{50}$ under the assay conditions described in the text.  All other values are calculated values.  Data provided by Pharmacopeia.

| Ligand | $IC_{50}$ | (1) Cheng-Pursoff | Munson-Rodbard | Huang | Kenakin |
|---|---|---|---|---|---|
| | | | Ki, nM | | |
| Cortisone | 8.0 | 3.0 | 0.24 | 0.24 | **0.6** |
| Dexamethasone | 3.6 | 1.3 | -0.16 | -0.16 | **0.3** |
| Estradiol | 815 | 306 | 74 | 74 | **63** |
| Testosterone | 229 | 86 | 20 | 20 | **18** |
| Compound 1 | 6.4 | 2.4 | 0.09 | 0.09 | **0.5** |
| **Compound 2** | **1000** | **375** | **91** | **91** | 77 |

A graphical representation of the data is shown below.



Application of Cheng-Pursoff under these conditions can lead to more than 10-fold overestimations of $K_i$. In many cases all three equations yield similar corrections and as mentioned above Munson & Rodbard and Huang yield identical values. However, one issue with the Munson & Rodbard and Huang type corrections is that certain combinations of $IC_{50}$, $K_d$ and bound tracer yield impractical negative values of $K_i$. This has been discussed in the literature as a breakdown in additional assumptions buried within these equations such as
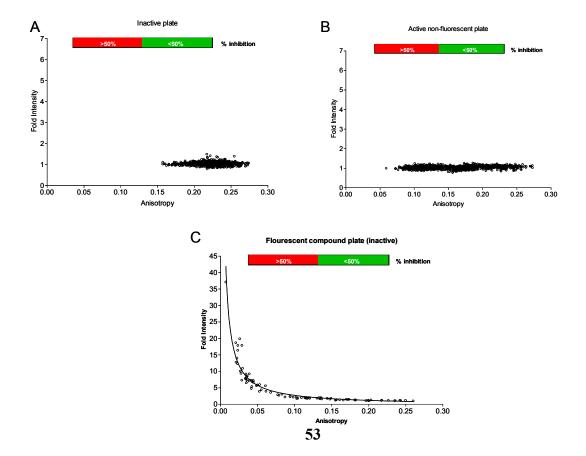
competitive inhibition with a single binding site. For this reason, the Kenakin equation is commonly chosen for performing this correction. Additionally, curve fitting to the equations given in Roehrl *et al*. (2004) can be used to examine if complete inhibition is achieved as well as the $K_D$ of the competitor compound.

## Detection of fluorescent interference from compounds in FP screens

All FP experiments start with measuring polarized prompt fluorescence from the assay well. This makes these experiments susceptible to fluorescence interference by compounds present in the well.  However, a helpful method to address this issue has been presented by Turconi *et al*. (2001). This paper calculates the total fluorescence intensity from a well (given by S + 2P; see references in above paper) and the observed anisotropy[1] from the each well to flag false positive wells due to fluorescence interference.

An example is provided below to illustrate the use of this method. Plots of the total fluorescence intensity (normalized to the control well values, *e.g.* the total fluorescence intensity of the assay in the absence of compounds) versus the anisotropy are shown below.

Three cases are illustrated in the figure above. In case A, the compounds in the wells

are not active or fluorescent. Therefore the measured Fluormone tracer is bound to GR ligand-binding domain (GR-LBD) and the anisotropy values are clustered around the 0% inhibition value. Furthermore, there is no change in fluorescence intensity in the compound-containing wells relative to the control wells. In case B, the compounds in the well are active in the assay but not fluorescent. Therefore, the tracer is being displaced from the GR-LBD and the anisotropy values distribute from high to low inhibition values. Again, there is no change in the total fluorescence intensity. In case C, the compounds appear active as they show a decrease in anisotropy values suggesting that the tracer has been displaced from the GR-LBD. However there is a correlation between decreasing anisotropy and increasing fluorescence intensity in the wells with the lowest anisotropy values showing more than a 35-fold increase in the fluorescent intensity relative to control values. This suggests that the measured FP is due to the compounds themselves rather than the tracer.

In typical FP-receptor binding experiments the tracer is kept at a low nM concentration while the compounds that are being screened are typically in the μM range. If these compounds are fluorescent at the detection wavelengths then their fluorescence can easily overcome that of the tracer. As compounds in screening campaigns are typically of low molecular weight (<500 Da) they will exhibit low anisotropy values. Compounds in case C were of this type and subsequent secondary assays showed them to be inactive. A final case not shown above is where the compounds are both fluorescent and active. Turconi *et al*. present an equation that can be used to fit the fluorescent intensity data to the case where anisotropy changes without displacement of the ligand (see Equation 4 and discussion therein of Turconi *et al*.). The solid line in case C above shows an example of this fit. One can then evaluate outliers from this curve fit in terms of potential active but fluorescent compounds.

It is also possible to observe changes in polarization that are due to fluorescent compounds present as aggregates. In this case, the fluorescence intensity will increase along with the polarization as long as the aggregation does not quench the fluorescence. Additionally, light scattering from particulates or compound participates can lead to apparently high polarization values. For receptor binding experiments as described above this superfluous increase in polarization may mask any decrease in polarization due to an active compound and thus result in a false negative. Careful examination of the fluorescence intensity versus polarization plots should identify these artifacts.

[1].Anistropy is derived by measuring the S and P channels as described above, however the fluorescence is expressed with the denominator representing the total fluorescence intensity from the sample. The equation for calculating anisotropy is given by:

$$a = \frac{(P-S)}{(S+2P)}$$

Anisotropy and polarization are related by the equations given below where P is the polarization and $a$ is the anisotropy:

$$P = \frac{(3a)}{(2+a)} \qquad\qquad \text{And} \qquad a = \frac{2P}{(3-P)}$$

In general, anisotropy is more useful analyzing complex systems or mixtures as the equations are simpler to express in terms of anisotropy. (Cantor and Schimmel, 1980). Arguably, screening data should be presented in terms of anisotropy rather than polarization but this convention has not been adopted as yet.

## Q. ABBREVIATIONS

[L] - Radioligand Concentration

[R] - Receptor Concentration

[RL] - Concentration of Receptor-Ligand complex

$K_d$ - equilibrium dissociation constant for radioligand ([RL] yielding $B_{max}/2$)

$K_i$ - equilibrium dissociation constant for an unlabeled compound

$IC_{50}$ - concentration of unlabeled drug which results in 50% inhibition of binding activity

$k_{on}$ - association rate constant

$k_{off}$ - dissociation rate constant

$k_{obs}$ - observed association rate constant

$B_{max}$ - maximum number of binding sites

NPE - Non-proximity Effects

NSB - Nonspecific binding

$K_i$ C-P = $K_i$ Cheng-Prusoff

$K_i$ T-B = $K_i$ Tight-Binding

# R. ADDITIONAL RESOURCES

**Web sites**

**GraphPad Prism**
http://www.graphpad.com/www/welcome.html

**Curvefit.com - related to GraphPad Prism web site**
http://www.curvefit.com/index.htm

**GE Healthcare (Formerly Amersham Biosciences)**
http://www.amershambiosciences.com/

**Perkin Elmer Life and Analytical Sciences**
http://las.perkinelmer.com/

**Receptor Binding Tutorial**
http://www.unmc.edu/Pharmacology/receptortutorial/home.htm


**Suggested Reading - General Receptor Binding**

Textbook of Receptor Pharmacology. (2003). Second Edition.  Foreman, J.C. and Johansen, T., Editors.  CRC Press, New York.

Kenakin, T.  (1997):  *Pharmacologic Analysis of Drug-Receptor Interactio*n. Lippincott-Raven Publishers, Philadelphia.

Receptor Binding Techniques. (1999). Methods *in Molecular Biology Series*, Volume 106, Keen, M., Editor.  Humana Press, New Jersey.

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Receptor Biochemistry:  A Practical Approach.  (1990).  *The Practical Approach Series*. E.C. Hulme, Editor.  Oxford University Press, New York.

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Kluwer Academic Publishers, Boston.

Winzor, D.J. and Sawyer, W.H.  Quantitative Characterization of Ligand Binding.  (1995).
Wiley-Liss, Inc., New York.

Lutz, M. W., Menius, J. A., Choi, T. D., Gooding-Laskody, R., Domanico, P. L., Goetz, A. S.
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Kahl, S. D., Hubbard, F. R., Sittampalam, G. S. and Zock, J. M.  (1997)  Validation of a High
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Sun, S., Almaden, J., Carlson, T.J., Barker, J. and Gehring, M.R. (2005). Assay development
and data analysis of receptor-ligand binding based on scintillation proximity assay. *Metab
Eng.* **7**:38-44.

**Suggested Reading – Fluorescence Polarization**

Cantor and Schimmel in Biophysical Chemistry Part II: Techniques for the study of
biological structure and function. pp. 454-465. (1980).

Huang, X. Fluorescence polarization competition assay: The range of resolvable inhibitor
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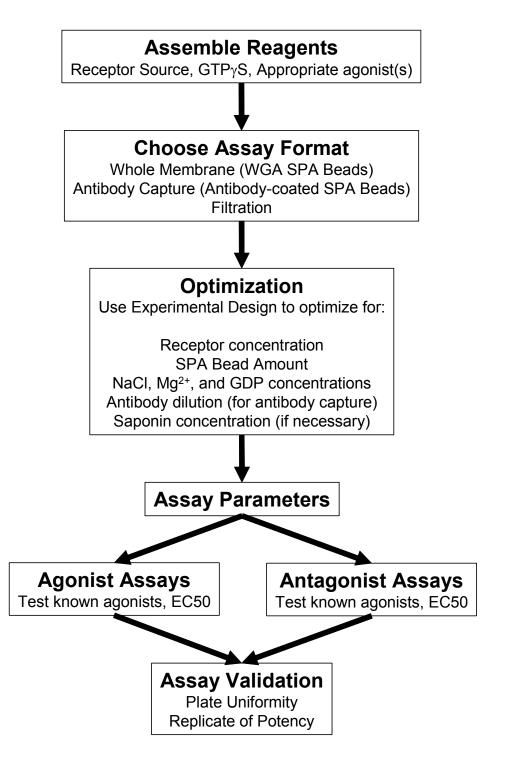Also see: http://www.invitrogen.com/downloads/FP8.pdf

# SECTION VI

# GTPγS BINDING ASSAYS

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## SECTION VII:  GTPγS BINDING ASSAYS

## A. STEPS TO ASSAY DEVELOPMENT

**Assemble Reagents**
Receptor Source, GTPγS, Appropriate agonist(s)

↓

**Choose Assay Format**
Whole Membrane (WGA SPA Beads)
Antibody Capture (Antibody-coated SPA Beads)
Filtration

↓

**Optimization**
Use Experimental Design to optimize for:

Receptor concentration
SPA Bead Amount
NaCl, $Mg^{2+}$, and GDP concentrations
Antibody dilution (for antibody capture)
Saponin concentration (if necessary)

↓

**Assay Parameters**

**Agonist Assays**
Test known agonists, EC50

**Antagonist Assays**
Test known agonists, EC50

**Assay Validation**
Plate Uniformity
Replicate of Potency

## B. INTRODUCTION

Binding of GTP to the alpha subunit of heterotrimeric GTP binding proteins is an early event in agonist-induced activation of G-Protein-Coupled Receptors.  Although GTPγS binding assays are carried out using membrane preparations in much the same way as radioligand binding assays, these are functional assays and can thus be used to differentiate agonist, antagonist, and inverse agonist activities.  Such assays are carried out using [$^{35}$S]guanosine-5'-O-(3thio)triphosphate which provides a radioactive ligand with high affinity for G-protein alpha subunits that is highly resistant to the inherent GTPase activity of alpha subunits such that it remains bound for sufficient periods of time to allow counting of radioactivity.

Although the classical method used for GTPγS binding has been filtration of radiolabeled membranes, scintillation proximity assays (SPA) are much more convenient and allow the use of an antibody capture technique which permits determination of receptor-mediated activation of specific G-protein families.  Thus there are two basic methods for running homogeneous SPA GTPγS binding assays in 96 well plate format: whole membrane binding in which labeled membranes are bound to wheat germ agglutinin (WGA)-coated SPA beads in the same way as these beads are used for radioligand binding assays, and antibody capture binding assays in which membranes are solubilized with detergent followed by isolation of the desired G-protein using a specific antibody along with capture of antibody-G-protein complexes onto anti-IgG coated SPA beads.

Advantages of GTPγS functional assays in comparison to determinations of second messengers produced as a result of receptor activation are:

1)  The assays are very simple to run and utilize membrane preparations which can be frozen at -80°C for periods of months

2)  Because GTP exchange is an event proximal to receptor activation these assays typically have lower degrees of receptor reserve than other functional assays and are thus useful for differentiating full from partial agonists

3)  The assays are useful for determination of antagonist inhibition constants since agonists and antagonists can be equilibrated prior to starting the incubation by addition of GTPγ$^{35}$S

4)  One can often measure specific coupling of receptor subtypes to different G-protein families, even in native tissues, under very similar assay conditions.

The major disadvantage is the relatively low signal to background which limits GTPγS binding to medium throughput evaluations.  The power of the antibody capture technique is its ability to easily generate multiple concentration response curves thus allowing true pharmacological evaluation of receptor-mediated coupling to individual G-proteins, an accomplishment that is prohibitive by older immunoprecipitation techniques.

## C. MATERIALS AND REAGENTS

The list below includes materials and reagents, which have been used successfully to enable GTPγS binding assays for a variety of G-Protein Coupled Receptors:

96 well plates: Costar 3632, white clear bottom
WGA SPA beads: Amersham SPQ0031
Anti-rabbit SPA beads: Amersham RPNQ 0016
Anti-mouse SPA beads: Amersham RPNQ 0017
GTPγ$^{35}$S: Perkin Elmer Life Sciences NEG030H
NP40 detergent 10%: Roche 1 332 473
Anti-Gs/olf: Santa Cruz SC-383, rabbit polyclonal
Anti-Gi3: Santa Cruz SC-262, rabbit polyclonal (recognizes Gi-1, Gi-2, and Gi-3)
Anti-Gq/11: Santa Cruz SC-392, rabbit polyclonal
Anti-Go: Chemicon MAB3073, mouse monoclonal

## D. MEMBRANE PREPARATIONS AND ASSAY BUFFERS

**Types of membrane preparations used**
1. Crude homogenates (2)
2. Plasma membrane preparations (P2 fraction, 3).
3. Sucrose density gradient enriched receptors (1)
4. Commercially available membranes, which includes:
   Receptors cloned into mammalian cells (Perkin Elmer, Euroscreen, Cerep)
   Receptors cloned into *Sf*9 insect cells co-expressing mammalian G-proteins.

**Types of Assay buffers**
1. Lazareno and Birdsall buffer (2) 20 mM HEPES, 100 mM NaCl, 5 mM MgCl$_2$, pH 7.4.
2. Buffers with HEPES replaced by 50 mM Tris HCl (1,9)

## E. BASIC ASSAY PROTOCOL

### Whole membrane assay using WGA SPA beads

1.  Incubate membranes, GTPγ[35]S, and compounds tested in 200 µl/well at room temperature for 30 – 60 minutes.
2.  Add 50 µl per well of suspended WGA beads (1 mg/well)
3.  Seal plates and incubate for one hour or more at room temperature
4.  Centrifuge at 200 x g and count plates in a Wallac microbeta
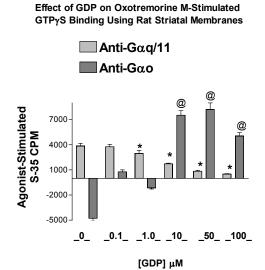
### Antibody Capture assay

1.  Incubate membranes as for whole membrane assay
2.  Add 20 µl per well of 3% NP40 and incubate for 15 minutes
3.  Add 20 µl per well of primary antibody and incubate for 15 minutes
4.  Add 50 µl per well of anti-rabbit or anti-mouse SPA beads (1 mg)
5.  Seal and incubate for three hours, centrifuge as above and count.

## F. ASSAY OPTIMIZATION

### Membrane protein/well and [GDP]

Using a starting buffer such as listed under assay buffer above, determine the optimal amount of membrane protein per well from 5 to 50 µg in the presence of varying concentrations of GDP (guanosine diphosphate) from 0 – 10 µM for transfected cell membranes and from 0 up to 300 µM for native tissue membranes using a concentration of 200 –500 pM GTPγ[35]S.  Note that Gi/o coupled receptors will require higher concentrations of GDP than Gs or Gq coupled receptors which may give optimal signals in the absence of added GDP.  Figure 1 illustrates the marked difference in GDP requirement for determination of muscarinic agonist-stimulated GTPγS binding in rat brain striatal membranes measured by anti-Gq/11 (M1 receptor) versus anti-Go (M4 receptor).

**Figure 1.  Difference in [GDP] required for Gq versus Go coupled GPCR's**



Effect of GDP on Oxotremorine M-Stimulated
GTPγS Binding Using Rat Striatal Membranes

* Significant reduction in binding compared to 0 GDP
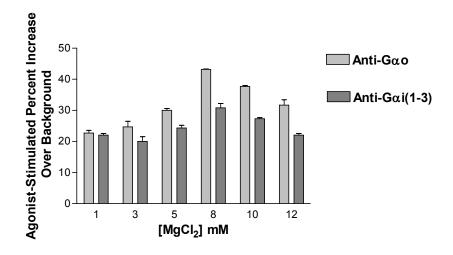
@ Significant agonist signal above basal binding

**Effect of Mg$^{++}$**

Determine the optimal Mg++ concentration for the best signal to noise over the range of
1 mM to 10 mM.  Figure 2 shows the variation of Mg++ on agonist-stimulated GTPγS
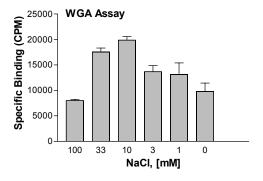binding mediated by GPCR receptors in rat striatal membranes.

**Figure 2.        Agonist-stimulated GTPγS binding in brain membranes**



**Effect of NaCl**

Determine the optimal amount of NaCl for best signal to noise over the range of 0 – 200
mM.  Although 100 mM NaCl is commonly used in these assays note that at times better
agonist stimulation may be achieved at lower Na++ and if higher constituitive activity is
desired (for evaluating inverse agonists) lowering Na++ will likely provide the best
opportunity.  Figure 3 demonstrates the effect of NaCl on the constituitive activity of an
orphan GPCR.

**Figure 3.  Optimization of NaCl to measure constituitive activity of an
                orphan GPCR**

**Effect of saponin**

The effect of adding saponin at 3 – 100 μg/ml can be explored, but recognize that while saponin may increase signal to background, it may also compromise the quality of concentration response curves.  Figure 4 demonstrates the optimization of saponin to achieve the highest signal to background for an orphan receptor where constituitive activity was measured to allow evaluation of inverse agonists.  Figure 5 shows how saponin may compromise the quality of some concentration response curves.

**Figure 4.     Optimization of saponin to measure constituitive activity of an orphan GPCR**
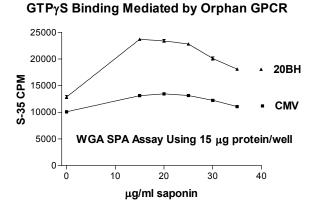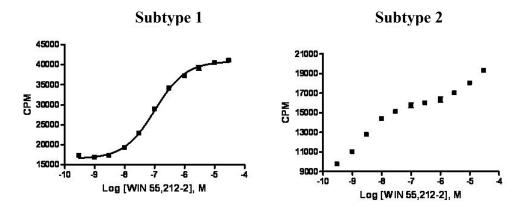


**Figure 5:     Effect of saponin on agonist concentration response curves for some receptor subtypes**



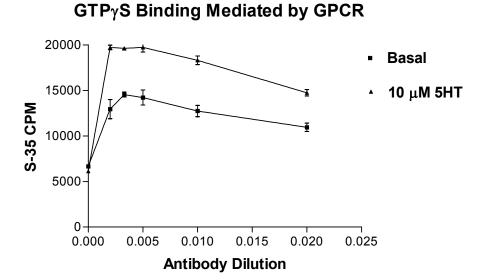100mM NaCl, 10mM MgCl2, 2uM GDP GDP, 0.2% BSA, 10ug/mL Saponin

## Incubation Time

The optimal incubation time for the best signal to background may be determined, but thirty minutes is usually satisfactory for cell membranes and one hour for native tissue membranes.

## Antibody dilution for antibody capture assays

If using the antibody capture method the optimal dilution will have to be determined for each lot of antibody. Figure 6 below illustrates the effect of various dilutions of anti-Gs/olf on GTPγS binding mediated by a specific GPCR receptor.
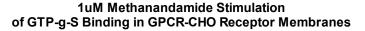
**Figure 6.      Effect of antibody dilution on basal and agonist-stimulated binding**

**The use of experimental design and JMP analysis for assay optimization**
Experimental design and JMP analysis are convenient tools for optimizing a variety of
conditions in a small number of experiments and determining if there are any interactions
among the factors. Figure 7A shows an example in which four factors were optimized in
a single experiment.  Figure 7B shows the two factor interaction profiles from JMP
analysis. Parallel lines indicate no interaction and intersecting lines indicate interactions.
For instance in this experiment there is virtually no interaction between NaCl and
saponin, but there is a significant interaction between the amount of protein and GDP
concentration.

**Figure 7A:     Experimental Design with 4 factors (GDP, Saponin, NaCl and
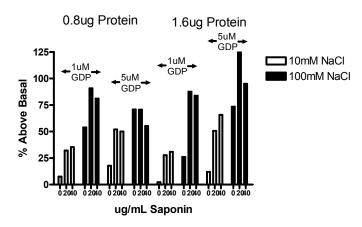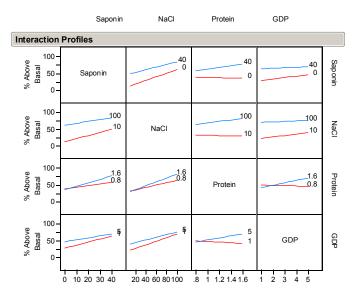Membrane protein)**



**Figure 7A:     Interaction Plots for Experimental Design (using JMP)**

**Signal window and Z' factor**
Determine the signal window for the assay under the optimal conditions by running background and maximal stimulation multiple times across assay plates on separate days. Calculate the Z' factor for the assay using the formula:

$$Z' = 1- (3(SDmax) + 3(SDmin)/Max-Min)$$

A Z' factor of > 0.5 indicates a useful assay. GTPγS binding assays can be quite reproducible and will give reliable results when signals are greater than 40-50% over background. Even with smaller signals, one can generate reliable concentration response curves by using 4 to 8 replicates per data point.

**Evaluation of standard compound concentration response curves**
After determining optimal conditions for the assay concentration response curves should be run for standard agonists and antagonists to determine variability and comparability to literature values if available. Most assays will require duplicate determinations per concentration but with exceptional signals one may be able to use single data points for each.

**Choice of whole membrane versus antibody capture**
Good assays for Gi/o may be developed using whole membranes and WGA beads. Use of antibody capture for Gi/o coupled receptors, however, may reduce assay variability. For Gq and Gs coupled GPCR's, the antibody capture assay will most likely be superior since most cells and tissues are dominated by inhibitory G-proteins and it is often not possible to develop reliable signals without the antibody technique unless receptors are fused to Gs or Gq (3, 13).


# G. DATA ANALYSIS

As with other functional assays, concentration response data may be fitted using a four-parameter logistic equation with variable slope to determine half maximal responses. Keep in mind that GTPγS assays will often show some degree of receptor reserve even though typically less than a cAMP or Ca++ mobilization assay and for this reason agonist EC50's may not agree with Ki values for agonists determined in radioligand binding assays. For antagonists, Kb values may be determined from rightward curve shifts in the presence of a fixed antagonist concentration or from antagonist concentration response curves run at a fixed agonist concentration (at or somewhat below the concentration that produces a maximal response). For curve shift at a single antagonist concentration the following equation may be used to determine the Kb:

EC50b = EC50a (1 + [antagonist]/antagonist Kb)

where EC50a is the agonist EC50 in the absence of antagonist and EC50b is that in the presence of antagonist.
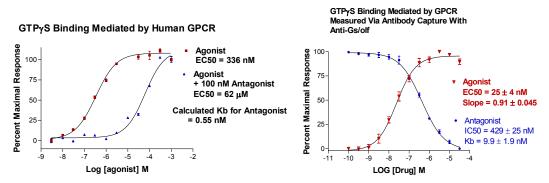
For antagonist concentration responses the following equation is used (14):

$$Kb = IC50/ (2 +([agonist]/agonist\ EC50)^n)^{1/n} - 1$$

where n is the slope of the agonist curve.

In antagonist concentration response experiments it is desirable to determine the agonist EC50 in each experiment along with the IC50 for the antagonist. Figure 8 below illustrates the use of both methods for measuring antagonist Kb values.

**Figure 8.       Examples of determining antagonist Kb values in GTPγS assays with a single antagonist concentration**
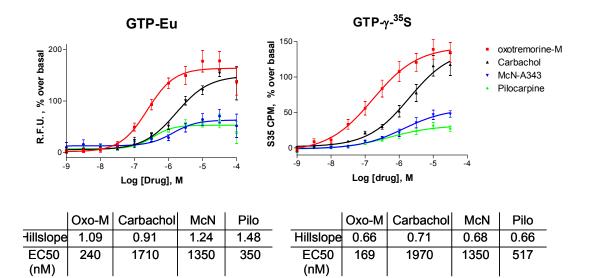


## H. FILTRATION ASSAYS

Filtration whole membrane assays may be used for GTPγS binding using the same methods employed for radioligand binding. The potential advantages of filtration assays are the lack of non-proximity effects, which are present in SPA assays and the ability to use higher concentrations of GTPγ$^{35}$S. Such advantages are not usually worth sacrificing the convenience of homogeneous SPA assays. There are many examples of the use of filtration for GTPγS binding in the literature (1,2,8, 9). As for WGA whole membrane binding, filtration assays are mostly limited to Gi/o coupled receptors since they cannot employ antibody capture.

## I.  NON-RADIOMETRIC GTPγS ASSAYS

Perkin Elmer Life Sciences has developed an assay based on the use of a europium-GTP complex, which has been used successfully by some scientists. This is a fluorescent whole membrane assay. It is not as convenient and is more expensive to use than GTPγ$^{35}$S method. However, it does not require radioactivity, there is no non-proximity effect, and the data are just as reliable (see figure 9 below).

**Figure 9:**     **Side by side comparison of GTP-Eu vs. GTPγ<sup>35</sup>S assay methods.**



|          | Oxo-M | Carbachol | McN  | Pilo |
|----------|-------|-----------|------|------|
| Hillslope | 1.09 | 0.91 | 1.24 | 1.48 |
| EC50 (nM) | 240 | 1710 | 1350 | 350 |

|          | Oxo-M | Carbachol | McN  | Pilo |
|----------|-------|-----------|------|------|
| Hillslope | 0.66 | 0.71 | 0.68 | 0.66 |
| EC50 (nM) | 169 | 1970 | 1350 | 517 |

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# SECTION VII


# TISSUE CULTURE ASSAYS

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## SECTION VII: TISSUE CULTURE ASSAYS

## A. CELL CULTURE GLOSSARY
Many of these definitions were obtained from Life Technologies, Inc. Cell Culture Course

- ## CELL CULTURE

  Establishment and maintenance of cultures derived from dispersed cells taken from original tissues, primary culture, or from a cell line or cell strain

- ## CELL LINE

  Immortalized cell, which have undergone transformation and can be passed indefinitely in culture

- ## CELL STRAIN

  Cells which can be passed repeatedly but only for a limited number of passages

- ## CELL CLONES

  Individual cells separated from the population and allowed to grow

- ## PRIMARY CULTURE

  Cells resulting from the seeding of dissociated tissues, i.e. Huvec cells.  Primary

  cultures often lose their phenotype and genotypes within several passages.

- ## CELL PASSAGE

  The splitting (dilution) and subsequent redistribution of a monolayer or cell suspension into culture vessels containing fresh media

- ## CONFLUENCY

  The confluency of a culture in a T flask or in a plate or dish is based on the amount of

   space between the cells.  The confluency of the culture often influences the growth of

  the culture and expression.

- ## ANCHORAGE DEPENDENT (ATTACHED) CELLS

  Cells which require a substratum to divide and produce a monolayer

- ### TRANSIENT TRANSFECTION

  The introduction of foreign DNA into a cell to allow the expression of the

  DNA into the host cell.  Protocols are available for opening transient "holes"

   in the cell membranes allowing plasmids, or siRNA to enter the cell.  Cells

  capable of being transfected or often referred to as "competent cells".  The

  DNA is not incorporated into the genome therefore, making the event

  transient referring to the transfection as a transient transfection.

- ### STABLE CELL LINE

  The selection of a stably transfected cell is where the transiently transfected cells
  are transfected with a co-expressed selection marker.  Typical systems that exist
  include resistance to antibotics such as neomycin phosphotransferase, conferring
  resistance to G418, etc.  The culturing of the cells can be done as a mixed
  population or by single cell culture to obtain cell clones from one single
  integration event.

- ## MONOLAYER

  A layer of cells one cell thick, grown in a culture.

- ## SUSPENSION CULTURE

  Cells which do not require attachment to substratum to grow, i.e. anchorage
  independent.  Cell culture derived from blood are typically grown in suspension.

  Cells can grow as single cells or clumps.  To subculture the cultures which grow as

  single cells they can be diluted.  However, the cultures containing clumps need to have

   the clumps disasociated prior to subculturing of the culture.

- ## DENSITY-DEPENDENT INHIBITION OF GROWTH

  Reduced response of cells upon reaching a threshold density. These cells recognize the

   boundaries of neighbor cells upon confluence and respond, depending on growth

patterns, by forming a monolayer. Usually these cells transit through the cell cycle at

 reduce rate (grow slower)

- **DIFFERENTIATION**

  Property of cells to exhibit tissue-specific differentiated properties in culture

- **HATCH**

  To bring cells out of the freezer; to start a culture from a freezer stock

- **THAW**

  Same as hatch

- **DEFREEZE(DEFROZE)**

  Same as hatch

- **SPLIT**

  To subculture/passage cells; see cell passage

- **PASS**

  See cell passage

- **CARRY**

  To maintain a cell line by subculturing in tissue culture medium containing nutrients that will maintain the phenotype and genotype of the cell line.

- **PLATE**

  To aliquot cells into microtiter plates; plates can be 6, 12, 96, 384, or 1536 well; as opposed to dishes of either circular or rectangular shape, commonly a 500 cm2 culture dish.
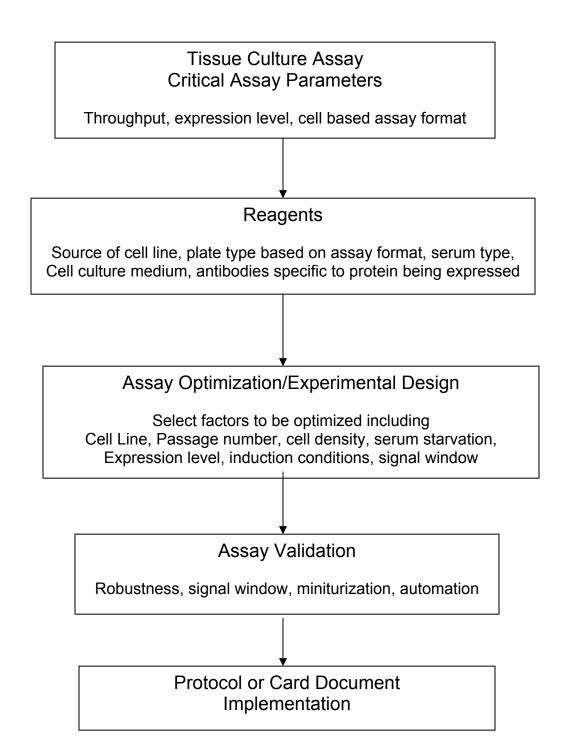
## B. INTRODUCTION TO CELL BASED ASSAYS

Cell based assays are a critical part of the flow scheme for discovering new chemical entities.  Cell based assays are typically used in the validation of a target.  Once the target is validated new chemical entities are screened for activity using numerous

assays including cell based assays as primary assays measuring activity against the target of interest or in cell based assays that determine the selectivity of the compound to the specific target.


## C. REQUIREMENTS FOR ASSAY PROOF OF CONCEPT FOR CELL BASED ASSAYS

1. Receptor of interest has been expressed in a suitable cell line and functional expression has been verified. Receptor expression has been verified by RT-PCR or Western blot.
2. Sample preparation has passed QC guidelines above.
3. Preliminary source for all reagents has been identified.
4. Early passage stable cell lines are available and free of mycoplasma contamination.
5. Transient transfection assays: appropriate cell line and transfection procedure demonstrated. Plasmids available and passes QC sequence and restriction mapping criteria (above).
6. Cell culture details are available as written SOP. This includes the *number* of cells (not dilution eg 1/10) used for passage, passage frequency, limit passage numbers for an assay, activity stability as a function of cell passage and density, and optimum cell density for target activity.
7. Biological activity (>90%) is target specific as demonstrated by transfection controls, comparison to parental cell lines, pharmacology, and/or tool compound activity.
8. Assay signal is dependant upon amount of cells present.
9. Assays enabled in DHT: Preliminary data showing a saturable activator and/or inhibitor response with sufficient signal window using a QB-supported assay format.

## D. TISSUE CULTURE ASSAY DEVELOPMENT, OPTIMIZATION AND VALIDATION FLOW CHART

```
┌────────────────────────────────────────────────────┐
│                Tissue Culture Assay                 │
│              Critical Assay Parameters              │
│                                                      │
│   Throughput, expression level, cell based assay    │
│                      format                          │
└────────────────────────────────────────────────────┘
                         │
                         ▼
┌────────────────────────────────────────────────────┐
│                     Reagents                        │
│                                                      │
│  Source of cell line, plate type based on assay     │
│  format, serum type, Cell culture medium,           │
│  antibodies specific to protein being expressed     │
└────────────────────────────────────────────────────┘
                         │
                         ▼
┌────────────────────────────────────────────────────┐
│        Assay Optimization/Experimental Design       │
│                                                      │
│          Select factors to be optimized including   │
│   Cell Line, Passage number, cell density, serum    │
│   starvation, Expression level, induction           │
│   conditions, signal window                         │
└────────────────────────────────────────────────────┘
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┌────────────────────────────────────────────────────┐
│                  Assay Validation                   │
│                                                      │
│  Robustness, signal window, miniturization,         │
│  automation                                         │
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│              Protocol or Card Document              │
│                  Implementation                     │
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## E.  TYPES OF TISSUE CULTURE ASSAYS

Listed below are types of cell-based assays frequently used as primary, secondary or profiling assays for screening compounds to find new chemical entities.

**Proliferation Assays:  Used to measure cell growth over a period of time and the effects of compounds on the cell growth**
      A.  Thymidine uptake is measured by using 96 well plates coated with scintillant or using filter binding methods.  Plates containing scintillant detect the thymidine taken up by the cells by using 14C or 3H.
      B.  WST reagents: a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (a soluble version of MTT or MTX)
      C.  Alamar Blue™: A colorimetric assay
      D.   There are several non-radioactive, fluorescence-based assays that are not dependent on cellular metabolism.  The fluorescent dye binds nucleic acids and the fluorescesences can then be measured quantitatively or qualitatively.  Propidium Iodide, Hoechst or other live dyes can be added to cells.  The fluorescence can than be detected using various high content imaging instruments.  The cell number can then be quantitated based on the fluorescence.  DNA content can also be quantitated using the tools available in the imaging instruments.
      E.  Chemiluminescent, non-separation assay kits for the determination of viable cell numbers are also available for either proliferation or cytotoxicity assays.

Apoptosis Assays:  Used to measure the various stages of cell death referred to as early, intermediate and terminal apoptosis. undergoing apoptosis. In just one of many scenarios of apoptosis, the process is triggered by another neighboring cell; the dying cell eventually transmits signals that tell the phagocytes, which are a part of the immune system, to engulf it.  There are numerous assays and markers available on the market to determine if the cells are undergoing apoptosis and where in the apoptosis pathway they are.  Some examples of these markers are listed below.

      A.  Annexin V staining
      B.  Tunnel staining
      C.  Caspase activity
      D.  Nuclear Fragmentation using microscopes, or other imaging platforms

**Gene Expression**

    A. Transient transfection of a gene of interest into a cell using various reporter systems. There are numerous transient transfection reagents and protocols available that have been optimized for various cell lines and promoters.

    B. Stable transfection of a gene, is where the gene of interest is stably integrated into the cell. Stable transfections can take a lengthy period of time to be generated.

**Activation assays are used to measure changes in the cellular mechanisms following treatment with compounds.**

    A. Measurements of intracellular second messengers: calcium flux, cAMP. There are numerous assay formats available for measuring cAMP including ELISA's, radioactive formats, homogenous formats including FRET assay formats.and alpha screen.

    B. Uptake of various cellular components measured by using radioactive ligands.

    C. Activation of metabolic pathways: measurement of phosphorylation events intracellular or intranuclear.

## F. BATCH CO-TRANSFECTION AND CELL BANKING PROTOCOLS

The use of transient transfection and co-transfections in cell based assays for screening compounds tends to have higher variability than non transfected cell based assays. The process of scaling up the cells and the transfection process also adds days to the process of the assay. By banking the transfected or co-transfected cells the process becomes less variable and more efficient. Below is the protocol for batch co-transfection and cell banking.

**A) Counting Cells for Transfection**

    • Allow both the 0.05% Trypsin and the Assay media to warm up at room temperature prior to detaching cells.

1. Remove the flask out of the incubator (37°C and 5% $CO_2$) that will be used for counting.
2. Aspirate off the media with an aspirating pipette attached to the vacuum source located within the culture hood.
3. Add 10mls of Dulbecco's PBS to each flask. Next, rock the flask(s) back and forth once making sure to wash the side of the flask with the cells attached.
4. Aspirate off PBS.
5. Add 3 ml of 0.05% Trypsin/flask. Rock the flask(s) back and forth to coat the cells with the Trypsin.
6. Let sit at room temperature for 3 minutes. Whack flask to detach cells.
7. Add 7mls media to flask to quench the Trypsin. Pipette up and down several times to break up clumps.
8. Transfer the cell mixture to a 50 ml blue-top centrifuge tube and mix well.

9.  Count the cells three times using a Coulter cell counter and calculate the average.
10. Next, calculate the number of cells using the following formula:

**(Avg. #of counts)/(vol. the counter will count, μl) X (vol. of isoton, μl )÷(vol. of the sample, μl) X (total vol. of the cells sampled from, μl)**

Example:  6279 counts/500 μl X 20,000 μl ÷ 100 μl X 10,000 μl = 25.11 million

(i)  B)  Transfections

1)  Prepare the Serum-free media and transfection reagent mix in a 250 ml orange-top conical centrifuge tube according to the optimized ratio for transfection.  Mix gently halfway through **and** after adding all of the reagent and incubate for 5 minutes. **DO NOT touch transfection reagent to the plastic sides of the tube.  Dispense directly into the SFM**.

2)  Add appropriate amounts of DNA to the tube with the SFM and transfection reagent mix.  Mix, by tapping the tube gently  after additions.

3)  Incubate for 30 minutes at room temperature.

4)  During this incubation period, aspirate media from the T225 flasks to be transfected and add back 38mls of Assay Media.  Place back in the incubator at 37°C, 5% $CO_2$.

## C)  Cell Banking

- Allow both the 0.05% Trypsin and the Assay media to warm up at room temperature prior to detaching cells.

1)  Remove the flasks out of the incubator (37°C and 5% $CO_2$).

2)  Aspirate off the media with an aspirating pipette attached to the vacuum source located within the culture hood.

3)  Add 10mls of Dulbecco's PBS to each flask. Next, rock the flask(s) back and forth once making sure to wash the side of the flask with the cells attached.

4)  Aspirate off PBS.

5)  Add 3 ml of 0.05% trypsin/flask.  Rock the flask(s) back and forth to coat the cells with the Trypsin.

6)  Let sit at room temperature for 3 minutes. Whack flask to detach cells.

7)  Add 7mls media to flask to quench the trypsin. Pipette up and down several times to break up clumps.

8)  Transfer the cell mixture to a 250 ml orange-top centrifuge tube and mix well. Divide cell suspension between orange-top tubes.

9)  Count the cells three times using a Coulter cell counter and calculate the average.

Next, calculate the number of cells using the following formula:

**(Avg. #of counts)/(vol. the counter will count, μl) X (vol. of isoton, μl )÷(vol. of the sample, μl) X (total vol. of the cells sampled from, μl)**

Example:  6279 counts/500 µl X 20,000 µl ÷ 100 µl X 10,000 µl = 25.11 million

10) Spin tubes in centrifuge at 1500 RPM's for 5 minutes.
11) Remove supernatant.
12) Divide total number of cells (see #9) by **50** (cell concentration/ vial frozen: **50X10e6**) = mls freezing solution to add to cell pellet.
13) Pipette up and down several times to break up cell clumps.
14) Aliquot 1 ml of cell suspension to a 2 ml cryogenic vial.
15) Place vials in a Mr. Frosty and place in -80°C freezer for approximately 4 hours.
16) Remove and place in Liquid Nitrogen tank for long term storage.

- **Freezing Solution**

  90% Fetal Bovine Serum (Charcoal Stripped FBS)
  10% DMSO

## D) Validation of Frozen Cells

1) Remove vial from liquid nitrogen storage.
2) Thaw quickly in 37°C water bath.
3) Bring up slowly to total volume of 20-30 mls of cold Assay media.
4) Spin at 1500 RPM's for 5 minutes.  Remove supernatant.
5) Re-suspend in 10 mls assay media.  Take a 100 ul aliquot of cell suspension and add to 20 mls Isoflow.
6) Count the cells three times using a Coulter cell counter and calculate the average cell count.

**Avg. #of counts)/ (vol. the counter will count, µl) X (vol. of isotonic, µl) ÷(vol. of the sample, µl) X (total vol. of the cells sampled from, µl)**

Example:  6279 counts/500 µl X 20,000 µl ÷ 100 µl X 10,000 µl = 25.11 million

7) Seed a 96 well or 384 well microtiter plate following one of the below equations to determine cell number in suspension.

384 well format:
(30,000 cells in 50 ul)
1 (11.5 mill. Cells/plate) (_____plates, included 2 extra) =_____mill. Cells needed
2 (____mill. Cells needed for assay accounting for extra) / (____mill. cells/ml) = _____ml of cells from cell stock
3 (____mill. cells/ml) (____ml of cells from cell stock) / (0.6 mill. cells/ml) = ml total
4 (____ml total) – (____ml of cells from cell stock) = ml of media

96 well format:
(50,000 cells in 80 ul)
1 (4.8 mill. Cells/plate) (____plates, included 1 extra) = ____mill. Cells needed
2 (____mill. Cells needed for assay accounting for extra) / (____mill. cells/ml) = ____ml of cells from cell stock
3 (____mill. cells/ml) (____ml of cells from cell stock) / (0.625) mill. cells/ml = ml total

4 (_____ml total) – (_____ml of cells from cell stock) = ml of media


8)  Incubate overnight overnight in 37°C, 5% CO$_2$
9)  Dose with appropriate dose response curve for each receptor


## G. CRYOPRESERVATION OF CELLS

Below is a protocol for the cryopreservation of cells for either the use of cell culture growth and maintenance of cell culture or for use in cell based assays.


## A. Cryopreservation

1.  Remove media from flask.
2.  Wash cells gently with 10mls D-PBS and aspirate.
3.  Add 3mls Trypsin-EDTA , let sit on cells for 2-3 minutes.
4.  Tap flasks to detach cells.
5.  Add 7mls of Growth Media (see recipe) to flask.  Pipet up and down several times and transfer to a 50ml conical centrifuge tube.  Take a 100ul aliquot of the cell suspension and add to 20mls Isoflow/Isoton and count using Coulter Counter.  Count cells three times.

**(Avg. #of counts)/(vol. the counter will count, μl) X (vol. of isoton, μl )÷(vol. of the sample, μl) X (total vol. of the cells sampled from, μl)**

e.g.  6279 counts/500 μl X 20,000 μl ÷ 100 μl X 10,000 μl = 25.11 million
Divide by the number of cells you want to freeze down and add that final number in mls of freezing solution.
Example:  25.11 million ÷ 5 million = 5.022 (number of mls of freezing solution to add to get 5 X 10e6 cells per vial)

6.  Spin cells in centrifuge at 1500rpms for 3 minutes.
7.  Remove supernatant and resuspend cells in freezing medium  (see below) @ a concentration of 5 X 10e6 cells per ml in a 1.5ml Cryo vial.
8.  Label vials with cell line, passage #, freeze down date, notebook number if possible and number of cells frozen.
9.  Place in a freezing container and place in a -80°C freezer for ~ 2 to 4 hours.
10. Remove and place in liquid nitrogen tank for long term storage.


Freezing Solution

10% DMSO + 90% Characterized FBS
(You want to use the FBS used in the Growth media for making up this solution)

## B. Thawing of Cryopreserved Cells

**Centrifugation Method:**

1.  Remove cells from liquid nitrogen storage and thaw quickly in a 37°C water bath.
2.  Remove cells from cryo vial and place in 50ml conical tube.
3.  Add ~20 to 30mls of cool Growth medium slowly to the tube.
4.  Centrifuge cells @ 1500rpms for 5 minutes.
5.  Discard supernatant.
6.  Resuspend cells in Growth media and count cells and seed flasks.

**Direct Plating Method:**

1. Remove cells from liquid nitrogen storage and thaw quickly in a 37°C water bath.
2. Plate cells directly with Growth medium.  Use 15-20mls of Growth Medium/ 1ml frozen cells @ 3 X 10e5.
3. Culture cells 6-8 hours.  Replace with fresh Growth Medium to remove the cryopreservative.

## H. CELL COUNTING PROTOCOLS

Traditionally a cell culture is counted prior to plating the cells or for culturing the cells in flasks, roller bottles, etc.  This is to determine the cell number of viable cells.  Counting cells consistently is important to the responsiveness of the cells.  Counting cells can be done numerous ways.  A common way of counting cells is by using a hemacytometer and light microscope.  This method can be subjective and is time consuming.  There are also several automated methods of counting cells including the Coulter Counter or the Vi-CELL.  The basis of the Coulter Counter is detects changes in electrical conductance of a small aperture as fluid containing the cells flows past the detector.  The deflection is then detected as a particle or cell.  The Vi-CELL is an automated method of counting cells using the trypan blue cell exclusion method.

## I. FACTORS FOR CELL BASED ASSAY DEVELOPMENT

When considering the factors for development of a cell culture assay there are two major parts that need to be considered: the cell growth conditions and the cell treatment conditions.  Many times variables may differ for the two parts of the assay so they need to be kept as separate parts of the assay when considering the factors.  In the table below is a list of the factors that one would consider when running an experimental design to develop an assay.  The range of the factor is based on literature reference, existing protocols, American Type Culture Collection (ATCC) recommendations, etc.  The rationale for why these factors are detailed below.  Once the factors and ranges of the factors are decided it is best to meet with a statistician to begin the experimental design usually with a fractional factorial using broad ranges.  The final outcome of the design will result in an optimized robust assay.

| Factor | Range /Rationale |
| --- | --- |
|  |  |
| Cell type | Primary cells vs. an established cell line<br>Primary cells are often difficult to culture.  Phenotype and viability may change within a few passages.  Established cell lines are typically easier to culture and usually maintain viability and phenotype over numerous passages. |
|  |  |
| Number of cells per | Cell density will depend on the cell type and the type of |

| | |
|---|---|
| well | assay being performed. Start with a broad range of cell concentrations and then narrow down the optimal cell number per well using experimental design |
| | |
| Cell passage number | Cells can change their responsiveness over passage number.  Primary cells in particular have a tendency to change over passage number.  Receptor number may be dependent on passage number or condition that the cells are maintained under. |
| | |
| Growth medium | Each cell type has specific medium optimized for growth.  This information can be found in ATCC or in publications. |
| | |
| Growth serum type | Serum type is usually specified in the literature or in ATCC.  Dextran Charcoal (DCC) treated serum removes proteins, which may interfere with the assay.  Cells may require Horse serum in addition to Fetal bovine serum. |

| Factor | Range /Rationale |
|---|---|
| | |
| Growth serum concentration | Serum is almost always required for growth conditions unless the medium has been optimized for serum free conditions.  Ranges are from 1-15% serum concentrations.  Serum free conditions are often used to prevent interference with compounds binding to protein or to increase the stimulation of the inducer. |
| | |
| Treatment medium | Medium being used during the treatment can differ from that used in growth medium. |
| | |
| Treatment serum type | Serum type is usually specified in the literature, by ATCC, or from the originator of the cell line.  However, compound testing can be done in serum free conditions. |
| | |
| Treatment serum concentration | Serum free conditions are often used during treatment of compounds once cells have been established in serum to remove effects of serum.  Low concentrations of BSA are often substituted to prevent non-specific protein binding from occurring. |
| | |
| Incubation time for pretreatment | Dependent on response desired for assay and biological relevance. |
| | |
| Treatment incubation | Dependent on desired assay response and biological |

| time | relevance. |
| --- | --- |
| | |
| Plate type | Plate type is assay dependent.  Fluorescent assay:  black or white; Luminescent: white or black;  Radioactive: Plates containing scintillant coated on the bottom or on the bottom and sides, or filter plates for filter binding. Colorimetric: clear tissue culture treated plates for assays measuring proteins in a cell culture supernatant like an ELISA, the supernatant is transferred to the ELISA plate. |
| | |
| Coating type | Some cells adhere better with some type of coating, reducing variability and enhancing the response.  The coating can be poly-D-lysine, fibronectin, collagen or other matrix, that enhances cell attachment to the plate. |
| | |
| pH | Neutral |
| | |
| Inducer/Inhibitor concentration | Dependent on experimental conditions. |
| | |
| Radioactive tracer concentration | Based on optimal signal window obtained during assay optimization. |

| Factor | Range /Rationale |
| --- | --- |
| | |
| Addition time of radioactive tracer | Based on optimal signal window obtained during assay optimization and biological relevance.  The simplest method is to add the radioactive tracer at the same time as the inducer and then measure the response over a period of time post induction. |
| | |
| Type of radiation | $^{14}$C is usually needed for plates containing scintillant coated on the bottom  to get an optimal signal window. Other types of radioactive tracers may be used for other assay platforms. |
| | |

## A. TYPICAL CELL CULTURE PROTOCOL

1.  Place media and trypsin in water bath (37degrees C).
2.  Take flask of cells out of incubator.
3.  Examine cells under microscope to determine health/condition.
4.  In hood, remove medium from flask by aspiration.

5.  Wash cells once with 5-10 ml of Phosphate Buffered Saline.
6.  Aspirate off PBS.
7.  Add 5-10ml trypsin or dissociation solution to cells.
8.  Rock flask to allow above to cover cells.
9.  Incubate 2-5 minutes or until cells begin sloughing off the flask.
10. Tap flask to see if cells have released from bottom of flask.
    -If not, incubate further 1-2 minutes or place flask in 37° incubator for few more minutes.
    If so, go to 11.
11. Add equal volume of medium to cells.
12. Pipet up and down several times to break up cell clumps.
13. Pool all cell into one container.
14. Take out 1ml sample to count.
15. Count on hemacytometer, or automated cell counters like a Coulter Counter or Vi-CELL.
16. Do calculations to determine cell density.
17. Calculate amount of pool needed for desired cell number for project.
18. Take off that amount of cells and centrifuge at 1000rpm for 5+minutes.
    Determine amount media to resuspend cell pellet in.
19. Resuspend pellet in appropriate amount of medium.
20. Seed or plate as needed.  Automated dispensers for cell plating will decrease variablility of cell seeding in 96, 384 or 1536 plates.

# B.  STANDARD HTS CELL CULTURE PRACTICES TO REDUCE CONTAMINATION RISK

- When moving things (media bottles, pipet tips, etc.) into a biosafety cabinet (hood) you should wipe them down with 70% EtOH. Be sure to cover the entire object.

- Wipe out hoods before and after use with 70% EtOH. UV weekly. Lysol (3%) weekly.

- Hoods should be completely cleaned at least 3 times a year. This entails taking the surface tray and grills out, washing them and the area beneath them with Lysol then EtOH, and removing any debris found below the tray. Autoclaving the tray and grill are acceptable.

- Bleach vacuum flasks and lines. Change the flask weekly even if not full.

- Wipe out incubators at least once a month with Lysol followed by 70% EtOH. This is a known source of fungal contamination. When opening an incubator, check for fungal growth on the shelves and around the seals.

- Empty biowaste containers regularly, preferably at least twice a week. All waste should be double bagged (bag into container then another bag inside the first).

- Do not carry large stacks of plates or flasks unless you use a cart.

- Wear gloves. Make sure your lab coat is not grimy.

- Bleach any container that has contained cells for a few minutes. The bleached media can be washed down the sink. However, do not open CONTAMINATED containers in the main lab area. If you have a contamination, autoclave it BEFORE opening.

- Fluid delivery lines/ drain lines should be rinsed with 70% EtOH chased with dH2O every day after use. This is a known source of contamination. This would include multidrop heads, multimek lines, MRD8 lines, etc. . If dispensing media with a multidrop, rinse head then autoclave the head. You should keep an autoclaved head in reserve if possible in case of failure of the daily one. If using the Multimek to aspirate or plate cells, rinse the lines and wash station then autoclave the wash station. The autoclaving does not apply to heads used for 384 well delivery.

- If you have a contamination event, DO NOT OPEN IT IN THE MAIN LAB! Contact your supervisor or a cell culture person to help in identifying the contamination and the source. Also, make others using cells aware that you had a contamination event.

- Be careful not to touch pipets, media bottle openings, etc. . Touch events are a leading source of contamination in cell culture. If you do happen to touch a pipet discard it and get a new one. If you touch a bottle opening, wipe it immediately with an EtOH swipe and then filter that media through a 0.2um filter apparatus.

- Cleanliness is next to Godliness especially in cell culture. Keep your hood free of unnecessary clutter. Wipe up spills promptly. Try not to sneeze inside your hood. Drips should be promptly cleaned up with EtOH.

Note the sash level limit on your hood. There should be a mark or arrow on one side of the sash (glass) to tell you how high not to go with the sash. Too high will disrupt the air flow and compromise your hoods sterility.

## C. EXAMPLE, TRANSIENT TRANSFECTION OF CELLS

Various methods of transient transfection are described in the literature and they differ in their efficiency of transfection and cell toxicity.  The primary decision for assay development involved the choice of a transfection method and reagent that yielded high efficiency, minimal toxicity and yet was compatible with the high cell volume requirements of HTS.  Following is a list of transfection methods that can be tested to determine the optimal transfection protocol for each cell type.  Measure the amount of toxicity, throughput and efficiency of transfection to determine the optimal method.

### TRANSIENT TRANSFECTION METHODS

Calcium Phosphate, Electroporation, Ballistic Particles, DEAE Dextran, Cationic Matrix, and Lipofection.

The Lipofection protocol is a relatively simple method that has been used for high throughput screening. Various lipid and matrix reagents can be compared for using the Lipofectamine protocol and measuring the transfection of enhanced green fluorescent protein (EGFP) and β-galactosidase (β -gal) marker plasmids to determine optimal reagents. Fugene 6 has been observed to work best in the presence of serum and resulting in little or no toxicity.  Following is a list of transfection cationic matrix reagents. Measurement of the efficiency and toxicity of the transfection can be used to determine the optimal transfection cationic matrix reagent.

### TRANSFECTION CATIONIC MATRIX REAGENTS

 Superfect (Qiagen), Lipofection, Fugene 6 (Boehringer), Transfectam (Promega), TransFast (Promega), Tfx (Promega), CLONfectin (ClonTech), Lipofectamine Plus (GIBCO)
Once the optimal transient transfection method and cationic matrix reagent have been selected then additional factors can be considered in developing a method for optimizing a transient transfection protocol.

| Day | Protocol | Variables |
|---|---|---|
| | | |
| 1 | Seed cells | Cell number is dependent on cell type<br>Type of culture flask<br>Optimal range is 50% density on day 2 (40-60%) |
| | | |
| 2 | Transfect cells | Protocol of transfection is dependent on reagents used (see list above on transfection reagents)<br>Variables to be tested:  DNA amount, Reagent amount, DNA:Reagent ratio, serum concentration, medium type, time of transfection. These variables are optimized according to cell type. |
| | | |
| 3,4 or 5 | Determine transfection efficiency, end product or reporter expression<br>Induction or stimulation | Depending on the cell type the assay for gene expression is usually measured between 24 to 72 hours after transfection.  Induction or stimulation is usually done 48 hours after transfection, and then determine expression levels.  Method for determining expression will also depend on type of expression- GFP can be monitored by visual inspection; luciferase is measured by cell lysis, addition of substrate and measurement of light output. |
| | | |

## D. GENERAL CELL BIOLOGY INFORMATION

Cell Biology has other general procedures inherent to the use of cells like the use of aseptic technique, culturing the cells, counting the cells, harvesting cells, maintaining the cell culture, preserving the cells, and types of cell culture vessels.  An excellent Cell Biology web site, which lists further details regarding cell biology, cell preservation, cell culture techniques, etc. is found in the web link below.  As always with cell culture, use the conditions stated in the literature, ATCC or in the most recent protocol as a starting point.

## E. REFERENCES

- http://www.atcc.org/
- http://www.protocol-online.org/prot/Cell_Biology/index.html
- http://www.protocol-online.net/cellbio/cell_culture/cell_culture.htm
  (Nice sight with cell culture protocols, etc)
- http://www.cellsalive.com/
- http://www.tissue-cell-culture.com/docs/libary/tc_trouble.html
  This site has excellent list of cell culture trouble shooting
- http://www.answers.com
- http://en.wikipedia.org/wiki/coulter_counter
- http://www.schuett-abraham.de/glossar-en.htm
- http://www.invitrogen.com/tissue_culture.htm
- http://www.amaxa.com/stable-transfection.html
- Apoptosis (Programmed Cell Death) - The Virtual Library of Biochemistry and Cell Biology
- Apoptosis Research Portal

Invitrogen Cell

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        Table of Contents for Invitrogen Cell website

Basic Mammalian Cell Culture

- Introduction

- Aseptic Technique for Cell Culture

- Media for Culture of Mammalian Cells

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- Isolation and Purification of Primary Schwann Cells

- Isolation and Generation of Human Dendritic Cells

- Isolation and Purification of Primary Oligodendrocyte Precursors

- Isolation and Purification of Primary Rodent Astrocytes

- Isolation and Generation of Oligodendrocytes by Immunopanning

Chinese hamster ovary (CHO) Cell Culture

- Transfection of DG44 cells and development of stable cell lines in defined medium

Preparation and Isolation of Human Cells

- Establishment of Fibroblast Cultures

- Preparation and Culture of Human Lymphocytes

- Preparation of Endothelial Cells

- Isolation of Whole Mononuclear Cells from Peripheral Blood and Cord Blood

- Isolation of B Cell Populations

- Isolation of Monocyte/Macrophage Populations

- Isolation of Human NK Cells and Generation of LAK Activity

- Isolation and Generation of Human Dendritic Cells

- Generation and Maintenance of Cloned Human T Cell Lines

- Isolation and Characterization of Human Natural Killer Cell Subsets


Preparation and Isolation of Murine Cells

- Mouse Embryo Fibroblast (MEF) Feeder Cell Preparation

- Production of a Heterozygous Mutant Cell Line by Homologous Recombination (Single Kno

- Isolation of Mouse Mononuclear Cells

- Isolation of Dendritic Cells

- Isolation of Mouse Small Intestinal Intraepithelial Lymphocytes, Peyer's Patch, and Lamina

- Isolation of Mouse Neutrophils

- Isolation of Mouse Intrahepatic Lymphocytes

- Isolation of Murine Natural Killer Cells

- Isolation of Murine Macrophages

- [Yeast Cell Culture](#)

  - [Media and Culture of Yeast](#)

- [Transfection and Selection](#)

**Transfection of DNA**
  - [Transfection of Cultured Eukaryotic Cells Using Cationic Lipid Reagents](#)

  - [Stable Transfer of Genes into Mammalian Cells](#)

  - [Lipofectamine™ 2000 Standard Plasmid Transfection](#)

  - [Lipofectamine™ 2000 siRNA - plasmid co-transfection protocol](#)

  - [Transfection of Insect Cells Using Cationic Lipid Reagent with Cellfectin](#)

**Transfection of RNA**
  - [Lipofectamine™ 2000 siRNA - plasmid co-transfection protocol](#)

  - [Lipofectamine™ 2000 Stealth™/siRNA transfection](#)

  - [Lipofectamine™ RNAiMAX Forward Transfections](#)

  - [Lipofectamine™ RNAiMAX Reverse Transfections](#)

  - [Oligofectamine™ Stealth™/siRNA transfections](#)

  - [Lipofectamine™ 2000 Transfection Protocol for Human Mesenchymal Stem Cells](#)

  - [Lipofectamine™ RNAiMAX Transfection Protocol for HUVEC](#)

  - [BLOCK-iT™ Fluorescent Oligo as RNA Transfection Control](#)

**Selection in Cultured Cells**

- [Selection of Transfected Mammalian Cells](#)

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# SECTION VIII

# CELL-BASED ELISA (c-ELISA) AND WESTERNS BLOTS FOR QUANTITATIVE ANTIGEN DETECTION

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## SECTION IX:
## CELL-BASED ELISA (c-ELISA) AND WESTERNS
## BLOTS FOR QUANTITATIVE ANTIGEN DETECTION

---

**Cell-based ELISA/Western Flow Chart:**
**Overview and Practical Considerations**

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**Reagents:**
Cell Line expressing protein
 Single specificity antibody
Stimulus to initiate protein modification (eg,
phosphorylation, degradation, induction)

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2

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**C-ELISA Variables or Paramters to Consider**

## A. INTRODUCTION
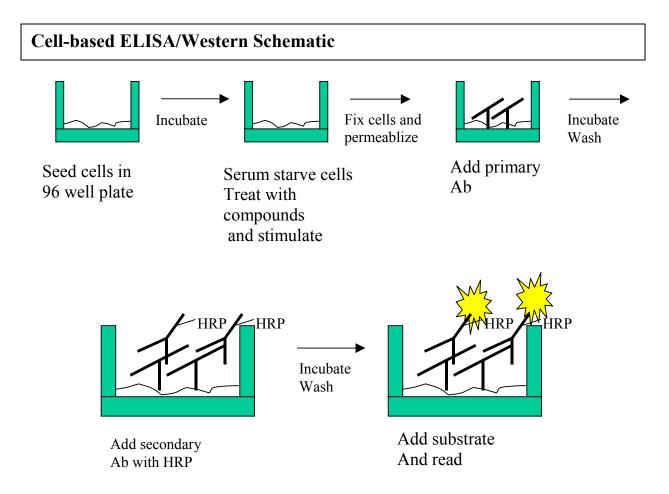
**Concept**
The cell-based ELISA or Western (cell-based ELISA or C-ELISA) is a moderate throughput format for detecting and quantitfying cellular proteins including post-translational modifications associated with cell activation (e.g., phosphorylation and degradation). Typically, these changes are monitored by Western blots. However, this procedure requires cell lysis, electrophoresis, blotting and staining of the gel with the appropriate antibody. Western blots are only semi-quantitative and have very low throughput. The C-ELISA does not require cell lysis, electrophoresis of the sample or membrane blotting. The C-ELISA does allow detection and quantitation of specific

cellular proteins in a 96 well plate format.  Furthermore, the C-ELISA is amenable to automation that facilitates the screening of large numbers of compounds.

In the past several years there has seen an explosion in the availability of commercial sources of antibodies to cell signaling molecules.  In addition, antibodies that selectively recognize post-translationally modified proteins (e.g., phosphorylated, acetylated) have also become available.  In many cases, these antibodies are very high quality as determined by strength of signal and specificity on immunoblots.  These same high affinity, specific antibodies can also be used to detect antigens in fixed cells by immunofluorescence.  The spatial and temporal information derived from these studies can be extremely valuable in delineating the function of post-translational modifications.  For example, phosphorylation can be a trigger for a change in subcellular localization and consequently, a change in protein function.

Unfortunately, for the purposes of high throughput drug discovery, both immunoblots and immunocytofluorescence have severe restrictions.  Immunoblots are not readily adaptable to 96-well plate formatted experimental designs. Although chemilumescent detection systems have made immunoblots extremely sensitive, quantitation is limited by the small linear range inherent in exposed film. More quantitative methods using newer instrumentation (e.g., phosphoroimagers) alleviate some of these difficulties; however, the laborious procedures for preparing cell lysates, determining protein concentrations, loading gels and blotting remain.  Immunocytofluorescence with conventional microscopy is also tedious, relatively insensitive and non-quantitative.  In order to take advantage of the high quality antibodies available for studying cell-signaling pathways, we have developed procedures for combining the best properties of immunocytofluorescence and immunoblots.  This cell-based western (also referred to as cell-based ELISA or C-ELISA) has proved to be extremely useful for medium throughput screens of kinase inhibitors and, in conjunction with biochemical assay data, has become an important SAR driving force.

The key ingredient for a successful cell-based Western assay is a high quality antibody.  Single band antibody specificity must first be established by conventional immunoblot procedures.  Once a suitable antibody is identified, it can be used to stain and quantitate the levels of antigen in cells in individual wells of a 96-well plate.  Cells are plated, treated according to experimental requirements and fixed directly in the wells, similar to an immunocytofluorescence experiment.  After fixing, individual wells go through the same series of steps used for a conventional immunoblot, including blocking, incubation with first antibody, washing, incubation with second antibody, addition of chemilumescent substrates and development.  Finally, results are read in a luminescence plate reader.  Other readouts including fluorescence can be used in this assay format.  In our experience, this procedure provides rapid, quantitative analysis of cellular antigens.  In general, the correlation between immunoblot and C-ELISA assays is quite good (Appendix 1).  The wide linear range of the C-ELISA allows for quantifying >5-fold changes in cellular protein levels in response to stimulant (Appendix 2).  The versatility is only as limited as the availability of high quality antibodies to the target proteins.

**Cell-based ELISA/Western Schematic**

Seed cells in
96 well plate

*Incubate* →

Serum starve cells
Treat with
compounds
and stimulate

*Fix cells and
permeablize* →

Add primary
Ab

*Incubate
Wash* →

Add secondary
Ab with HRP

*Incubate
Wash* →

Add substrate
And read

## B. CELL CULTURE AND TREATMENTS

Culture conditions can be adapted to any cell type. The following recommendations are
based on our collective experiences with primary cells, normal and tumor cell lines,
attached and suspension cells. Validated multi-channel pipettors can be used throughout.
For experiments involving more than 8 to 10 plates, a multi-drop is useful.

1.  Seed cells in a 96-well plate at near confluence 1 day prior to the experiment in
    100 ul of growth medium. Optimal seeding density should be experimentally
    determined (see below).

2.  For poorly adherent cells, it is useful to plate cells in wells coated with poly-D-
    lysine or other extracellular matrix components.

3.  To avoid chemiluminescent signal spillover between wells, cells should be plated
    on white plates. Clear bottoms are convenient because cells can still be viewed
    under the microscope. However, there is a tendency towards "edge effects" with

these plates.  Opaque bottom white plates  can minimize this problem.  Alternatively, outside rows and columns can be excluded from the experimental format.  When a fluorescent readout is being used a black plate should be used.

4.  For growth factor-stimulated responses, the signal window is often increased if cells are serum starved prior to the experiment.  Starvation can be for 2-4 h or overnight depending on the cell type and should be optimized using the experimental design templates (see example below).

5.  Preincubate with compounds ~1-2hr before adding the activating stimulus.  Compounds should be added such that the final DMSO concentration is $\leq$0.25%.  The compounds are diluted into medium containing serum or if in serum free conditions add BSA to a final concentration of 1%.  Dilute the stocks serially to a final concentration of 10X.  You will be adding 10 ul to a total of 100 ul to give a final concentration of the compound of 1X.

6.  Prepare activators as 10x stocks in medium + 1.0% BSA.  This is usually enough BSA to prevent peptides and small molecules from sticking to the sides of the test tubes. Return cells to incubator for the appropriate time.

7.  Stimulate the cells with the reagent that is known to specifically produce the desired response.  The concentration of the stimulant and treatment times will be determined by experimental design.


## C. FIXING AND STAINING

1.  *Stop the reactions by inverting plate and dumping media into appropriate waste container.  Tap gently on absorbent paper several times to remove residual liquid. *For suspension cells, see note 1 below.*

2.  Add fixative (100 ul/well). The standard fixative is 3.7% formaldehyde diluted from commercially available 37% stock solution into PBS.  Alternatively, non-toxic fixatives have recently become available (See reagents below).  Usually a 10-minute incubation at room temperature is sufficient, but this may vary with cell type. Other types of fixatives include glutaraldehye, methanol, etc.

3.  Invert plate and dump fixative into appropriate waste container. Tap gently on absorbent paper several times to remove residual liquid.

4.  Rinse 3 times with PBS + 0.1% Triton X-100 to permeabilize the cells.  Incubate 5 min each time.

5.  Invert, dump and blot. Add blocking buffer for one hour to block non-specific sites.  We have normally used 10%FBS in PBS. *At this point, plates can be*

**stored at 4⁰ overnight ( for several days).**

6.  Invert, dump and blot.  Add first antibody (50-100 ul/well) diluted in Blocking Buffer or PBST + 1%BSA. Incubate 2 hr at room temperature or overnight at 4$^o$. As a guideline, use first antibody concentrations the same as or 2-fold more concentrated than the optimal immunoblot dilution. The optimal concentration will be determined by experimental design.  Incubate the cells with the primary antibody for one hour at room T or overnight at 4$^o$.

7.  Invert, dump and blot.  Wash 3 times with 100 ul/well PBS + 0.1% Triton (Wash Buffer).  One-minute incubation is sufficient at this step.

8.  *Invert, dumb and blot.  Add 100 ul/well of horseradish peroxidase coupled second antibody of appropriate species specificity.  In our hands, a 1:1000 dilution of Amersham Pharmacia antibodies in Blocking Buffer is optimal. Incubate 1hr at room temperature. ***See note 8 for alternative second antibodies.***

9.  Towards the end of the 1hr incubation, prepare the commercially available chemiluminescent substrate solution. Mix equal volumes of the luminol/enhancer and stable peroxide reagents.  Protect from light while solutions equilibrate to room temperature.

10. *Remove second antibody (invert, dump and blot). Wash wells 2 times with 100 ul Wash Buffer and then 3 times with 100 ul PBS. It is important to completely remove the Triton, which interferes with the peroxidase activity.

11. * If the experiment involves more than 1 plate, process only 1 plate at a time. Leave others in the final PBS wash. Starting with the first plate, invert, dump and blot.  Add 100 ul/well of substrate solution.  Wait 1 min. Read luminescence (relative light units, RLU) on standard plate reader at 0.1 second (or longer)/well.

12. While first plate is being scanned, develop second plate, etc.

*Notes:*

*1\*.  For suspension cells, aliquot cells into wells of a poly-D-lysine-coated plate. We normally spin the cells in the plate for 5 minutes at 1000-1500RPM and wait one hour to allow the cells to rest prior to compound addition or stimulation. The alternative method would be to proceed with the reaction after plating the cells and then stopping the reaction by spinning cells at 1000-rpm for5 min.  Invert, dump, and blot.  Proceed with fixation as for adherent cells. At this point the cells will be well stuck to the plate.*

*8\*.  Fluorescent secondary antibodies can also be used with detection on:*
- ♦ *High information/content laser scanning detection instruments are commercially available and have been used extensively for these applications.*
- ♦ *The use of infrared fluorescent labels can be used in the cELISA assays by using commercially available infrared imaging systems.*

*10\*.  The development time significantly influences the luminescence.  Therefore, it is not practical to compare the absolute values of RLU between plates.  Each plate must contain the appropriate controls, such as min and max or standard curve.*

*11\*.  One minute is a convenient reaction time.  Although the absolute values of the RLU increase with time, the relative values are consistent for 5 to 10 minutes.*

## D. REAGENTS

**Plates**

      White plates for chemiluminescent readout
      Black plates for fluorescent readout
      Clear plates if reading colorimetric

**Coating**

      Poly-D-lysine
      Collagen
      Gelatin
      Fibronectin

**Fixatives**

      3.7% Formaldehyde (Sigma catalog #F1635 37% solution) in PBS
      Commercially available non toxic fixative reagents
      Methanol

**Antibodies**

      Primary antibody specific for protein of interest

Secondary antibody:
- ♦ For chemiluminescence: anti-rabbit or anti-mouse IgG HRP conjugate
- ♦ For fluorescence detection using the high content imaging technology use: anti- mouse or anti-rabbit IgG Alexa 488
- ♦ For fluorescence detection using the the infrared use the reagents labeled with IR tags

**Buffers**

Phosphate buffered saline
Wash buffer: PBST (PBS with 0.1% Triton X-100
Antibody dilution and blocking buffer:  PBS with 0.5% BSA and 0.1% Triton X-100

**Substrates**

Colorimetric: TMB
Chemiluminescent: Commercially available chemiluminescent reagents


# E. ASSAY VALIDATION

1. Check all pipettors to ensure that each channel is precise and accurate.

2.  Determine signal window. For statistical analysis, it is important to verify that well-to-well variation is minimal and that the signal window (i.e., difference between min and max values) is large enough to yield reproducible analyses. Perform the C-ELISA using the template provided below

# Plate Variability Layout:

**Plate 1**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | H | M | L | H | M | L | H | M | L | H | M | L |
| 2 | H | M | L | H | M | L | H | M | L | H | M | L |
| 3 | H | M | L | H | M | L | H | M | L | H | M | L |
| 4 | H | M | L | H | M | L | H | M | L | H | M | L |
| 5 | H | M | L | H | M | L | H | M | L | H | M | L |
| 6 | H | M | L | H | M | L | H | M | L | H | M | L |
| 7 | H | M | L | H | M | L | H | M | L | H | M | L |
| 8 | H | M | L | H | M | L | H | M | L | H | M | L |

**Plate 2**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | L | H | M | L | H | M | L | H | M | L | H | M |
| 2 | L | H | M | L | H | M | L | H | M | L | H | M |
| 3 | L | H | M | L | H | M | L | H | M | L | H | M |
| 4 | L | H | M | L | H | M | L | H | M | L | H | M |
| 5 | L | H | M | L | H | M | L | H | M | L | H | M |
| 6 | L | H | M | L | H | M | L | H | M | L | H | M |
| 7 | L | H | M | L | H | M | L | H | M | L | H | M |
| 8 | L | H | M | L | H | M | L | H | M | L | H | M |

**Plate 3**

| Row | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
|-----|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| 1 | M | L | H | M | L | H | M | L | H | M | L | H |
| 2 | M | L | H | M | L | H | M | L | H | M | L | H |
| 3 | M | L | H | M | L | H | M | L | H | M | L | H |
| 4 | M | L | H | M | L | H | M | L | H | M | L | H |
| 5 | M | L | H | M | L | H | M | L | H | M | L | H |
| 6 | M | L | H | M | L | H | M | L | H | M | L | H |
| 7 | M | L | H | M | L | H | M | L | H | M | L | H |
| 8 | M | L | H | M | L | H | M | L | H | M | L | H |

where:

"H" is the maximum (HI) signal, "M" is the mid-level signal, and "L" is the minimum (LO) signal.

See the QB Handbook for definitions of each signal for each assay format

Edge effects, which are not uncommon in this type of assay, will be seen by comparing min and max values throughout the 96 well plate.  Consistent deviations in rows or columns will be noted, and should be corrected or accounted for before validation proceeds. The signal window size is also calculated and should be $\geq 2$.

3.  Response Surface Design: *Example of Experimental Design to Develop and Optimize a C-ELISA*
    To optimize the assay using experimental design it is best to work directly with a statistician..  They will help in setting up the design and with analysis of the data.
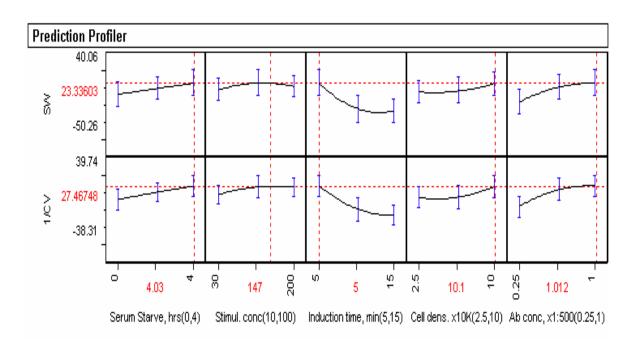
**Experimental factors**

- •HUVEC cells with ERK p42/p44 Antibody
- •Serum Starve time (0 - 4 hrs)
- •VEGF conc. (originally 10-100.  Changed to 30-200)
- •Cell Density (2.5 - 10 x$10^3$ cells)
- •Antibody conc. (1:2000, 1:1000, 1:500)

**Results from Response Surface Experiment**

- Serum Starvation greater than 4 hours
- VegF conc. higher than 100 ng/ml
- Induction time less than 5 minutes
- Cell Density 20,00 cells per well



11
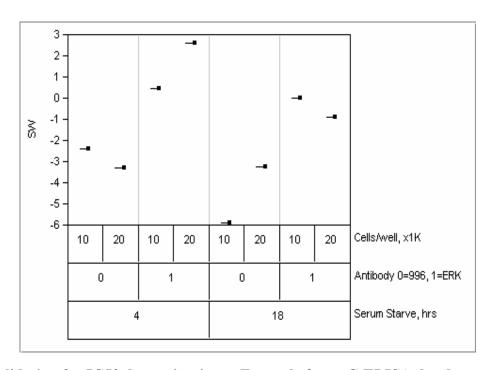
**Optimization experiment - Example from  C-ELISA development**


## Variables evaluated

- Plating density: Cells at 10 or 20,000 cells per well
- Antibodies: Two antibodies pKDR (996) and pERK p42/44
- Culture conditions: Serum starvation for 4 hours or overnight


## Optimal Conditions Results

HUVEC cells at 20,000 cells per well stimulated for 4 minutes at 150 ng/ml
VEGF using 1:500 of pERK p42/44
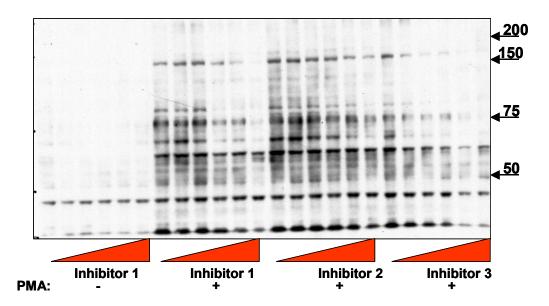


**Assay validation for IC50 determinations - Example from  C-ELISA development**

After optimal conditions are determined test control compounds to determine if IC50's
can be obtained in the assay.
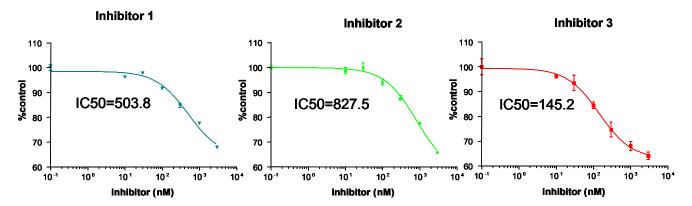
Follow this with plate to plate and within plate variability using the plate validation
templates.  If the assay passes plate validation then proceed with analyzing compounds in
a test retest assay.  See section X11 of the QB manual for test retest.

## APPENDIX 1.  IMMUNOBLOT VS. CYTOBLOT ASSAYS FOR ENZYME INHIBITOR POTENCIES

### ENZYME INHIBITION:  WESTERN ANALYSIS
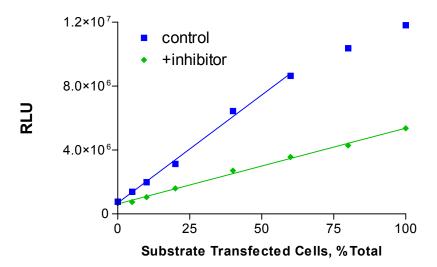


### ENZYME INHIBITION:  C-ELISA ANALYSIS



**Enzyme inhibitor profiling.**  A. Immunoblot analysis.  Confluent HEK293 cells were serum-starved for 2h before incubating with increasing concentrations of specific enzyme inhibitors 1, 2 &3 for 1h.  Where indicated, PMA was added for 10 min.  Cell lysates were collected, electrophoresed and blotted.  Blots were stained with a degenerate antibody to the specific enzyme phosphorylation consensus sequence that recognizes the phosphorylated form of several substrates in these cells. B. C-ELISA was performed on parallel cultures using duplicate wells per data point.

## F.  APPENDIX 2.  LINEARITY AND SENSITIVITY OF CELL-BASED WESTERN ASSAYS



**Cell-based Western:
Linearity with Cell Number**

HEK293 cells were transfected with a specific enayme substrate using standard procedures.  The next day, transfected and control (not transfected) cells were harvested and mixed together in the proportions indicated.  The mixed populations were seeded into individual wells of a 96-well plate and cultured for an additional 24h.  Cells were serum starved for 2 h in medium containing 0.1% BSA before adding a specific enzyme inhibitor. After additional 1 h incubation, PMA (200 nM) was added for 10 min.  Cells were then processed according to the C-ELISA protocol.  Phosphorylated substrate was measured using an antibody that selectively recognizes the exogenous enzyme substrate expressed only in the transfected cells.

References
http://www.licor.com/
http://www.activemotif.com/
\http://www.selectscience.net/users-views/ttp-labtech/acumen-explorer
http://www.cellomics.com/

# SECTION IX

# FLIPR™ ASSAYS FOR GPCR
# AND ION CHANNEL TARGETS

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# Table of Contents

## SECTION IX:
## FLIPR™ASSAYS TO MEASURE GPCR
## AND ION CHANNEL TARGETS

# OVERVIEW: FLIPR™ ASSAY DEVELOPMENT

**Reagents Needed:**
Cell line(s) expressing GPCRs, ion Channels, and coupling proteins.
Control cell line without target.
Suitable fluorescent dye (e.g. Fluo-3AMA, Calcium 4, etc).
Suitable agonist or ion channel modulators.
Standard antagonists, potentiators, and control compounds.
Appropriate buffer solutions, additives, etc.

**Determine Preliminary Assay Conditions and Design**
(based on literature references, previous assays run, etc)

**Cell Culture Conditions:**
Culture medium and conditions
 (timing, temp, stability)
Plating (starvation) medium
Seeding density
Dye loading conditions
DMSO tolerance
Trypsinization vs enzyme-free

**FLIPR™ Optimization:**
Calibration and performance
 check
Temperature control
Liquid handling calibration
Instrument linearity check
Kinetic read-out conditions

**Plate type Selection:**
96 vs 384 wells
Poly-D-lysine coated
Clear-bottom, black sides
Cell plating conditions

**Assay Development and Optimization:**
Determine volume of additions for agonist, buffers & compound to plated cells.
Determine timing of compound addition to cells (i.e. pre-incubation desired?)
Set height and speed of FLIPR™ pipettor. Is mixing step needed for consistency?
Compare results for a practical range of cell densities.
Establish baseline signal with control cells & determine signal window/Z' factor.
Test agonist, antagonist, potentiator responses with control molecules as
 appropriate.
Determine day-to-day reproducibility of agonist $EC_{10}$ or $EC_{90}$ response.
Statistical analysis of preliminary data.
Statistical experimental design if appropriate.

**Assay Validation (Pre-Study):**
Plate-uniformity experiments to establish mid-points and detect edge effects.
Concentration-response curve reproducibility ($EC_{50}/IC_{50}$) of known agonists,
 antagonists or potentiators.
Estimate day-to-day and within-plate variabilities and potential signal drift.
Compile pre-study assay validation and development documents containing
 detailed experimental procedures.

## A. **INTRODUCTION**

The introduction of FLIPR™ (Fluorescence Imaging Plate Reader) in the 1990's provided biologists with a fast and easy method of detecting GPCR activation through changes in intracellular calcium concentration.  By coupling receptors to Gq proteins which stimulate intracellular calcium flux upon binding, a functional response can be measured using calcium-sensitive dyes and a fluorescence plate reader. The FLIPR™ instrument has a cooled CCD camera imaging system which collects the signal from each well of a microplate simultaneously.  The FLIPR™ can read at sub-second intervals, which enables the kinetics of the response to be captured, and has an integrated pipettor that may be programmed for successive liquid additions.
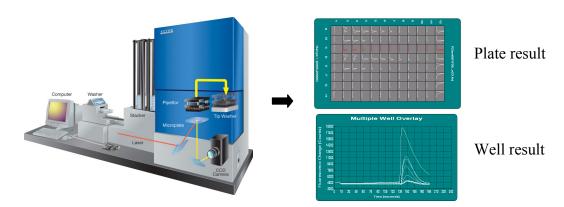


**Figure 1**.  Diagram of a FLIPR™ instrument and typical kinetic tracings.  The FLIPR™ collects a signal from each well of a multi-well plate at sub-second intervals, which captures and records a kinetic tracing of the calcium flux response.  By successive additions to the same well, the FLIPR™ instrument allows one to distinguish between agonist, antagonist and allosteric modulators.

The integrated pipettor capabilities of the FLIPR™ provide an opportunity to detect agonists, antagonists, and allosteric modulators of GPCRs all in one assay.  In the first addition, compounds of screening interest are added.  The timing can be adjusted to allow for a pre-incubation period with the compounds, and agonist activity is detected by monitoring the calcium flux response in this step.  In the second addition, a small amount of a known agonist that results in ~10% of maximal response is added to detect potentiator activity.  The third addition consists of a maximal concentration of known agonist (~90% of the maximal response) to test for antagonism.  This experimental design can encompass either two or three additions depending on the specific responses to be detected.

The FLIPR™ has also been utilized to screen ion channel targets using membrane permeable fluorescent dyes, such as the bis-oxanol dye DiBAC$_4$(3), to measure changes in membrane potential.  Compared to the rapid sub-second kinetics of

channel opening observed by electrophysiology approaches, redistribution of the dye often takes minutes to produce a measurable response, and has prompted the development of more rapid dyes compatible with the FLIPR™.

## B. **TYPES OF FLIPR™ FORMATS**

### I. <u>GPCR Targets Coupled to Ca$^{\pm 2}$ Mobilization</u>

GPCR targets that naturally couple via $G_q$ produce a ligand-dependent increase in intracellular Ca$^{+2}$ that can be measured using a calcium-sensitive dye. $G_{I/o}$-coupled GPCR receptor activation can be "switched" to induce an increase in intracellular calcium in two ways: by the use of chimeric G-proteins (G$\alpha_{qi5}$ or G$\alpha_{qo5}$), or by engineering the cells to over-express a promiscuous G-protein (G $\alpha_{16}$ or G$\alpha_{15}$).



*Adapted from Nature Reviews - Drug Discovery*
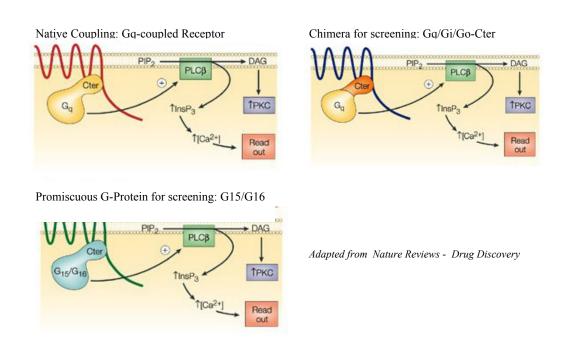
**Figure 2**. GPCR targets that couple via $G_q$ naturally produce an increase in intracellular Ca$^{+2}$ that can be measured using calcium-sensitive dyes and a FLIPR™ instrument. GPCR targets that naturally couple via $G_{I/o}$ can be adapted to respond to agonist with a ligand-dependent increase in intracellular calcium by the use of chimeric G-protein or by the introduction of an over-expressing promiscuous G-protein (G $\alpha_{15}$ or G $\alpha_{16}$).

The integrated pipettor capabilities of the FLIPR$^{TM}$, as well as internal software modifications, provide an opportunity to detect agonists, antagonists, and allosteric modulators all in one assay.  One-, two-, or three-addition assays may be performed depending on the desired assay format.  A one-addition assay can be performed to detect agonists, where the compound of interest is added to look for a response.  This mode could also be used to look for allosteric modulators or antagonists if the test compounds are added "off-line", although this is not the preferred method of operation.  Until 2006, the two-addition assay was the standard assay format.  In this method, the test compounds are added in the presence of an EC$_{10}$ dose of the agonist in the first addition to detect agonists or allosteric modulators.  The second addition is an EC$_{90}$ dose of the max control to identify antagonists.  While this scheme works, it requires a secondary assay to distinguish the agonists from the allosteric modulators; this need was abolished by the advent of a three-addition assay.  In the three-addition mode, you can detect all three modes of activity in a single assay, saving considerable time and reagents.  Another advantage found during testing of the three-addition assay was better mixing and a pre-incubation of the cells with compound resulting in better identification of potentiators.  Typical assay formats and the resulting curves are summarized below (Table 1).

**Table 1.  Typical FLIPR™ Assay Formats**

| Agonist | Potentiator | Antagonist | First Addition | Second Addition | Third Addition | Inactive Cmpd Profile | "Hit" Profile |
|---------|-------------|------------|----------------|-----------------|----------------|------------------------|----------------|
| X | | | Compound | None | None |  |  |
| X | X | | Compound | 10% Agonist | None |  |  |
| X | | X | Compound | 90% Agonist | None |  |  |
| X | X | X | Compound | 10% Agonist | 90% Agonist |  |  |

## II.  Ion Channels with Significant Ca2+ Permeability

Ion channel targets with significant $Ca^{+2}$ permeability, such as the iGluRs, produce an increase in intracellular calcium that can be measured using calcium-sensitive dyes and the FLIPR™ instrument.  The methodology used is analogous to that for the GPCRs.
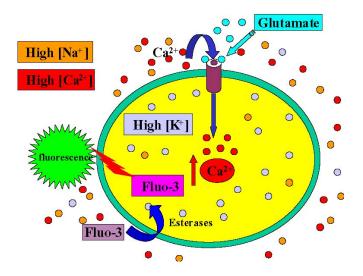


**Figure 3.**  Schematic of the calcium flux response in ion channel targets.

## III.  Ion Channels which Produce Significant Changes in Membrane Potential

Ion channel targets such as the iGluRs with ion permeability that significantly affects the membrane potential can be measured using a membrane potential dye and the FLIPR™ (see Baxter, *et al*).
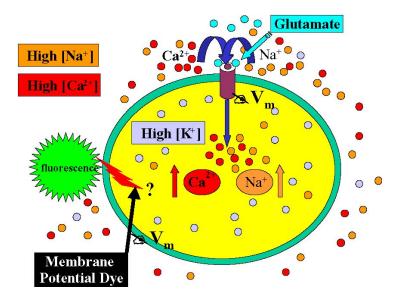


**Figure 4.**  Measuring changes in membrane potential of ion channel targets.

## C. REAGENTS AND BUFFERS FOR METHOD DEVELOPMENT

It is critical to ensure the appropriate cell lines expressing the target, control agonist and antagonist standards are available before beginning method development and validation.  The minimal requirements are:

1.  Transfected cell line with the Gq-coupled hGPCR target. (eg. HEK293, CHO, THP-1 etc.). Receptors coupling through Gi, Go, Gs or Gz can be coupled to Gq via promiscuous G proteins as previously described.

2.  Parental cell line control without the target and grown under identical conditions.

3.  Agonist, antagonist, and allosteric modulator reference standards (with a wide range of potencies, if available).

4.  Poly-D-lysine coated 96- or 384-well plates.

5.  Appropriate cell growth media, buffer solutions, trypsinizing reagents.

6.  The reagents for ion channels are the same as for GPCRs, with the exception of the FLIPR™ buffer.  It is recommended that 5mM calcium be used in the buffer for ion channel experiments.  Since HBSS contains 1.3mM calcium, 3.7mM calcium chloride (Sigma) must be added prior to use.

7.  Additional reagents needed for a FLIPR assay:

| Reagent | Manufacturer |
|---|---|
| Calcium dyes (Fluo-3, Fluo-4, Calcium 3, Calcium 4, etc) | Molecular Probes, Molecular Devices |
| HBSS | BioWhittaker, Invitrogen |
| HEPES | BioWhittaker, Invitrogen |
| Probenecid (if needed) | Sigma |
| Pluronic Acid | Sigma, Molecular Devices |

## D. METHOD DEVELOPMENT AND OPTIMIZATION

### I. Optimization Experiments for GPCR Targets Coupled to Ca+2 Mobilization

Early method development should include the following experiments to demonstrate the validity of the assay concept:

1. Gq coupling (or promiscuous G-protein coupling) of the cells expressing the GPCR should be demonstrated.  Load selected cell clones with Fluo-3AM or other suitable dye, trigger Ca$^{+2}$ flux with a known agonist, and measure fluorescence signal.  Select the clones with the most robust response.

2.  Determine whether cells need to be constantly maintained in culture or whether they can be prepared as frozen aliquots to be thawed and plated the day prior to the assay.  The use of frozen cell stocks is a convenient and efficient alternative if it can be shown that the FLIPR™ signal is sufficiently robust and stable.

3.  Conduct dye-loading experiments.  Select the combination of cell line, agonist and dye concentrations that produces the most significant signal window.  Use a control cell line without receptor expression to establish signal base line.  Choose between use of cells in culture and frozen cell stocks.

4.  Conduct preliminary experiments to establish a reasonable cell density that could be further optimized in subsequent experiments as described below.

5.  Using a known antagonist or potentiator, demonstrate that the $Ca^{+2}$ mobilization induced by the agonist can be blocked or enhanced, respectively.

6.  Test poly-D-lysine coated plates with selected cell lines and conditions demonstrated in preliminary experiments.  Select the plate with a stable and acceptable signal window.

7.  Establish preliminary growth conditions and DMSO tolerance for the selected cell line.

Statistical experimental design can be employed to optimize these conditions and the following factors should be included:
1.      Cell clones
2.      Cell seeding density/well
3.      Type of dye (wash vs. no-wash)
4.      Dye loading concentration
5.      Dye loading temperature
6.      Dye loading duration
7.      Coated plate type
8.      Buffer additives: eg: probenecid, concanavalin A, etc.
9.      Height, speed and mixing of FLIPR pipettor
10.     Volume of addition


**<u>Notes on optimization experiments for GPCR targets coupled to $Ca^{+2}$ mobilization</u>:**

Some general points regarding a FLIPR™ assay for GPCRs need to be noted:

- Some receptors contain trypsin-sensitive sites in their extracellular domain that results in a loss of response if the cells are harvested by trypsinization.  In these instances, cells should be harvested by either scraping or using enzyme-free dissociation buffer.

- Care should be taken when removing media and dye from the cell plate.  It is common for mechanical aspiration to disrupt the cell monolayer, resulting in a

deterioration of the assay performance.  It is recommended to manually invert the plate and shake or "flick" the liquid out of the plate and blot onto paper towels if you are using a dye that requires washing.

- Several no-wash dyes are commercially available.  Testing of multiple dyes is strongly recommended, as signals differ widely.  Depending on the receptor studied, media may interfere with the no-wash dyes, so testing both with and without media may be required.  An example of the difference between the signal obtained from the traditional Fluo-3 dye and the new Calcium 4 no-wash dye is shown in Figure 5.
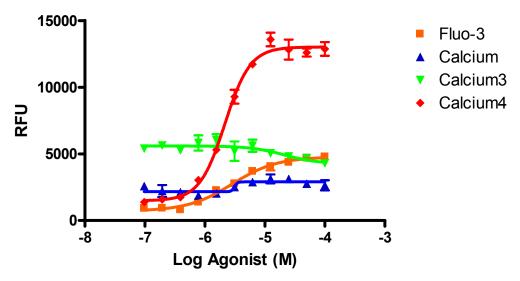


**Figure 5:**  Comparison of different $Ca^{+2}$ dyes on maximum response of a GPCR.  In this example, a no-wash dye produced a significantly larger signal window than the traditional Fluo-3 dye.  Signal windows are specific to receptors and cell lines, so it is recommended that testing be done during the initial optimization to ensure the appropriate choice of dye.

- Probenecid should be included in the dye and the buffer following dye loading whenever using CHO cells (5mM probenecid is sufficient).  This prevents the release of dye from the cells back into the medium.  AV12 and HEK293 cells do not require probenecid.

- CHO cells are dye-loaded at 37°C, whereas AV12 and HEK293 cells can be dye-loaded at 25°C.

- Poly-D lysine coated plates can improve the results obtained from some cell lines.

- Variability in the signal obtained on the FLIPR™ can sometimes be improved by adjusting the tip height or dispense speed on the FLIPR™.

- The standard assay buffer used in FLIPR™ experiments is HBSS with 20mM HEPES.

- The most common fluid addition volumes for a FLIPR assay are:

| | Volume per Well | |
|---|---|---|
| | 96-well Format | 384-well Format |
| Dye | 50 µl | 20 µl |
| Buffer | 50 µl | 20 µl |
| 1$^{st}$ Addition | 50 µl | 20 µl |
| 2$^{nd}$ Addition | 100 µl | 20 µl |

The development of a FLIPR assay generally requires the following experiments:

*1. Cell density determination and incubation time:*
This is typically the first parameter that is examined. The best way to assess cell density requirements is to seed an entire assay plate at a single density; therefore, several plates are required to examine multiple cell seeding densities. The cells should be examined on the FLIPR™ using buffer in the first addition and a maximal concentration of agonist in the second addition. This will allow one to assess the extent of variability within the plate and detect any patterns in variability. The most common variability pattern we have observed is an edge effect which can usually be resolved by increasing the cell density or the humidity during incubation. We recommend examining the following cell densities for the indicated cell types:

| | Seeding Densities (cells/well) | |
|---|---|---|
| **Cell Line** | **96-well Format** | **384-well Format** |
| AV12 | 30K, 40, 50K, 60K | 20K, 30, 40K, 50K, 60K |
| CHO | 10K, 20K, 30K, 40K | 5K, 10K, 15K, 20K, 30K |
| HEK293 | 30K, 40K, 50K, 60K | 20K, 30K, 40K, 50K, 60K |

Some assays will perform best with a 24-hour incubation time prior to assay, while others may need a 48-hour incubation time.

*2. Dye loading time, dye concentration and temperature:*
The optimal dye loading can range from 30 minutes to 2 hours depending on the cell line and the dye used. The concentration of Fluo-3 used in the majority of FLIPR assays is 8µM. Lower concentrations can be examined in order to reduce the cost of the assay. The no-wash dyes have been shown to be effective at lower concentrations as well. CHO cells are dye loaded at 37°C, whereas AV12 and HEK293 cells can be dye loaded at 25°C.

*3. DMSO tolerance:*
> DMSO can alter the response of the cells as well as shift the dose response curve for agonist. It is recommended to perform an agonist dose response curve in the presence of different concentrations of DMSO in order to assess the DMSO tolerance of the assay. Extreme care should be taken if a DMSO concentration >0.1% is required.

*4. Agonist/antagonist dose response curves:*
> The reproducibility of the assay can be examined by performing two independent days of agonist/antagonist/or potentiator dose-response curves. The $EC_{50}/IC_{50}$ values should remain relatively constant over the course of the two experiments.

*5. Full plate variability and Z' factor determination:*
> The variability of the assay is determined by running triplicate max/mid/min plates on three days and then calculating the Z'factor.

**Considerations when performing 384-well FLIPR™ assays:**

   384-well FLIPR assays have a number of challenges that are not apparent in the 96-well format. The first is mixing in the well. Most 96-well experiments are designed to allow a larger volume to be added to a larger space where mixing is not a concern. In a typical 96-well assay, 50µl of test compound are added to 50µl of buffer in the cell plate at a height of approximately 80 to 95µl. The height is the liquid height in the well at which the tips dispense. The 384-well plate is limited to a maximum volume of a 30-µl addition in a much smaller diameter well, and using the 96-well technique will result in variable response. When adding to a 384-well plate, the tips are typically in the buffer solution of the cell plate when the dispense takes place. In a number of cases, the speed of dispense has to be increased as well. These heights and speeds should be tested with buffer to check for unwanted "pre-firing" of the cells. Another issue that arises with the 384-well format is the limited amount of diluent that can be added to the compound plate. This limitation can result in having to create intermediate dilution plates off-line, thereby slowing throughput and adding costly consumables. This has been eliminated by using an in-tip dilution on the FLIPR™ (Figure 6). Although the final DMSO concentration is the same, the bolus of DMSO in the bottom of the tip can have an effect on the cells (Figures 7a and b). In our hands, a ratio of 15µl buffer/5µl compound was found to have the least DMSO effect. However since this result can be variable, different combinations should be tested during development. This in-tip dilution method can be used in both the two- and three-addition FLIPR™ methods.
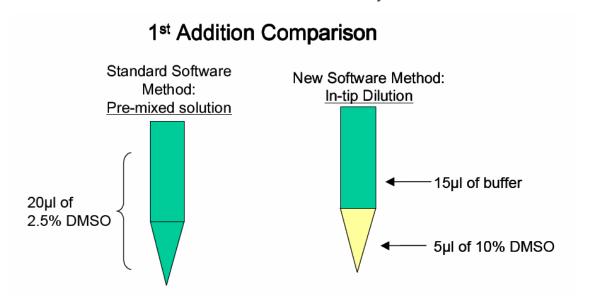
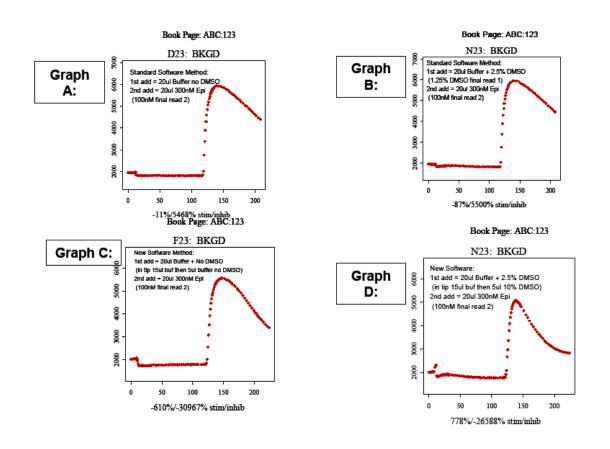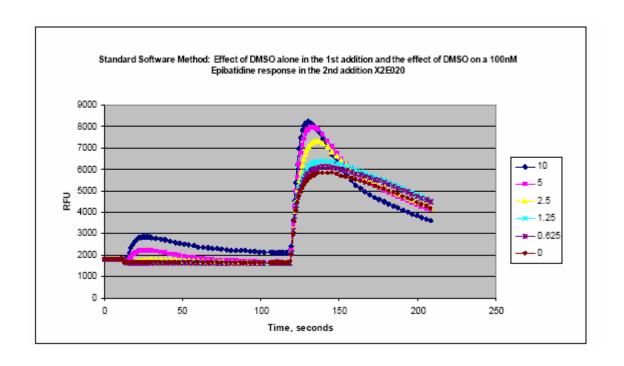**Figure 6**.  Schematic of in-tip dilution method.



**Figure 7(a).**  Effects of bolus of DMSO on shapes of kinetic tracings.
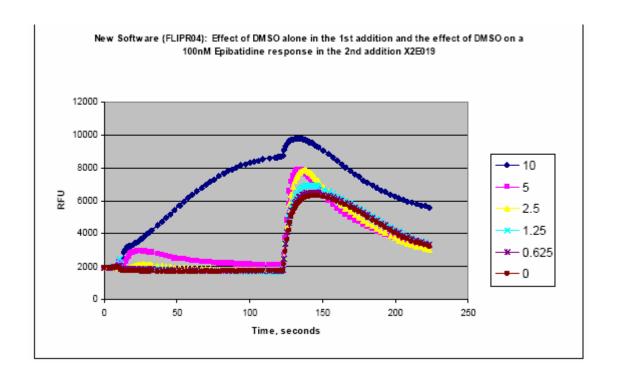
**Figure 7(b).** Effects of bolus of DMSO on shapes of kinetic tracings.

**Notes on tip washing:**

The FLIPR™-2 and FLIPR™-3 have tip wash stations that can be incorporated into the assay to eliminate the need to change tips. This allows one to use reservoirs without fear of cross contamination among the test compounds. In addition, a DMSO pre-wash can be performed at the tip load station with the proper adapter. When running a single-point screen of more than 100K compounds, tip washing should be tested first to minimize cost and maximize throughput. Occasionally, the compound used for the EC$_{90}$ addition cannot be washed off the tips, resulting in significant carry-over of active compounds in to the subsequent plate (example in Figure 8); in these cases, the tips will have to be changed. This typically happens when peptides are added as the EC$_{90}$ dose.
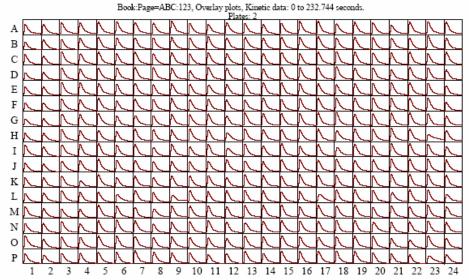


**Figure 8(a).** Example of max addition with tip wash in agonist/potentiator assay.
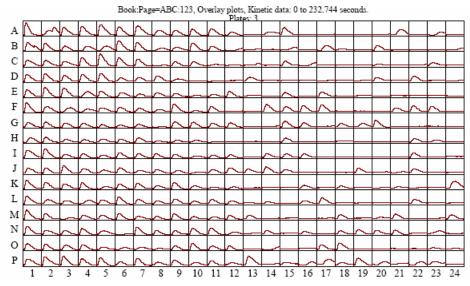


**Figure 8(b).** Carry-over from tips in (a) in subsequent plate (buffer addition only).

## II.  Optimization Experiments for Ion Channel Targets with Ca$^{+2}$ Permeability
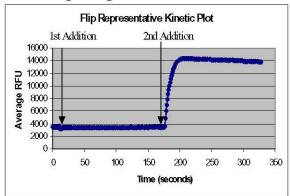
Some ion channels (e.g. ionotropic glutamate receptors) differ from GPCRs in that they desensitize very quickly to agonist exposure, and in most cases, it is not possible to see a response in FLIPR™ with agonist alone.  Such targets require the use of agents that decrease the rate of desensitization, which are called channel modulators or "clamps".  The choice of which channel modulator to use is dependent upon the receptor.  The following is a brief summary of modulators that we have used:

| Receptor | Channel modulator |
|---|---|
| iGluR1 flip | Cyclothiazide (CTZ) |
| iGluR1 flop | LY compound |
| iGluR4 flip | Cyclothiazide (CTZ) |
| iGluR4 flop | LY compound |
| iGluR5 & 6 | Concanavalin A (Con A) |
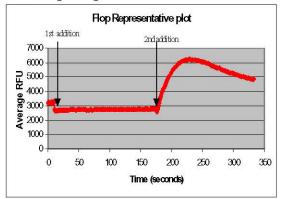
Since ion channel modulators are needed to decrease the rate of desensitization of the channel to agonist, the assay design is somewhat different than for GPCRs.  Like for GPCRs, the ability of the FLIPR™ to make two fluid additions to the cells enables the detection of agonists, antagonists, and allosteric modulators in one assay.  Representative kinetic profiles for iGluR1 flip and flop are shown in Figure 9A.  Test compounds are added in the first addition along with a 90% dose of the known agonist, in this case glutamate, which normally does not generate a measurable Ca$^{+2}$ response because the rate at which the receptor desensitizes is too fast to be detected on the FLIPR™.  A response in the first read will indicate that the test compound is either a non-desensitizing agonist or a positive allosteric modulator (Figure 9B).  The second addition consists of an optimal concentration (~90%) of a known allosteric modulator which results in maximal response by clamping the channel open and decreasing receptor desensitization.  A reduced response in the second read will indicate that the compound is an antagonist (Figure 9C).  The question of whether the compound is a non-desensitizing agonist or an allosteric modulator will be answered in the secondary assay in which the compound is added in the absence of any glutamate in the first read.  If the compound alone elicits a response, it is a non-desensitizing agonist.  Alternatively, if the compound only gives a response in the presence of glutamate (read 2), then it is a potentiator.

In the case of the Kainate receptor iGluR6, the allosteric modulator ConA needs to be incubated on the cells for a minimum of 5 minutes prior to adding agonist.  ConA takes longer to bind and has an effect on receptor desensitization.
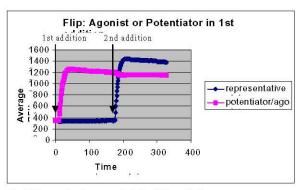
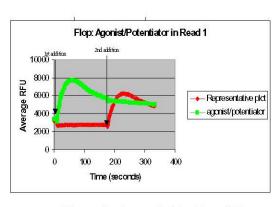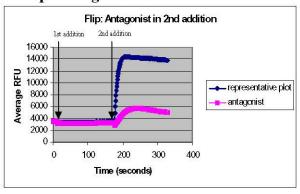**A.  Flip:  Representative Kinetic Plot**        **Flop: Representative Kinetic Plot**



**B.  Flip: Agonist or Potentiator in Read 1**        **Flop: Agonist or Potentiator in Read1**



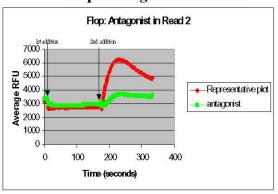**C. Flip: Antagonist in Read 2**        **Flop: Antagonist in Read 2**



**Figure 9.**  Expected kinetic profiles of iGluR1 Flip and Flop receptors.
(A) Expected kinetic profile of 0.5mM glutamate (agonist) in the 1$^{st}$ addition followed by 20µM LY (allosteric modulator) in the 2$^{nd}$ addition.  (B) Expected kinetic profile of an agonist or an allosteric modulator where 20µM LY (control potentiator) and 0.5mM glutamate are added in 1$^{st}$ addition.  (C) Expected kinetic profile of an antagonist where 10µM NBQX (control inhibitor) and 0.5mM are added in the 1$^{st}$ addition, followed by 20µM LY in the second addition.  In B and C, the test compounds will be added at the 1$^{st}$ addition with 0.5mM glutamate, followed by 20µM LY in the 2$^{nd}$ addition.

## III.  Optimization Experiments for Ion Channel Targets with Ion Permeability that Significantly Impacts Cell Membrane Potential

Changes in membrane potential associated with ion channel activity may be measured on the FLIPR™ instrument using a voltage-sensitive dye available from Molecular Devices.  The following are some of the parameters that need to be considered in developing a FLIPR™-based membrane potential assay:

*Cell Density*:

Optimal cell conditions for the FLIPR membrane potential assay require the creation of a confluent cell monolayer.  The cell seeding density depends on the cell type and the time in culture following the plating of the cells.  Receptor expression levels can change with the cell passage number or as a result of the drug-selection conditions used for cell maintenance.  Thus, it is critical to monitor changes in functional activity over time.  Refer to the previous in this chapter for optimizing the cell seeding density.

*Assay Buffer:*

HBSS + 20mM HEPES + added $CaCl_2$ (5mM final concentration).

*Preparation of Membrane potential dye:*

We recommend dissolving the dye in assay buffer.  After formulation, the loading buffer can be stored frozen in aliquots for several months without loss of activity.

*Method of Dye Loading Cells:*

Dilute the loading buffer 1:1 with assay buffer.  Aspirate the media from the cells and add 100µl of diluted buffer per well for 96-well plates.  (Note: We have not had success following the Molecular Devices recommendation of adding the dye directly to the media with the iGluR targets.)  The dye:buffer ratio can be optimized to reduce cost of the assay.  Dye-loading the cells should be tested at 37C and at ambient temperature.  The optimal dye loading time, on average, for HEK293 cells is 60 minutes, but the range can be wide (5-60min).

*Antagonist Assays – Results Export Range:*

The kinetic profile of the calcium response to ion channel activation is prolonged when compared to the typical profiles generated by GPCR activation.   As a result, agonists introduced in the first addition, read frame I, will lead to a baseline shift which will not return to baseline prior to the second addition, read frame II (see figure 9B).  This baseline shift within read frame II is due to the prolonged activation of receptor when agonists are introduced.  Because the $EC_{90}$ challenge dose for antagonists assays is added within the initial portion of read frame II, the read frame I baseline shift due to agonists will lead to antagonist assay interference if exporting data from read frame II only (Max-Min).  For this reason, one should consider exporting both read frames I and II for ion channel antagonist assays, which includes the pre-compound addition portion of read frame I, to capture the pre-compound addition or actual assay baseline (Figure 9B,

time 0-350 seconds).  By utilizing the pre-compound addition baseline of read frame I, false positive agonist interference in antagonist ion channel targets can be avoided.

***Clamp:***

Clamping agents such as Concanavalin A may be required to prevent rapid desensitization of ion channels.  Depending on the incubation time required for the clamp, it could either be added with the loading buffer or it could be added with the compound.

***FLIPR setting:***

Choose filter #2 in the experiment setup of the FLIPR™ software to measure membrane potential. Set the background reading ~ 20000 RFU.  The following are some of the recommended setup parameters for the compound ($1^{st}$ addition) and agonist ($2^{nd}$ addition) additions to a 96-well plate.

|  | Volume | Addition Speed | Pipettor Height |
|---|---|---|---|
| $1^{st}$ Addition | 50µl | 50µl/sec | 100µl |
| $2^{nd}$ Addition | 50µl | 50µl/sec | 150µl |

***Control:***

We recommend running a KCl dose curve as a positive control to measure changes in membrane potential independent of the ion channel activity.  The following is an example of time course tracings observed with the iGluR6 assay (Figure 10).
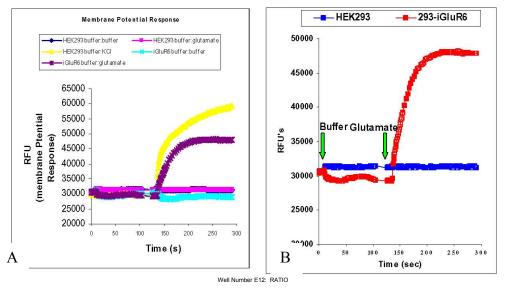


Figure 10(A).  The HEK293 response to KCl vs the 293-iGluR6 response to glutamate.  (B) HEK293 and 293-iGluR responses to glutamate.

## Performing FLIPR™ using a non-adherent cell line:

So far, we have been describing methods appropriate for adherent cells cultures.  In these cases, dye can be loaded directly onto cells grown to confluency in microtiter plates.  In contrast, when the transfected cell line is weakly adherent or grows in suspension culture the following procedures should be followed:
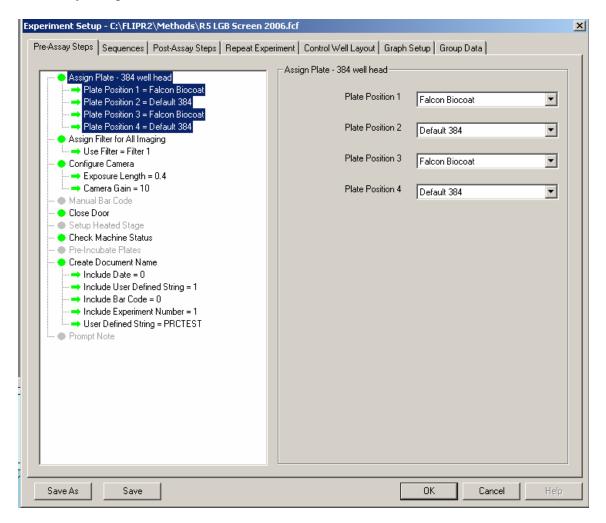
1.  Remove growth media from cell culture flask.
2.  Add 10ml PBS to each flask to rinse.
3.  Remove PBS and repeat rinse step.
4.  Add 10ml cell dissociation buffer to each flask.
5.  Rock flask gently.
6.  Add 10ml Alpha-MEM and discard the rinse.
7.  Transfer cells to 50ml centrifuge tube.
8.  Add 30 ml buffer.
9.  Pellet cells for 5 min at 2000 rpm.
10. Remove supernatant.
11. Add 30ml buffer with 30 µL Fluo-3 AM (1:1000 dilution) and 30 µl pluronic acid.
12. Cover tube with foil and shake gently.
13. Place on shaker for 60 min at 180 rpm at room temperature
14. Fill up tube with buffer and spin for 5 min at 2000 rpm and remove supernatant.
15. Repeat step #14.
16. Resuspend cells at $1 \times 10^6$ cells/ml.
17. Plate 50 µl/well of Poly-D-Lysine pre-coated plates.
18. Wait 20 min and centrifuge plates for 3 min at 1500 rpm.
19. Place plates in FLIPR until ready for use.

Notes:
- If cells are weakly adherent, start at step #1.
- If cells are in suspension, start at step #7.
- If using a no-wash dye, skip steps #14-15.

## E.  FLIPR INSTRUMENT SET-UP

Pre-Assay Setup for FLIPR™-2 and -3:



In this screen, which is the same for 96- or 384-well assay set-up, the user defines the labware used in the experiment from a drop-down list.  The other options on this screen are the filter selection, camera configuration, and the output file setup.
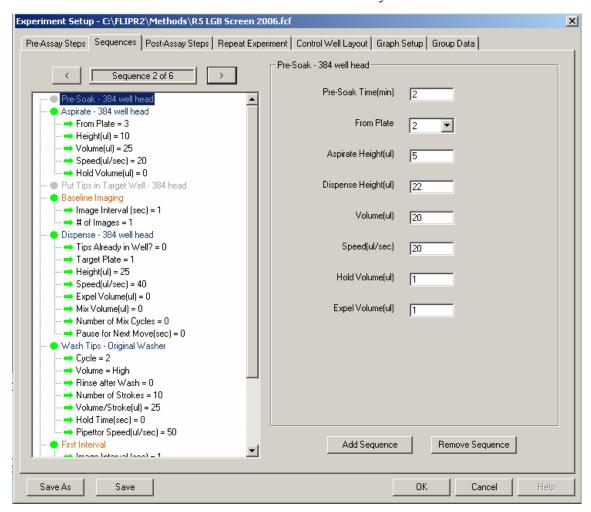
1. Assign Plate:  This is where the user configures the deck layout. If the plate you are using is not included, there is a default 96-well and default 384-well that can be used until the correct plate is defined.

2. Camera configuration:  The exposure length is typically set to 0.4 secs.  The gain is only applicable to The FLIPR™-3 with the Andor camera.

Note: To adjust the baseline signal of the plate, first adjust the laser intensity from the keypad before adjusting the exposure time. This should be done for each plate to set the same baseline over a run.

3. Filter selection: The FLIPR™ has a two-position filter slide. Typically, filter #1 is a 488-nm filter used for calcium assays, and filter #2 is either blank or a 535-nm filter for membrane potential assays.

4. Create document name: This is where the filename is created. A "1" in the field means this will be included in the file name and a "0" means it will not. A few issues deserve a warning here: If you use the date only, it is very possible that the data generated will be overwritten if another run is made on the same date. Therefore, it is a good practice to include a user-defined string in your file name. ALWAYS include the experiment number in the output. This is the flag that assigns the _n1, _n2, etc to the plates in the run. Failure to include this will result in every plate being labeled _n1, thereby overwriting all previously generated data. The best practice here is to use a lab notebook number and page as the filename. An example would be: D00567_143, where D00567 is the notebook number and 143 is the page.

Sequences Setup:

The sequence setup is where the entire experiment is defined. This includes defining the number of reads to be taken as well as all liquid handling steps, wash sequences, automated tip unload, etc. These settings should be done with the assistance of an automation engineer or an experienced FLIPR™ user.
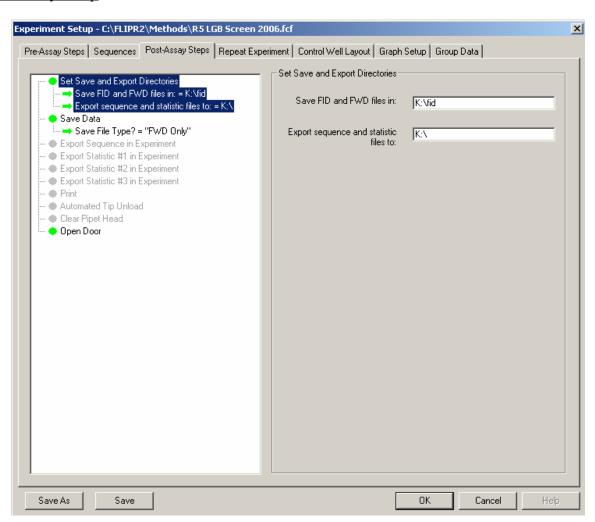
By double clicking on the circle beside each step, the user can activate/deactivate that part of the sequence. A green circle indicates the step is active while grey indicates inactive. By single clicking on the sequence step, the step's setup box appears on the right side of the window with all parameters that can be accessed by the user.

1. Pre-Soak: This is typically not used.
2. Aspirate: The FLIPR™ can aspirate from any of the four deck positions as long as a plate has been defined there in the initial setup page.
3. Put tips in target well:  This will move the tips into the target plate before dispensing. Typically not used.  NEVER use this if dispensing at a low height where the tips are in contact with the buffer.  We have observed that this can cause a response from the compound on the outside of the tips.
4. Baseline imaging:  The pipettor head will not move to the cell plate until the baseline imaging is complete.  A typical setting is 1 to 5 secs.
5. Dispense: The FLIPR can dispense to any of the four deck positions as long as a plate has been defined there in the initial setup page.
6. Wash tips:  This will wash tips in the wash station at position 6 if the unit has a wash station installed.  A pre-wash can be performed at position 5 by
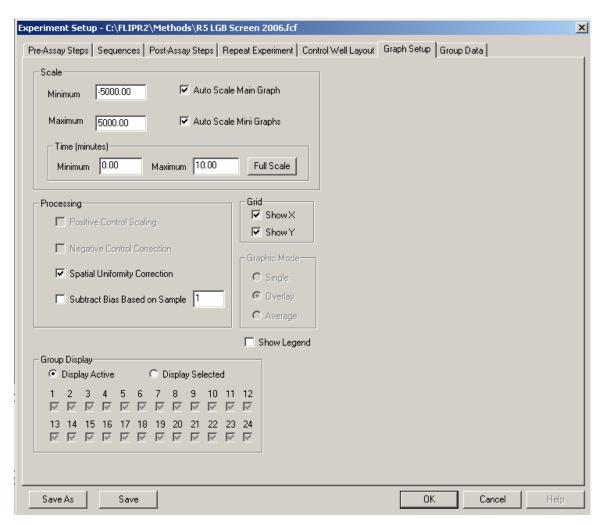
clicking the "rinse after wash" button.  This will use the same wash parameters defined, only perform them at position 5.

7.  First Interval:  This sets the number of images to be acquired and the interval between each image.  Typically, the interval is short (1 sec) and the number of images are 30 to 60 to capture the compound addition.  This should be set long enough to capture past the peak response.

8.  Second Interval:  This set the number of images to be acquired and the interval between each image.  Typically, the interval time is longer (3-5 secs) and the number of images is sufficient to capture when the response decreases to background.  In some cases, the signal will never return to background and it is the judgment of the scientist to set this range.

9.  Automated Tip Unload:  This will automatically unload the tips to the rack when all pipetting steps are completed.  This should only be done in the last sequence.

10. Clear Pipette Head:  This return the pipettor head to the home position.

Post Assay Setup:

In the post-assay setup section, the user selects where data will be saved, what type of data to save, and considers the option to automatically export and print data at the end of each plate.  When setting the save location, you must type in the exact path to the save directory.  The software will generate an error if the location is invalid or if it is a network location that is not available.  In most instances, only FWD files should be saved.  This saves storage space, as the FID files are larger image files.  In some instances, such as when a heated stage is used, the open door may need to be turned off to maintain better temperature control in the FLIPR™.

Graph Setup:



Typically, Spacial Uniformity Correction is used without subtracting the background.  Spacial Uniformity Correction is basically a software normalization that sets all wells to the average RFU of the plate when starting the experiment.
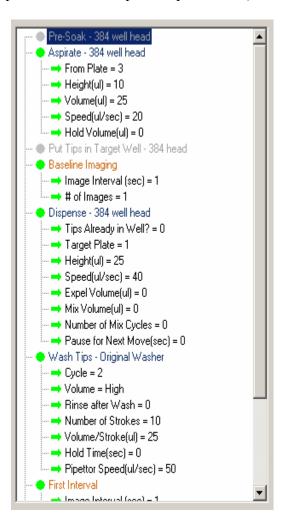
In most cases, subtract bias is not used.  This will background subtract the data set which can mask the assay window.  An example would be to start with a baseline of 5000 RFU

and the max signal response being 6000 RFU.  In most situations, this is not a screenable window, but if the 5000 RFU background is subtracted, the window "looks" good (0 to 1000).
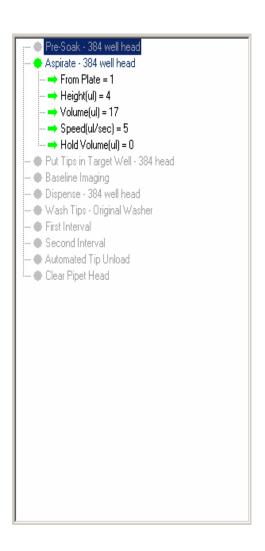
One-, Two- and Three-Addition Assay Examples:

All three of these formats will require the same initial setup described above.
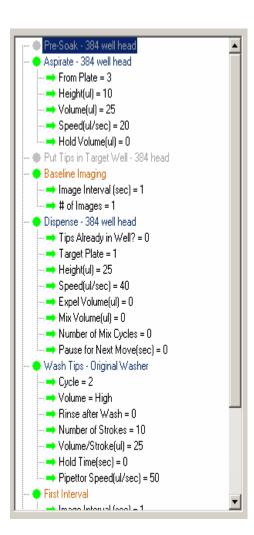
One-addition assays will need one or two sequences dependent upon the use of an in-tip dilution.  The example below shows a 384-well aspiration from position 3 with a dispense into the cell plate at position 1 (Read Position), followed by a wash.

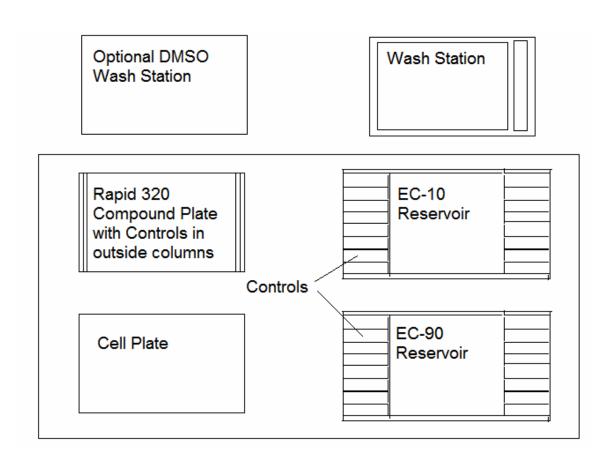A one-addition assay with an in-tip dilution is shown below. The first step aspirates 17µl from plate 1 and then 8µl from plate 3.

Note:  When performing an in-tip dilution, the volume in the second step is the final total volume aspirated (17µl + 8µl).  This is a result of the way the FLIPR™ software keeps track of the pipettor head.
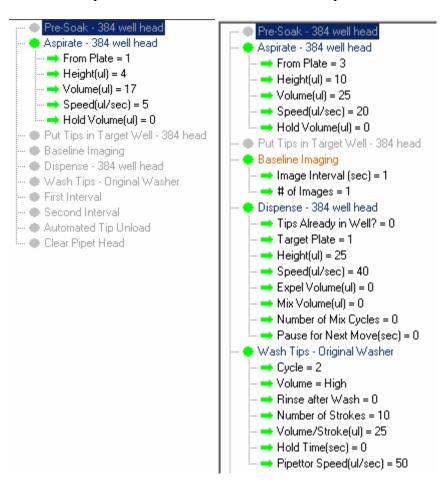
A two-addition or three-addition assay can be run by simply adding sequences.  It is recommended that if the assay is targeting potentiators, the in-tip dilution and pre-incubation time be used to maximize the sensitivity of the assay.  Below is the complete liquid handling setup for a three-addition assay. Volumes and read times will vary.

Deck Layout for a three-addition assay

Sequence 1     Sequence 2

Sequence 1:

- Pre-Soak - 384 well head
- Aspirate - 384 well head
    - From Plate = 1
    - Height(ul) = 4
    - Volume(ul) = 17
    - Speed(ul/sec) = 5
    - Hold Volume(ul) = 0
- Put Tips in Target Well - 384 head
- Baseline Imaging
- Dispense - 384 well head
- Wash Tips - Original Washer
- First Interval
- Second Interval
- Automated Tip Unload
- Clear Pipet Head

Sequence 2:

- Pre-Soak - 384 well head
- Aspirate - 384 well head
    - From Plate = 3
    - Height(ul) = 10
    - Volume(ul) = 25
    - Speed(ul/sec) = 20
    - Hold Volume(ul) = 0
- Put Tips in Target Well - 384 head
- Baseline Imaging
    - Image Interval (sec) = 1
    - # of Images = 1
- Dispense - 384 well head
    - Tips Already in Well? = 0
    - Target Plate = 1
    - Height(ul) = 25
    - Speed(ul/sec) = 40
    - Expel Volume(ul) = 0
    - Mix Volume(ul) = 0
    - Number of Mix Cycles = 0
    - Pause for Next Move(sec) = 0
- Wash Tips - Original Washer
    - Cycle = 2
    - Volume = High
    - Rinse after Wash = 0
    - Number of Strokes = 10
    - Volume/Stroke(ul) = 25
    - Hold Time(sec) = 0
    - Pipettor Speed(ul/sec) = 50

Sequence 3                          Sequence 4



Note that in sequence 3 and 5, the order of aspiration is reversed.  This is due to the fact that unknown test compounds have been added to the cell plate and to aspirate from there first would be a source of contamination to the EC$_{10}$ reservoir.  This is not the case for the 4$^{th}$ and 6$^{th}$ sequence as the tips have been washed.

Sequence 5            Sequence 6



## F. POTENTIAL ARTIFACTS

Although the FLIPR™ has facilitated advances in cellular calcium mobilization screens, these assays remain difficult to configure, relatively slow, and fraught with potential artifacts. Blocked FLIPR™ tips will lead to false positives in an inhibitor screen, or false negatives in an agonist screen. Fluorescent compounds, $Ca^{+2}$ ionophores, and compounds that permeabilize the cell membrane can all contribute to false positives in the agonist read (Figure 11). These types of nuisance or interference compounds can often be identified from the kinetic traces of the response, but this kind of in depth data review is time consuming and requires experience to correctly recognize strange response profiles. In addition, compounds with agonist activity may interfere with antagonist

reads due to desensitization or internalization of the receptor, resulting in false positives.

The utility of the FLIPR™ and calcium dye approach for screening GPCR targets has been greatly enabled by the use of over-expression of promiscuous and chimeric G proteins that provide a method to "switch" $G_{I/o}$-coupled receptor activation to an increase in intracellular calcium. However, screens designed to detect receptor activity against a backdrop of stable, high-level promiscuous G protein expression are also susceptible to artifacts - - false positives derived presumably from other cell surface receptors hi-jacking the promiscuous G proteins. Indeed, even in the absence of a promiscuous G-protein, any endogenous GPCR that couples through Gq and induces a $Ca^{+2}$ response may show up as an agonist or interfere with antagonist reads. It is well documented that GPCRs, particularly those in heterologous expression systems, can activate multiple signal transduction pathways, and indeed there is also evidence for cross-talk between recombinant and native receptors that may also complicate the responses to compounds. Thus, we recommend routinely performing a secondary screen against the parent cell line that lacks the receptor of interest in order to definitively identify false positives.
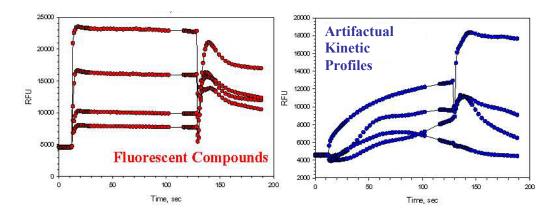


**Figure 11**. Typical kinetic traces that can result from FLIPR™ artifacts.

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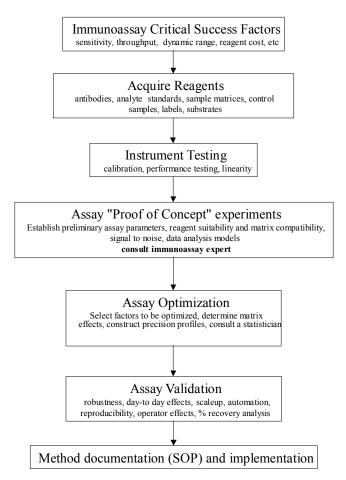# SECTION X

# IMMUNOASSAY METHODS

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## SECTION X:  IMMUNOASSAY METHODS

# A. IMMUNOASSAY DEVELOPMENT, OPTIMIZATION AND VALIDATION FLOW CHART

**Immunoassay Critical Success Factors**
sensitivity, throughput, dynamic range, reagent cost, etc

**Acquire Reagents**
antibodies, analyte standards, sample matrices, control samples, labels, substrates

**Instrument Testing**
calibration, performance testing, linearity

**Assay "Proof of Concept" experiments**
Establish preliminary assay parameters, reagent suitability and matrix compatibility, signal to noise, data analysis models
**consult immunoassay expert**

**Assay Optimization**
Select factors to be optimized, determine matrix effects, construct precision profiles, consult a statistician

**Assay Validation**
robustness, day-to day effects, scaleup, automation, reproducibility, operator effects, % recovery analysis

**Method documentation (SOP) and implementation**

# B. INTRODUCTION

The intent of this document is to provide general guidelines to aid in the development, optimization and validation of an immunoassay. Following these guidelines will increase the likelihood of success in developing a robust immunoassay that will measure consistent values for unknown samples.

Immunoassays are used when an unknown concentration of an analyte within a sample needs to be quantified. To obtain the most accurate determination of the unknown concentration, an immunoassay must be developed based not only on the usual assay development criteria (SD window) but also on how well the immunoassay can predict the value of an unknown sample. First, one needs to establish the assay critical success factors. Then the immunoassay needs to be developed which establishes proof of concept. During the optimization phase the quantifiable range of the immunoassay method is

determined by calculating a precision profile in the matrix in which the experimental samples will be measured. If the precision profile is within the desired working range, then assaying spiked recovery samples over several days completes the validation of the immunoassay.  If the precision profile limits are not within the desired working range further optimization of the immunoassay is required prior to validation.

### Basic Steps for Developing and Running an Immunoassay

1. Establish assay critical success factors.
2. Ensure appropriate antibody and antigen reagents are available.
3. Adsorb antigen or primary antibody to a solid surface.
4. Block nonspecific binding sites to reduce background.
5. Incubate the primary antibody with the sample.
6. Wash off unbound reagents.
7. Incubate secondary antibody-conjugate with sample.
8. Wash off unbound reagents.
9. Incubate substrate to generate signal.
10.  Calibration curve fitting, data analysis and quantitation by non linear regression.

### Basic Steps in Using Immunoassays for HTS

Immunoassays are used in screening to quantify the production or inhibition of antigens/haptens related to a disease target. These antigens or haptens are characteristic of the disease process, mediated by the target such as *cytokines or growth factors*. Hence the screening procedure will involve incubating compounds with specified target, usually expressed in cells, and collecting the cell medium or lysates to quantify the activity of the compounds. Several examples of this approach for using immunoassay procedures have been described in the literature (1-4). The critical steps in setting up a screen are as follows:

1. Develop a validated immunoassay as described above.
2. Acquire antibody, antigen/calibrator, label and buffer reagents in quantities needed for HTS.
3. Establish liquid handling and automation procedures for screening and immunoassay methods.
4. Establish stability of the primary capture antibody bound to a plate or antigen plate stability.
5. Determine compound collections to be tested.
6. Develop and validate a method for incubation of compounds with relevant target in the screening mode.
7. Develop sample collection procedure from screening experiments.
8. Develop data analysis procedures to use immunoassay data to derive compound potency such as $IC_{50}$ or $EC_{50}$.

## C.  IMMUNOASSAY PARAMETERS

It is important to define the relevant immunoassay parameters before one begins the development, optimization and validation of an immunoassay:

1. Analyte (hapten or antigen) to be measured.
2. Sample matrices in which measurements will be made (serum, plasma, cell lysates, culture media etc.)
3. Source of antibody, analyte standards and detection reagents (labeled antibody, enzyme substrates etc). Availability of these reagents is a critical requirement.
4. Detection mode (colorimetric, fluorescence or chemiluminescence) and appropriate plate readers.
5. Type of immunoassay to develop: Sandwich, competitive or antigen-down formats.
6. Expected analyte concentration ranges to be measured: pg/ml, ng/ml or μg/ml in the sample matrix of choice. This would determine the detection limits and the measurable range that should be achieved in a validated assay.
7. Data analysis models and format for reporting results.
8. Validation and optimization criteria using statistical experimental design tools.
9. Recovery, accuracy and precision expected at the limits of quantification and the measurable range.
10. Sample throughput, frequency of use, automation and the number of laboratories that would run the assay.
11. Control samples that would be used for optimization, validation and quality control runs.

## D. REAGENTS

Reagents are a critical piece of any assay development process.  This refers to all of the reagents that will be used in the assay.  There are certain items that need to be considered when obtaining reagents:
1. Quality of standards and antibodies.
2. Quantity of standards and antibodies.
3. Purity of standards and antibodies (when possible antibodies are affinity purified).
4. Selectivity and specificity of antibodies.

**Example Plate Types**
Greiner high binding plates
Costar EIA/RIA high-low binding plates
Immunotech
Falcon

**Note:** Other plate types can also be used based the experience of the investigators and appropriate quality control to demonstrate acceptability.

## Coating Buffers

0.05 M sodium bicarbonate, pH 9.6
0.2 M sodium bicarbonate, pH 9.4
PBS-50 mM Phosphate, pH 8.0, 0.15 M NaCl
Carbonate-bicarbonate
Phosphate Buffer: 1.7 mM $NaH_2PO_4$, 98 mM $Na_2HPO_4.7H2O$, 0.1% $NaN_3$, pH 8.5
TBS - 50 mM TRIS, pH 8.0, 0.15 M NaCl


## Blocking Buffers

1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20
Phosphate Buffer: 73 mM Sucrose, 1.7 mM $NaH_2PO_4$, 98 mM $Na_2HPO_4.7H_2Om$ 0.1%
NaN3, pH 8.5
1% HSA in PBS
Casein Buffer: Pierce Blocker cat # 37528
Pierce has many blocking buffers that are available in their catalog


## Wash Buffers

PBS, 0.05% Tween-20, Chimeras
TBS, 0.05% Tween-20, Chimeras


## Antibody Diluents Buffers

1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20
1% BSA or 10% host serum in PBS, or PBS with 0.05% Tween-20
50 mM HEPES, 0.1 M NaCl, 1%BSA, pH 7.4


## Matrix Diluent

1. Serum from the host animal-mouse serum, human serum, etc
2. 0.1 M HEPES, 0.1 M NaCl, 1% BSA, 0.1 % Tween-20
3. Tissue culture medium for samples.
4. Cell lysates will contain SDS or other denaturing reagents that may interfere with the assay


## Enzymes and Substrates

1. HRP: horseradish peroxidase
2. TMB: 3,3', 5,5'-tetramethyl benzidine
3. OPD: o-phenylene diamine
4. ABTS: 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

### Stop Solutions
1. HRP/TMB: 2M $H_2SO4$ solution (at a 1:1 volume with the HRP/TMB substrate/enzyme solution)
2. OPD:    3M $H_2SO4$ solution, (at a 1:1 volume with the OPD substrate/enzyme solution)
3. ABTS: 1%SDS

### Absorbance Readout
1. HRP TMB: 450 nm
2. OPD: 490 nm
3. ABTS: 405 nm

### Specific Antibodies
1. Sandwich Immunoassay: matched pair of antibodies, one for analyte capture on a solid surface and one for detection that binds to the antigen/hapten/analyte. Antibodies need to be affinity purified for optimal results.
2. Competitive Immunoassay; a single antibody specific for the hapten/analyte. For optimal results affinity purified reagents are preferred.

### Standards or Antigen (Analyte)
1. The analyte to be measured is typically a recombinant form of the natural analyte or peptide.
2. Enough standard should be obtained for use in the development phase, validation phase and the continued support of the method to avoid changing lots and or running out of standard.
3. Standard quality: Can vary from vendor to vendor and from lot to lot from a vendor.
4. Standard stability: Information on the stability of a standard can be obtained from the vendor and their recommendations should be followed in storing the standards.

### Control Samples
1. Spiked controls are created by adding a known concentration of the standard analyte into the matrix (for example: tissue culture, serum, cell lysates). Spiked controls can be used to determine assay performance based on calculating the percent recovery.
2. Control samples are real samples where the antigen analyte level has been determined by another validated method. Samples are aliquoted, frozen and used as control samples in each experiment to track assay performance.

# E. INSTRUMENTATION

## Instrument Linearity and Performance

The instrument used to read the output of the immunoassay should be tested initially for both linearity and performance.  Instrument performance should be regularly calibrated according to manufacturer's specifications.  The majority of plate readers employ UV-Vis Absorbance, fluorescence or chemiluminescence signals as the measured response, since the products of enzyme labels are chromophores, fluorophores or emit luminescent signals.  Linearity in response of the specific enzyme product of an ELISA should be checked at the appropriate wavelengths and instrument settings.

## Spectrophotometric/Colorimetric Plate Readers

Lamp sources and Photomultiplier tubes (PMT) vary in quality and performance in many plate readers. The linear range of many plate readers is generally between 0-2.5 Absorbance units (AU), but other instruments have a linear range up to 4.0 AU. A malfunctioning lamp source or photomultiplier tube can significantly affect the linear response range.

## Fluorescence Plate Readers

These readers employ excitation and emission filter sets in addition to excitation lamp sources and photomultiplier tubes (PMT's). In addition to the lamps and PMT's, the filter sets also vary in quality, light throughput and bandwidth. Fluorescence signals are generally in Relative Fluorescence Units (RFUs) and linearity should be verified with appropriate filter sets for the fluorophores employed according to instrument specifications.

## Chemiluminescence Readers

These instruments have sensitive photomultipliers to detect light emitted from a chemical reaction. No Lamp sources are necessary. These readers usually have a much larger dynamic range, thus allowing for the increase in sensitivity. Signals or responses are measured in Relative Light Units (RLU) and can be significantly different depending on their instrument design.

# F.  IMMUNOASSAY FORMATS

An enzyme-linked immunoassay (ELISA) is one of several methods used in the laboratory to detect and quantify specific molecules.  ELISA's rely on the inherent ability of an antibody to bind to the specific structure of a molecule.  In order to optimize an ELISA and obtain the sensitivity and dynamic range required for the particular assay

being developed, all the various components of the assay must be evaluated.  The components will vary depending on the immunoassay format selected.
Following is a description of the various types of ELISA formats as well as reagents that needed to be optimized in order to obtain a robust assay.
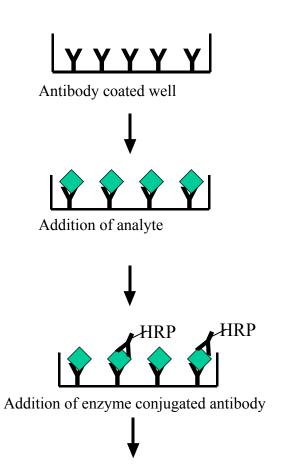

## Types of ELISA Formats

Three frequently used types of ELISA are: sandwich assays, competitive assays and antigen down assays.  The format selected depends on the reagents that are available and the dynamic range required for the particular assay.  Sandwich assays tend to be more sensitive and robust and therefore tend to be the most commonly used.


## Sandwich Immunoassay (ELISA)

 A Sandwich Immunoassay is a method using two antibodies, which bind to different sites on the antigen or ligand.  The primary antibody, which is highly specific for the antigen, is attached to a solid surface.  The antigen is then added followed by addition of a second antibody referred to as the detection antibody.  The detection antibody binds the antigen to a different epitope than the primary antibody.  As a result the antigen is 'sandwiched' between the two antibodies. The antibody binding affinity for the antigen is usually the main determinant of immunoassay sensitivity.  As the antigen concentration increases the amount of detection antibody increases leading to a higher measured response.  The standard curve of a sandwich-binding assay has a positive slope. To quantify the extent of binding different reporters can be used.  Typically an enzyme is attached to the secondary antibody which must be generated in a different species than primary antibodies (i.e. if the primary antibody is a rabbit antibody than the secondary antibody would be an anti-rabbit from goat, chicken, etc., but not rabbit). The substrate for the enzyme is added to the reaction that forms a colorimetric readout as the detection signal. The signal generated is proportional to the amount of target antigen present in the sample.
The antibody linked reporter used to measure the binding event determines the detection mode. For an ELISA, where the detection is colorimetric, a spectrophotometric plate reader is used. Several types of reporters have been recently developed in order to increase sensitivity in an immunoassay.  For example, chemiluminescent substrates have been developed which further amplify the signal and can be read on a luminescent plate reader. Also, a fluorescent readout where the enzyme step of the assay is replaced with a fluorophor tagged antibody is becoming quite popular.  This readout is then measured using a fluorescent plate reader.

**Figure 1**

# Sandwich ELISA



Antibody coated well

Addition of analyte

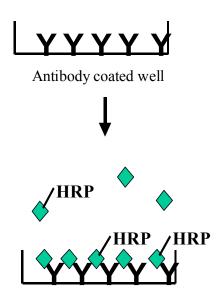Addition of enzyme conjugated antibody

Addition of enzyme's substrate leads to the development of color.
The signal generated is directly proportional to the concentration
of antigen present.

## Competitive Binding Assay

A competitive binding assay is based upon the competition of labeled and unlabeled ligand for a limited number of antibody binding sites.  Competitive inhibition assays are often used to measure small analytes.  These assays are also used when a matched pair of antibodies to the analyte does not exist. Only one antibody is used in a competitive binding ELISA. This is due to the steric hindrance that occurs if two antibodies would attempt to bind to a very small molecule.  A fixed amount of labeled ligand (tracer) and a variable amount of unlabeled ligand are incubated with the antibody. According to law of mass action the amount of labeled ligand is a function of the total concentration of labeled and unlabeled ligand. As the concentration of unlabeled ligand is increased, less labeled ligand can bind to the antibody and the measured response decreases.  Thus the lower the signal, the more unlabeled analyte there is in the sample. The standard curve of a competitive binding assay has a negative slope.

**Figure 2**



# Competitive ELISA

Antibody coated well

Addition of analyte and analyte conjugated with enzyme.
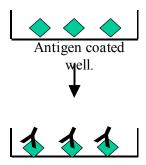Competition occurs between the analyte and the conjugated analyte.

Addition of the substrate allows color development.
In a competitive assay the signal is inversely proportional
to the concentration of analyte in the sample.

**Antigen-Down Immunoassay or Immunometric Assay**
An antigen-down immunoassay or immunometric assay involves binding the antigen to a solid surface instead of an antibody. Antigen-down immunoassays are used to bind antibodies found in a sample. When the sample is added (such as human serum), the antibodies (IgE for example) from the sample bind to the antigen coated on the plate. A species-specific antibody (anti-human IgE for example) labeled with HRP is added next. The signal is directly proportional to the amount of antibody present in the sample; the more antibodies there are in the sample the higher the signal.
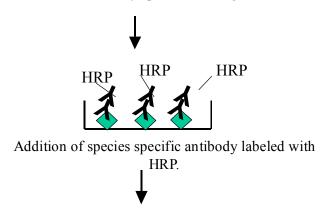
**Figure 3**

# Antigen-Down Immunoassay

Antigen coated
well.

Addition of antibody specific to antigen.

HRP          HRP          HRP

Addition of species specific antibody labeled with
HRP.

Substrate is added to allow color.

## G. IMPORTANT PARAMETERS FOR DEVELOPMENT OF AN IMMUNOASSAY

1.  Primary capture antibody

2.  Secondary detection antibody

3.  Plate type

4.  Coating buffer

5.  Blocking buffer/diluent buffer

6.  Wash buffer

7.  Coating antibody concentration

8.  Coated antibody stability

9.  Timing of each step in the immunoassay

10. Secondary antibody concentration

11. Reporter concentration

12. Readout

13. Instrument linearity


# H. INITIAL CONCEPT AND METHOD DEVELOPMENT OF A SANDWICH IMMUNOASSAY

**Initial Development Experiment**

*Goal:*      Develop a basic working method by determining the antibody which should be the primary/capture antibody and which antibody should be the secondary or detection antibody.  Determine the optimum antibody concentrations for both the primary and secondary antibody.

*Experiment:*   Coat the ELISA plate with several dilutions of both antibodies that will be used as part of the sandwich assay.  Add the standard to be measured at a high, low and zero concentration.  Use both of the antibodies at several concentrations as a secondary antibody.  The results of this experiment will determine which antibody is best for both the capture antibody and the secondary antibody.  The dilution needed for both antibodies will also be determined.

*Reagents:*
Listed below is the plate type and buffers that will work for the majority of immunoassays.  Use these buffers as a starting point.
1.  Two antibodies that recognize different epitopes on the analyte.
2.  A monoclonal for the capture and polyclonal for the detection antibody tend to yield the best sandwich assay.
3.  Greiner immunoassay plate.
4.  Coating buffer:  50mM sodium carbonate pH 9.6.
5.  Blocking buffer:  1% BSA, TBS, 0.1% Tween-20.
6.  Antibody diluent buffer:  1% BSA, PBS or TBS, 0.1 % Tween-20.
7.  Wash buffer:  PBS or TBS, 0.1% Tween-20.
8.  TMB and HRP are used for enzyme/substrate readout.
9.  Acid stop buffer.

*Protocol:*
1.  Titrating both the primary capture antibody and the secondary detection antibody are made across a plate using a high, low and zero level of the analyte.
2.  Determine the desired working range of the analyte.  This will give you the high and low concentrations.
3.  Dilute both antibodies in coating buffer at 0.5, 1 and 2 mg/ml and add 100 μl to each well of the 96-well microtiter plate.
4.  Incubate the plate containing the primary capture antibody overnight at 4 ° C then use the next day.
5.  Stability of the primary capture antibody bound to the plate can be determined in later experiments.
6.  Remove the primary capture antibody solution from the microtiter plates by aspirating or dumping the plate.
7.  Add 200 μl of blocking buffer to each well of the 96-well microtiter plate.
8.  Incubate the plate for one hour at RT.
9.  Remove the blocking buffer from the plate by aspirating or dumping the plate.
10. Dilute the standard in dilution buffer to give a high and low concentration.
11. Zero concentration will give you the NSB.
12. Add 100 μl of the standard to each well in the microtiter plate and incubate for 2.5 hours at RT.
13. Wash the plates 3 times with wash buffer.
14. Dilute the secondary antibody serially at 1:200, 1:1000, 1:5000 and 1:25,000 in diluent.
15. Add 100 μl of detection antibody to each well of the microtiter plate and incubate for 1 1/2 hours at RT.
16. Wash the plates 3 times with wash buffer.
17. Dilute streptavidin-HRP according to manufacture instructions in antibody diluent and add 100 μl to each well in the microtiter plate and incubate for 1 hr at RT.
18. For HRP readout add either OPD or TMB as substrate to allow color development and incubate for 10-20 minutes at RT.
19. Add acid stop reagent to stop the enzyme reaction.
20. Read at 405 nm for TMB/HRP.

**15**

# Plate Layout for Initial Experiment

**Plate 1**

### *Primary capture antibody A*

| | 5 µg/ml | | | 2 µg/ml | | | 1 µg/ml | | | 0.5 mg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Secondary** | | | | | | | | | | | | |
| **Antibody** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:200** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:1000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:5000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:25000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |

**Plate 2**

### *Primary capture antibody B*

| | 5 µg/mg | | | 2 µg/ml | | | 1 µg/ml | | | 0.5 mg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Secondary** | | | | | | | | | | | | |
| **Antibody** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:200** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:1000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:5000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |
| **1:25000** | H | L | 0 | H | L | 0 | H | L | 0 | H | L | 0 |

### *Results*

H=High and L=Low: High ligand concentration in combination with the low ligand
concentration will give an indication of the dynamic range.

L=Low: Low ligand concentration will give indication of sensitivity.

0=Zero: Zero ligand will give the non-specific binding (NSB) and indicate if there are
background issues.

Determine the Absorbance units (A.U.) that yield the maximum signal to noise
ratio or the greatest difference between the high and low analyte concentrations with
the lowest variability. These are the conditions that will be selected for the antibody
to be used as the primary capture antibody and the dilution of the antibodies to be
used in the next experiment.

- If the background signal is unacceptably high (greater than 0.2 A.U.) then run
  additional experiments varying the plate type, blocking buffers, blocking buffers with
  a diluent agent like species specific IgG, antibody diluent buffers, wash buffers, and
  the reporter type.
- If the above general conditions have an acceptable NSB then determine if the
  dynamic range and sensitivity are in the appropriate range. To improve the sensitivity
  of the assay, the buffers, timing of incubations and matrix conditions can be varied in
  the next experiment.
- Antibodies are the reagents that play a major role in the sensitivity and dynamic range
  of an immunoassay. This is due to the actual antibody affinity for the analyte. If
  after attempting to develop the assay the sensitivity is still not in the desired range,
  different antibody pairs will need to be evaluated.

***Example:*** An ELISA was set up to measure the amounts of an LP protein where there is
only one polyclonal antibody available. The polyclonal antibody was used as both the
primary capture antibody and the secondary detection antibody. Biotinylated antibody
was used as the detection antibody.

Reagents:
1. Pab- 0172B   140μg/ml affinity pure antibody.
2. LP276   230 μg/ml.
3. LP276BT   400μg/ml biotinylated affinity pure antibody.

Experiment: Follow the above protocol and plate layout
1. Coated the affinity purified antibody at 3 levels:  2, 1 and 0.5 μg/ml
2. Diluted the biotinylated antibody at 3 levels: 1:1000, 1:5000, and 1;25,000
3. Diluted the standard LP276 protein in buffer to 50 ng/ml and 1 ng/ml

## I. RESULTS

# <u>Plate Layout</u>

*Primary capture antibody*

| 2 µg/ml | | | 1 µg/ml | | | 0.5 µg/ml | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| H | L | 0 | H | L | 0 | H | L | 0 | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

**Secondary Antibody**
1:1000 (rows 1–2)
1:5000 (rows 3–4)
1:25000 (rows 5–6)

Average A.U. readings at A490
Actual results from initial experiment to determine the coating antibody and detection antibody

*Primary capture antibody*

| 2 µg/ml | | | 1 µg/ml | | | 0.5 µg/ml | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3. | 1. | 1. | 3. | 1. | 1. | 3. | 1. | 1 | | | |
| 4 | 1 | 1 | 4 | 1 | 1 | 3 | 1 | 1 | | | |
| 3. | .7 | .5 | 3. | .8 | .5 | 3 | .8 | .4 | | | |
| 3. | .7 | .5 | 3. | .8 | .5 | 3. | .8 | .4 | | | |
| 1. | .2 | .1 | 1. | .2 | .1 | 1. | .3 | .1 | | | |
| 1. | .2 | .1 | 1. | .3 | .1 | 2 | .3 | .1 | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

**Secondary Antibody**
1:1000 (rows 1–2)
1:5000 (rows 3–4)
1:25000 (rows 5–6)

Data summarized for the high, low and zero values for the LP276 protein concentration
across the detection and primary antibody concentrations.
Values below are averages at an A.U. of A490.

**LP 276 ng/ml**

|  | 50 | 1 | 0 | 50 | 1 | 0 | 50 | 1 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| Sec Ab conc |  |  |  |  |  |  |  |  |  |
| 1:1000 | 3.69 | 1.81 | 1.37 | 3.63 | 1.89 | 1.33 | 3.3 | 1.79 | .99 |
| 1:5000 | 3.22 | .7 | .49 | 3.24 | .81 | .47 | 3.1 | .83 | .36 |
| 1:25000 | 1.61 | .21 | .15 | 1.75 | .25 | .16 | 1.72 | .26 | .12 |
| Primary Ab ug/ml | 2 | 2 | 2 | 1 | 1 | 1 | 0.5 | 0.5 | 0.5 |

*Conclusions:*  Lowest NSB and best signal to noise ratio from low to high LP276
   concentration are the 0.5 µg/ml concentration for the coating antibody and the
   1:25,000 dilution of the biotinylated antibody as the detection antibody.


**Second Experiment-Matrix Compatibility**

*Goal:* To determine the matrix effect or sample type on the immunoassay method.
The matrix is based on what the sample is found in, for instance tissue culture media,
serum, cell lysate, buffers, etc.  Serum matrix, due to its complexity, can have a
significant effect on the method.  In this example the samples are in rat serum so the
matrix effect of rat serum needs to be determined.

*Experiment:*  The samples that need to be measured in this assay will be in either mouse
or rat serum.  Use the conditions established in the first experiment for the concentration
of the primary capture antibody and the secondary detection antibody.  Serially dilute the
standard to obtain a full standard curve in 3 different matrices (10% rat serum, 30% rat
serum and the original buffer diluent used in the first experiment).  This will determine
the matrix effect that will be used for the experimental samples.

*Reagents:*
1. Use all of the reagents and buffers listed in the first experiment.
2. Matrix diluent:  10% rat serum in antibody diluent or 30% rat serum in antibody
   diluent.

*Protocol:*
Follow the standard protocol changing only the matrix diluent to include rat serum.

1.  Dilute the primary antibody in coating buffer at 0.5 μg/ml and add 100 μl to each well of the 96-well microtiter plate.
2.  Incubate the plate containing the primary capture antibody overnight at 4 ° C and use the next day.
3.  Stability of the primary capture antibody bound to the plate can be determined in later experiments.
4.  Remove the primary capture antibody solution from the microtiter plates by aspirating or dumping the plate.
5.  Add 200 μl of blocking buffer to each well of the 96-well microtiter plate.
6.  Incubate the plate for one hour at RT.
7.  Remove the blocking buffer from the plate by aspirating or dumping the plate.
8.  Serially dilute the standard in antibody dilution buffer containing either 10% or 30% rat serum.
9.  Add 100 μl of the standard to each well in the microtiter plate and incubate for 2.5 hours at RT.
10. Wash the plates 3 times with wash buffer.
11. Dilute the detection antibody to 1:25,000 in antibody diluent.
12. Add 100 μl of detection antibody diluent to each well of the microtiter plate and incubate for 1 1/2 hours at RT.
13. Wash the plates 3 times with wash buffer.
14. Dilute streptavidin-HRP according to manufacturer instructions in antibody diluent and add 100 μl to each well in the microtiter plate and incubate for 1 hr at RT
15. For HRP readout add either OPD or TMB as substrate to allow color development and incubate for 10-20 minutes at RT.
16. Add acid stop reagent to stop the enzyme reaction.
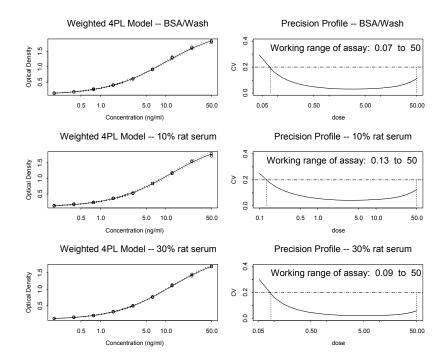17. Read at 405 nm for TMB/HRP.

Results:  Use the standard curve data and construct a precision profile. Check background levels.  See page 138 for standard or calibration curve model fitting.
Note that the standard curves under all three matrix diluent conditions give the dynamic range and sensitivity necessary for the intended use. For this particular assay, there is no further development needed (based on the standard curve, low background and precision profile).

Precision Profile: Generate the precision profile for the standard curve of the appropriate matrix for the experiment. A web-based tool developed internally is available for computing the calibration curve and the precision profile that gives the estimated working range of the assay. The web address of this tool is:

**http://pascal/statmath/calibration/prd/html/calibration.html**

## Calibration Curve and Precision Profile for the Three Different Matrix Conditions

## Calibration Curve Model Selection

A significant source of variability in the calibration curves can come from the choice of the statistical model used for the calibration curve. It is therefore extremely important to choose the correct calibration curve model. For most immunoassays, the models commonly available in software are the following.

Linear Model:
$$Response = a + b*(Concentration) + error,$$
where a and b are the intercept and slope respectively, and "response" refers to optical density or fluorescence reading from an immunoassay.

Quadratic Model:
$$Response = a + b*(Concentration) + c*(Concentration)^2 + error,$$
where a, b and c are the intercept, linear and quadratic term coefficients respectively of this quadratic model.

Four Parameter Logistic Model:

$$Response = Top + \frac{(Bottom - Top)}{1 + \left(\dfrac{concentration}{EC_{50}}\right)^{Slope}},$$

where the four parameters to be estimated are Top, Bottom, $EC_{50}$ and Slope. Top refers to the top asymptote, Bottom refers to the bottom asymptote, and $EC_{50}$ refers to the concentration at which the response is halfway between Top and Bottom.
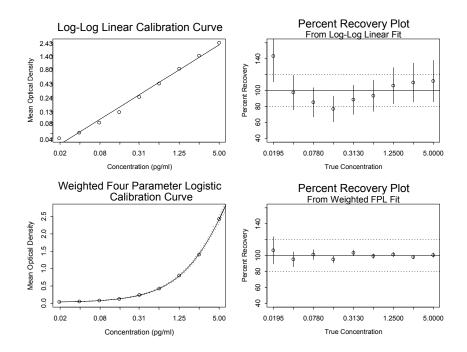
Five Parameter Logistic Model:

$$Response = Top + \frac{(Bottom - Top)}{\left[1 + \left(\dfrac{concentration}{EC_{50}}\right)^{Slope}\right]^{Asymmetry}},$$

Asymmetry is the fifth parameter in this model. It denotes the degree of asymmetry in the shape of the sigmoidal curve with respect to "$EC_{50}$". A value of 1 indicates perfect symmetry, which would then correspond to the four-parameter logistic model. However, note that the term referred to as "$EC_{50}$" in this model is not truly the $EC_{50}$. It is the $EC_{50}$ when the asymmetry parameter equals 1. It will correspond to something very different such as $EC_{20}$, $EC_{30}$, $EC_{80}$, etc., depending on the value of the asymmetry parameter for a particular data set. Further details are beyond the scope of this chapter.

For most immunoassays, the four or five parameter logistic model is far better than the linear, quadratic or log-log linear models. These models have recently become available

in several software packages, and are easy to implement even in an Excel-based program. As illustrated in the plots below, the quality of the model should be judged based on the dose-recovery scale instead of the lack-of-fit of the calibration curve ($R^2$). In this illustration, even though the $R^2$ of the log-log linear model is 0.99, when assessed in terms of the dose-recovery plot, this model turns out to be significantly inferior to the four parameter logistic model. Before the assay is ready for production, the best model for the calibration curve should be chosen based on the validation samples using dose-recovery plots.



## Importance of Weighting in Calibration Curves

The default curve-fitting method available in most software packages assigns equal weight to all the response values, which is appropriate only if the variability among the replicates is equal across the entire range of the response. However, for most immunoassays, the variability in the calibration-curve data between replicates increases proportionately with the response mean. Giving equal weight can lead to highly incorrect conclusions about the assay performance and will significantly affect the accuracy of results from the unknown samples. It is therefore extremely important to use a curve-fitting method/software that has appropriate weighting methods/options. See Appendix for more details.

**Third Development Experiment**

The two-step experiment detailed above is a very simple example of how to develop a sandwich ELISA method.  If the dynamic range and sensitivity of the assay does not meet the experimental needs then further experimental parameters should be tested using experimental design. With experimental design all of the factors involved in the ELISA including buffers, incubation time and plate type can be analyzed.

In a sandwich ELISA method the antibodies chosen are the major drivers of the assay parameters.  If at this point in the method development the precision profile of the standard curve is extremely far from the desired dynamic range and sensitivity, instead of continuing with development experiment, antibodies should be further characterized. Changing some of the variables such as the Ab concentrations can significantly improve the calibration curve and hence it's precision profile.

*Goal:*          Determine the optimal conditions for the variables in the immunoassay including incubation steps, buffers, substrate, etc. Also, determine the optimal antibody concentrations and the stability of the primary capture antibody bound to the plate.

*Experiment:*  Dilute the standard in the matrix compatible to the sample (as determined in the second experiment). Vary the incubation times, dilution buffers and other variables in order to optimize the immunoassay.  Analyze by using experimental design software and precision profiles.

*Reagents:*
    1. Coating buffers
    2. Blocking buffers
    3. Wash buffers
    4. Antibody diluents
    5. Substrate

*Protocol:*
1. Coat the microtiter plate with the primary capture antibody at the concentration determined in the initial experiment.  Incubate overnight at 4° C.
2. Discard the primary capture antibody solution from the microtiter plate.
3. Block the plate for 1 hour at RT using various blocking reagents.
4. Store plates at 4° C, desiccated, for several periods of time 0-5 days.
5. Repeat steps 1-3 the day of the actual experiment.
6. Serially dilute, using an 8-point standard curve, the known standard in the appropriate matrix for the experiment.  For the control also dilute the standard in the same buffer as was used in the initial experiment.  Add 100 μl of standard to each well in the 96-well microtiter plate.

7. Incubate the diluted standard with the primary capture antibody for 1 hour and 3 hours at RT and overnight at 4°C. Each time point will have to be run in a separate plate.
8. Wash plates 3 times (If background or NSB is high try different wash buffers)
9. Add 100 µl of diluted secondary detection antibody.  If background is high again different diluents can be tested.
10. Incubate the secondary detection antibody for different time periods and again different plates will have to be used for each time condition.
11. Wash plates 3 times.
12. Add 100 µl of substrate to the plate containing the detection antibody conjugated to the enzyme and allow to incubate according to the manufacturers conditions
13. Add 100 µl of stop buffer.
14. Read at 405 nm.

***Data Analysis***: Compute the standard curves and their precision profiles for all the experimental design conditions. Derive the optimization endpoints using the precision profiles. Then analyze the optimization endpoints using software such as JMP to determine the optimum levels of the assay factors. See next section for the details and illustration.


## Experimental Designs for Increasing Calibration Precision

Step 1:

Identify all the factors/variables that potentially contribute to assay sensitivity and variability.  Choose appropriate levels for all the factors (high and low values for quantitative factors, different categories for qualitative factors).  Then use fractional-factorial experimental design in software such as JMP to derive appropriate experimental "trials" (combinations of levels of all the assay factors).  Run 8-point calibration curves in duplicate for each trial.  With each trial taking up two columns in a 96-well plate 6 trials per plate can be tested.  All trials should be randomly assigned to different pairs of columns in the 96-well plates.  However, certain factors such as incubation time and temperature are inter-plate factors.  Therefore, levels of such factors will have to be tested in separate plates.

|   | Trial 1 | | Trial 2 | | Trial 3 | | Trial 4 | | Trial 5 | | Trial 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 8pt calibration curve; duplicate | | 8pt calibration curve; duplicate | | 8pt calibration curve; duplicate | | 8pt calibration curve; duplicate | | 8pt calibration curve; duplicate | | 8pt calibration curve; duplicate | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |

| H | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|

After the above experiment is run the calibration curves should be fit for each trial using an appropriately weighted-nonlinear regression model. Then the precision profile for the calibration curve of each trial should be obtained along with the important optimization end-points such as working-range, lower quantitation limit and precision area. Now analyze these data to determine the optimal level of all qualitative factors and determine which factors should be further investigated. See Appendix for the definition of these terms/concepts and details on the computations.

<u>Step 2</u>:

We now need to determine the optimum levels for the factors determined in the previous step. Choose appropriate low, middle and high levels for each of these factors based on the data analysis results from step 1. Now use software such as JMP to generate appropriate trials (combinations of low, middle and high levels of all the factors) from a central-composite design. Then run duplicate 8-point calibration curves for each trial using a similar plate format as in step 1.
Now obtain the precision profile and the relevant optimization end-points of the calibration curve of each trial. Perform the response-surface analysis of these data to determine the optimal setting of each of the quantitative factors run in this experiment.

**Illustration of Experimental Design and Analysis for Sandwich ELISA Optimization**

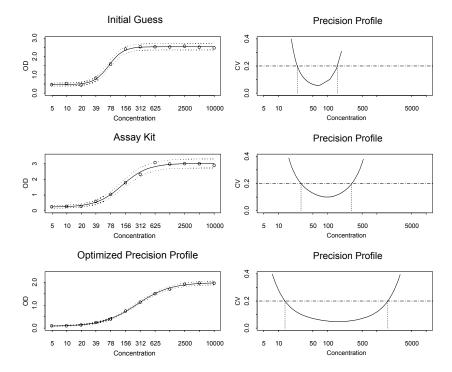In this table, we have the experiment plan from the second step of the optimization

| Trial # | Pattern | CaptureA | BiotynA | EnzCult | Volume |
|---|---|---|---|---|---|
| 1 | ---+ | 250 | 250 | 300 | 100 |
| 2 | 0000 | 500 | 425 | 525 | 75 |
| 3 | -+++ | 250 | 600 | 750 | 100 |
| 4 | -++- | 250 | 600 | 750 | 50 |
| 5 | ---- | 250 | 250 | 300 | 50 |
| 6 | 0-00 | 500 | 250 | 525 | 75 |
| 7 | 0000 | 500 | 425 | 525 | 75 |
| 8 | 0000 | 500 | 425 | 525 | 75 |
| 9 | 00+0 | 500 | 425 | 750 | 75 |
| 10 | --++ | 250 | 250 | 750 | 100 |
| 11 | +--+ | 750 | 250 | 300 | 100 |
| 12 | 0000 | 500 | 425 | 525 | 75 |
| 13 | 0000 | 500 | 425 | 525 | 75 |
| 14 | +++- | 750 | 600 | 750 | 50 |
| 15 | 0000 | 500 | 425 | 525 | 75 |
| 16 | 0000 | 500 | 425 | 525 | 75 |
| 17 | 0000 | 500 | 425 | 525 | 75 |
| 18 | -+-- | 250 | 600 | 300 | 50 |
| 19 | +000 | 750 | 425 | 525 | 75 |
| 20 | 0000 | 500 | 425 | 525 | 75 |
| 21 | 0+00 | 500 | 600 | 525 | 75 |
| 22 | -+-+ | 250 | 600 | 300 | 100 |
| 23 | ++-- | 750 | 600 | 300 | 50 |
| 24 | 000- | 500 | 425 | 525 | 50 |
| 25 | 00-0 | 500 | 425 | 300 | 75 |
| 26 | 000+ | 500 | 425 | 525 | 100 |
| 27 | 0000 | 500 | 425 | 525 | 75 |
| 28 | 0000 | 500 | 425 | 525 | 75 |
| 29 | 0000 | 500 | 425 | 525 | 75 |
| 30 | +-++ | 750 | 250 | 750 | 100 |
| 31 | ++-+ | 750 | 600 | 300 | 100 |
| 32 | --+- | 250 | 250 | 750 | 50 |
| 33 | -000 | 250 | 425 | 525 | 75 |
| 34 | +-+- | 750 | 250 | 750 | 50 |
| 35 | +--- | 750 | 250 | 300 | 50 |
| 36 | ++++ | 750 | 600 | 750 | 100 |

process using experimental design for a sandwich ELISA. These four factors (primary capture antibody, secondary detection antibody, enzyme and volume) were picked out of the six factors considered in the first step of this optimization process (screening phase) for further optimization. We use a statistical experimental design method called central composite design to generate the appropriate combinations of the high, mid and low levels of the four factors in this second step. For example, trial #6 in this table refers to the middle level of the first, third and the fourth factors, and the low level of the second factor.

8-point standard curves in duplicate were generated for each of these trials, in adjacent columns of a 96-well plate. This resulted in six trials per plate, and with 36 trials in 6 plates.

We computed the precision profiles of the calibration curves of each of these 36 trials. From these precision profiles, we computed the working range (lower and upper quantification limits), CV and related variability and sensitivity measures. We then used a statistical data analysis method called "response surface analysis" on these summary measures. This resulted in polynomial type models for all the factors. Using the shape of the curve and other features from this model, the optimum levels for these factors were determined. This gave us the most sensitive dynamic working range possible for this assay.

An experiment was then performed for this ELISA to compare these optimized levels to the pre-optimum levels and the assay kit manufacturer's recommendation. The results from this comparison are summarized below.



The optimized levels derived from statistical experimental design for this ELISA resulted in the following improvements over the pre-optimum and assay kit manufacturer's recommendation.

- Lower quantification limit decreased more than two-fold to 13.6 nM.
- Upper quantification limit by up to 10-fold to 1662.3 nM.
- Precision area increased by 2-fold and the working range increased by 2-fold to two log cycles.

This improvement is evident from the precision profiles given above for these three conditions.

## J.  INITIAL CONCEPT AND METHOD DEVELOPMENT OF A COMPETITIVE ASSAY

**Competitive Binding Immunoassay**

Development and validation of a competition immunoassay requires considerable expertise in reagent characterization and method development.  Sandwich and antigen-down immunoassays formats should be explored before attempting the competitive immunoassay format.

**Drawbacks Using a Competitive Immunoassay**

1.  A competitive immunoassay is not as sensitive as a sandwich ELISA.
2.  A competitive immunoassay is more sensitive to matrix issues, especially serum matrix, which can affect assay performance.
3.  Timing of the various incubation steps is less robust in a competitive assay.  That is the $IC_{50}$ of the standard curve will shift with minor changes in incubation of the various steps of the immunoassay.
4.  The labeling of the hapten or analyte can change the analyte binding affinity for the antibody.  Experiments need to determine the effect of the label on the binding affinity of the antibody to the analyte.

## K. DEVELOPMENT OF A COMPETITIVE IMMUNOASSAY

**Initial Development Experiment**

*Goal:*  Determine the optimal coating concentration of the antibody used for capture and the labeled ligand.

*Reagents*
1.  Antibody- mono or polyclonal, specific to the analyte.
2.  Buffers- same as for a competitive assay.
3.  Labeled ligand-the enzyme or biotin is labeled directly to the analyte or ligand.

*Experiment:*  Coat the ELISA plate with various antibody concentrations to determine the optimal concentration of antibody and labeled ligand.

Protocol:
1.  Determine the desired analyte working range.
2.  Titrate capture antibody using high, low and zero analyte concentration levels.

3. Dilute the capture antibody in coating buffer at 0.1, 0.5, 1 and 2 mg/ml and add 100 µl to each well of the 96-well microtiter plate.  The capture antibody may need to be titrated down further.  The amount of antibody coated on the plate will be proportional to the sensitivity of the assay.
4. Incubate the plate containing the primary capture antibody overnight at 4 ° C and use the next day.
5. Stability of the primary capture antibody bound to the plate can be determined in later experiments.
6. Remove the primary coating antibody solution from the microtiter plates by aspirating or dumping the plate.
7. Add 200 µl of blocking buffer to each well of the 96-well microtiter plate.
8. Incubate the plate for one hour at RT.
9. Remove the blocking buffer from the plate by aspirating or dumping the plate.
10. Dilute the labeled standard in antibody dilution buffer over a wide range.  The desired result is the condition, which gives a readable signal with the least amount of antibody coated, in combination with the least amount of labeled standard.
11. Zero concentration will give you the NSB.
12. Add 100 µl of the labeled standard to each well in the microtiter plate and incubate for 2.5 hours at RT.  (The standard can either be directly labeled with the enzyme or biotinylated).
13. Wash the plates 3 times with wash buffer.
14. If a biotinylated standard is used, Dilute streptavidin-HRP according to manufacturer's instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hr at RT.
15. For HRP readout add either OPD or TMB as substrate to allow color development and incubate for 10-20 minutes at RT.
16. Add acid stop reagent to stop the enzyme reaction.
17. Read at 405 nm for TMB/HRP.
18. Determine the linearity of the instrument being used for the readout the same way as described for a sandwich ELISA.

**Second Development Experiment**

*Goal:* Determine the potential dynamic range and sensitivity.  Take the conditions established in the initial experiment for the concentration of the antibody and labeled ligand and incubate with a wide range of unlabeled analyte.  The resulting standard curve and precision profile calculation will give an estimate of the sensitivity and dynamic range of the assay.

*Reagents:*
Reagents are the same as in initial experiment.

*Protocol:*

1. Dilute the capture antibody in coating buffer at the concentration determined in the initial experiment. Add 100 μl to each well of the 96-well microtiter plate.
2. Incubate the plate containing the primary capture antibody overnight at 4 ° C and use the next day.
3. Remove the primary capture antibody solution from the microtiter plates by aspirating or dumping the plate.
4. Add 200 μl of blocking buffer to each well of the 96-well microtiter plate.
5. Incubate the plate for one hour at RT.
6. Remove the blocking buffer from the plate by aspirating or dumping the plate.
7. Dilute the labeled standard in antibody dilution buffer at the concentration determined in the initial experiment.
8. Dilute the unlabeled ligand in antibody dilution buffer over a wide range of concentrations.
9. Add 100 μl of the labeled standard to each well in the microtiter plate and 100 μl of the various dilution of the unlabeled ligand. Incubate for 2.5 hours at RT. This is the competitive part of the assay and will allow for the competition between the labeled and unlabeled ligand to compete for the sites on the antibody.
10. Wash the plates 3 times with wash buffer.
11. If a biotinylated standard is used, Dilute streptavidin-HRP according to manufacturer's instructions in antibody diluent and add 100 μl to each well in the microtiter plate and incubate for 1 hr at RT.
12. For HRP readout add either OPD or TMB as substrate to allow color development and incubate for 10-20 minutes at RT.
13. Add acid stop reagent to stop the enzyme reaction.
14. Read at 405 nm for TMB/HRP.


**Third Development Experiment**


Goal: Determine the optimal buffers, incubation periods, temperatures, matrix effects, and other variables that may affect the assay.


*Reagents:*
Reagents are the same as in initial experiment.

*Protocol:*
Same as in previous experiment except for the following changes at steps 8 and 9:

8. Dilute the unlabeled ligand in antibody dilution buffer, and the matrix appropriate for the experiment, over a wide range of concentrations. Again the dilution buffer can be varied here according to the experimental design.
9. Add 100 μl of the labeled standard to each well in the microtiter plate and 100 μl of the various dilution of the unlabeled ligand. Incubate for 2.5 hours at RT. This incubation time can be varied for longer and shorter periods of time to potentially increase the sensitivity and dynamic range of the assay.

Results:  Analysis of the results is by use of JMP or other appropriate statistical software to determine the optimal conditions for incubation timing, buffers for dilution, and matrix effects.

# L. METHOD VALIDATION (PRE-STUDY)

It is important to note that the precision profile is based on just the calibration curve. Consequently only the calibration curve factors (quality and stability of reference standards, quality and stability of reagents, statistical validity of the calibration curve model) are taken into account for deriving these quantitation limits. Sample factors such as analyte (similar physicochemical substances), matrix (other substances that can affect analytical result) and operational factors can affect the performance of the assay/method as well. Thus the quantitation limits derived from the precision profile of a calibration curve is an optimistic assessment of method performance. If these limits are not satisfactory, we need to re-optimize the assay further.

If the quantitation limits from the precision profile are close to the limits desired for the method's intended use, proceed to a full validation experiment as outlined below. This validation experiment is used to <u>establish</u> the method quantitation limits using the analysis of recovery data from validation samples (spiked standards). This experiment will take into account the three major sources of variation described above (calibration curve factors, sample factors and operational factors).

For the full validation experiment, generate the following data in at least three independent runs.

- Calibration curve in each run, preferably in triplicate.

- Validation/QC samples (independent set of samples spiked with known amount of standards) in each run at six concentrations with four replicates; three concentrations near the precision profile estimates of lower and upper quantification limits, and three more equally spaced between lower and upper quantification limits.

- Estimate the concentrations of the validation samples of each run using the respective calibration curves. Then compute the % recovery of these validation samples using the following formula:

    % Recovery = 100*(Estimated Concentration)/True Concentration

- Now compute the average and standard error of the % recovery data of the validation samples from all runs for each concentration. Then standard error should be based on a separate variance component analysis of the multiple runs of validation data, and it should include the sources of variability relevant during the use of the assay in production. At the minimum, it will include inter-run and intra-run variability. Some of the other sources to consider may be analyst, plate, equipment, etc.

- Plot the average % recovery values along with the standard error (as calculated above) versus the true concentrations. Note that the % recovery along with the standard error as determine above reflects the Total Error of the assay.

- The %recovery and the standard error limits must be within +/- X% of the nominal value. If X is 30 of the nominal value at each concentration (i.e., 70% to 130%). That

is, this means that the Total Error of the assay must be within 30%. The value of X should be set based on the intended use of the assay. Recommendations on the acceptance criteria are discussed later in this chapter.

- If X is set at 30%, the lower quantification limit is the lowest concentration at which the % recovery is within 70% to 130%. The upper quantification limit is the highest concentration at which the % recovery is within 70% to 130%.

# M. METHOD VALIDATION (IN-STUDY)

The in-study validation phase is about making sure that the assay continues to perform per pre-defined specifications in each study run. During production phase, when the assays are being used for screening the unknowns, it is important to run validation/QC samples in every run with at least 2 replicates at high, middle and low concentrations (just one or two columns of a 96-well plate). Compute the average % recovery of these samples to make sure that the average recovery is within a reasonable range of accuracy (say, 80% to 120%). This might be adequate for quality control and is a reasonable compromise for any loss in assay throughput. Various methods may be considered for setting criteria for accepting or rejecting a study run during production run (in-study validation). This is addressed in a subsequent section in this chapter.

## Example of an Immunoassay Validation Experiment

Set up numerous aliquots of the standard and store frozen at – 70 C.  If the standard concentration is much higher than the first point on your curve, pre-dilute it so that a single, simple dilution can be made in order to set up the standard curve.

Dilute the standards serially to obtain an 8 point standard curve in the matrix appropriate for the samples that need to be measured.  For example if measuring tissue culture samples then the standards should be diluted in the same tissue culture medium that the samples are in.  For serum samples, the standards should be diluted in serum diluted with an optimized buffer to the same dilution that the samples will be diluted.

Set up a series of spiked samples, again in the matrix appropriate for the samples that will be measured.  The spiked control samples should not be the same concentration as in the standard curve and should cover the detectable range that the samples are thought to cover.

Follow the immunoassay protocol established during the optimization experiments. Set up the plate with 3-4 replicates of the standard curve and 4 or more replicates of the spiked control samples.

Assay at least 3 plates over 3 different days for a complete validation.

**Validation Plate Layout**

| C+ | C+ | C+ | C+ | CP | CP | CP | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C+ | C+ | C+ | C+ | CP | CP | CP | CP | | | | |
| C+ | C+ | C+ | C+ | CD | CD | CD | CD | | | | |
| C+ | C+ | C+ | C+ | CD | CD | CD | CD | | | | |
| C+ | C+ | C+ | C+ | CD | CD | CD | CD | | | | |
| C+ | C+ | C+ | C+ | CD | CD | CD | CD | | | | |
| C+ | C+ | C+ | C+ | CD | CD | CD | CD | | | | |
| C+ | C+ | C+ | C+ | CP | CP | CP | CP | | | | |

**Validation Results from an IL-10 Immunoassay**

The %recovery and the standard error that takes into account of the relevant sources of variation are plotted below. If X is 30%, then the quantification limits are the lowest and highest concentrations where the %recovery are within 70% to 130%. So for this assay, the lower quantification limit is the lowest concentration tested in this validation study (6.2 pg/ml), and the upper quantification limit is 3265 pg/ml.

| IL-10 | expected value | average 4,5,6 | % recovery |
|---|---|---|---|
| | 40000 | 36924.96 | 92.31 |
| | 11428.57 | 13846.57 | 121.16 |
| | 3265.306 | 2966.42 | 90.85 |
| | 932.9446 | 892.76 | 95.69 |
| | 266.5556 | 242.70 | 91.05 |
| | 76.15874 | 71.65 | 94.08 |
| | 21.75964 | 22.32 | 102.60 |
| | 6.21704 | 6.06 | 97.50 |

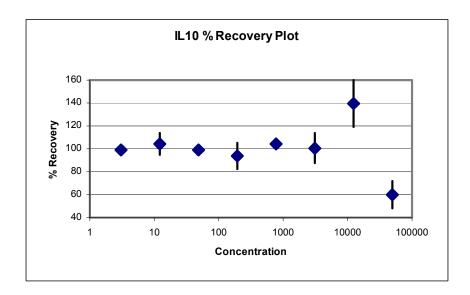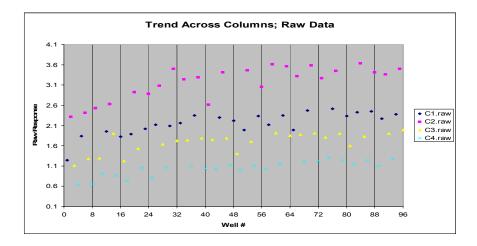**IL10 % Recovery Plot**

## Plate Uniformity & Variability Experiment

It is important to check whether there is any systematic data trend across rows or columns of the 96-well plate and whether there is any significant variability between plates. An experiment with three plates and four concentrations of the standard can be done using the plate-layout given below. In this layout, C1, C2, C3 and C4 denote the standard concentrations from lowest to highest. For the purpose of illustration, data from one of the plates and a plot of the data from this experiment are given below for a sandwich ELISA. A systematic trend across columns is evident from this plot. For determining the statistical significance of this trend and the plate to plate variability, further statistical analysis of the data can be done with the help of a statistician.

If the plate-format is not the same as indicated below, type in the necessary changes below:

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 |
| B | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 |
| C | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 |
| D | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 |
| E | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 |
| F | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 |
| G | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 |
| H | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 | C4 | C1 | C2 | C3 |

Paste the plate-data below:

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1.24 | 2.53 | 1.23 | 0.81 | 2.16 | 2.61 | 1.41 | 1.02 | 1.99 | 3.27 | 1.60 | 1.10 |
| B | 2.31 | 1.29 | 0.73 | 2.12 | 3.24 | 1.75 | 1.00 | 2.12 | 3.32 | 1.81 | 1.14 | 2.27 |
| C | 1.11 | 0.91 | 1.89 | 3.08 | 1.74 | 1.03 | 1.99 | 3.61 | 1.88 | 1.30 | 2.43 | 3.36 |
| D | 0.63 | 1.95 | 2.92 | 1.64 | 1.09 | 2.30 | 3.46 | 1.92 | 1.21 | 2.51 | 3.63 | 1.90 |
| E | 1.84 | 2.63 | 1.53 | 1.06 | 2.35 | 3.41 | 1.70 | 1.15 | 2.47 | 3.45 | 1.83 | 1.27 |
| F | 2.41 | 1.90 | 1.05 | 2.09 | 3.29 | 1.79 | 1.11 | 2.35 | 3.58 | 1.90 | 1.24 | 2.38 |
| G | 1.28 | 0.86 | 2.02 | 3.50 | 1.79 | 1.13 | 2.33 | 3.56 | 1.91 | 1.24 | 2.45 | 3.50 |
| H | 0.65 | 1.82 | 2.88 | 1.72 | 1.04 | 2.22 | 3.05 | 1.85 | 1.23 | 2.33 | 3.41 | 1.99 |

## N.  PRE-STUDY & IN-STUDY ACCEPTANCE CRITERIA

Different methods of quality control are available and routinely used in analytical methods. It is important that the methods used for assessment of method performance are suitable for the intended purpose.

The use of QC methods used in PK assays (eg 4-6-x) and clinical diagnostics (confidence limits) may both be applicable. It is up to the laboratory performing the analysis to choose the most relevant method to use and justify it scientifically based on statistical and clinical criteria. This will be critical when using 4-6-x in order to assign an appropriate value to 'x'.

Shah et al (1990) proposed the 4-6-X rule for in-study validation phase that has become popular and widely used. This rule states that 4 out of the total 6 samples should be within X% of the nominal/reference value, and at least one out of the two samples at each level must be within X% of the reference value. The choice of X is specified a priori based on the intended use and purpose of the assay, and it was set at 20% by Shah et al. DeSilva et al (2003) proposed the following criteria for pre-study and in-study validation phase of ligand-binding assays for assessing pharmacokinetics of macromolecules.

| Characteristic | Pre-Study Validation | In-Study Validation |
|---|---|---|
| Trueness (%Relative Bias) | ≤ ± 20 (± 25 at LQL) | - |
| Intermediate Precision (%CV) | ≤ 20  (25 at LQL) | - |
| Total Error | ≤ 30% | "4-6-30" rule |

It should be noted that the acceptance criteria for biomarker assays will depend heavily on the intended use of the assay and should ideally be based on physiological variability as well. According to the criteria listed in this table, X is set at 30% for in-study validation, and the total error is set to be within 30% for the pre-study validation, along with the 20% limits for each component of total error (bias and precision). The pre-study criteria (Total Error < X%) and the in-study criteria (4-6-X rule) are not entirely consistent because it does not take into account total error estimate variability and the consequent decision error rates. Thus the uncertainty in these estimates will depend on the magnitude of the errors and the number of measurements, and will in turn impact the level of decision error rates (Kringle, 1994). The appropriate value of X in 4-6-X can be determined based on the variability of the total error estimates in pre-study validation. When it is feasible to use more QC samples in each run, 8-12-X or 10-15-X will have much better statistical outcomes than the 4-6-X criteria. In addition, the use of control

charts as described by Westgard or tolerance limits based on pre-study validation data may be considered when possible.

The concept of total error as the primary parameter, and with bias and precision as additional constraints is very useful. This is because total error has a more practical and intuitive appeal as it relates specifically to our primary question of interest about the assay; How far are my observed test results from the reference/nominal value? Since this is the primary practical question in the minds of most laboratory scientists, the criteria on the assay performance for the in-study phase is defined with respect to this question. Therefore the primary criteria for the pre-study phase are also defined with respect to this question, that is, the total error.

Given that this total error approach is very intuitive and practical, it is important to consider a rule that will provide better consistency between our expected performance for the assay to the in-study and pre-study validation criteria.

Consideration of Physiological Variation for Acceptance Criteria:

One of the most important considerations for defining the performance criteria of most biomarker methods is the physiological variability in the study population of interest. That is, in order to determine whether a biomarker method is 'fit-for-purpose', we should determine whether it is capable of distinguishing changes that are statistically significant based on the intra-subject and inter-subject variation. The term "subject" here may refer to animal or human. For example, an assay with 50% total error during pre-study validation may still be adequate for detecting a 2-fold treatment in a clinical trial for a certain acceptable sample size. Thus whenever possible, the acceptance criteria for pre-study validation should be based on physiological variation in the study. An example of the use of intra-subject and inter-subject variation for defining the pre-study acceptance criteria can be found in http://www.westgard.com/guest17.htm.

When the relevant physiological data (say, treated patients of interest) are not available during the assay validation phase, then healthy donor samples should be used to estimate the intra and inter subject variation, and hence the desired specifications on the pre-study assay validation. This can be updated at a later time when there is access to the relevant patient data. If access to healthy donor samples is also not feasible, then other flexible biological rationale should be considered and updated periodically as more information become available over time. In the absence of physiological data or other biological rationale, the acceptance criteria for pre-study validation should not be strictly defined. Instead, only the performance characteristics from pre-study validation such as the bias, precision and total error should be reported. Any decision regarding the acceptance of the assay (pre-study acceptance criteria) and consequently the determination of the dynamic range (LQL, UQL) should be put on hold until adequate information related to the physiological data become available.

Assessment of analytical batches/runs in terms of acceptance (in-study validation) needs to take into account of the study need. Setting critical acceptance criteria a priori may not be appropriate (or even possible) to take into account all possible outcomes in the analytical phase – especially since the values seen in the incurred samples may not be what is expected or predicted. This is especially the case in new or novel BM's as opposed to those where historical information in normal and diseased populations is available.

It is advised that when constructing batches for analysis, ALL levels of QC's are analyzed at each QC interval. For example, a batch of 96-well microtiter plates may include 3 sets of QC's at start, middle and end of the plate, and all QC's (Low,Medium,High) are assayed at all three intervals. This will help in the assessment of method performance and batch acceptance for incurred samples.

In studies with large numbers of samples, assessment of method performance between batches may help before rejecting data. For example, it is of no value to reject batches when large numbers of high concentration QC's fail but where the low and medium QC's are good AND when all the study sample results are in the low to medium range. Here the positioning of the high QC based on expectation before the analysis of incurred samples has been flawed – but it does not necessarily make the study sample results invalid.

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http://www.brendan.com/

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# SECTION XI


# DATA STANDARDIZATION FOR RESULTS MANAGEMENT

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## SECTION XI:  DATA STANDARDIZATION FOR DISCOVERY RESULTS MANAGEMENT

## A. INTRODUCTION

## Definitions of Result Levels

**Raw** data

**Normalized** well level or individual data values (Inhibition, Stimulation, etc; in most cases Inhibition and Stimulation are expressed as a % of the dynamic range of the assay)

**Aggregate** median (preferred) or mean normalized well level data when replicates exist in a single run (Inhibition, Stimulation, etc…). This level provides for a consistent determination of n as it applies to *in vitro* results.

**Derived** data (ICx, Relative ICx, ECx, Ki, Kb, etc…)

**Summarized** data (geometric mean ICx, Relative ICx, Ki, Kb or average Inhibition, Stimulation, etc…)

## Abs IC50, Rel IC50 or Rel EC50

For assays described in this chapter, Absolute IC50, Relative IC50 and Relative EC50 are predominantly used to derive a value that can be used to compare results within and across runs as well as between assays.  Abs IC50 and Rel IC50 are used when different assumptions are applied; the selection of either is at the discretion of the scientist but should be applied consistently and not changed for a defined assay.

For consistency, Rel IC50 is used for inhibition assays while Rel EC50 is used for stimulation assays, even though there is no fundamental difference between them. Because of their relative simplistic composition, biochemical in vitro assays can be easily labeled as either Stim or Inh, while every biochemical whole cell assay can be either as Stim or Inh depending on multiple factors.  Therefore, the guideline for defining whole cell biochemical assays is to use the label that better reflects the perceived pharmacology, regardless of the direction (increasing or decreasing with test substance concentration) of the raw signal.  How an assay is defined can also drive which result type label to use.  For instance, if an assay categorized by Cell Cycle Modulation is attempting to <u>inhibit</u> the cell cycle, the Rel IC50 should be used.

## Guidelines for Curve Fitting

- Three or four parameter logistic curve fits are acceptable.
- Under appropriate conditions, the Top may be fixed to 100 and the Bottom may be fixed to 0.
- It is recommended that the Hill Coefficient not be preset to any fixed number, unless supported by a statistician.
- Cubic spline curve fits are not recomended, unless supported by a statistician.
- The Fitting Error of the IC50/EC50 should be less than 40% of the IC50/EC50 and not exceed 100%, unless supported by a statistician.  (*It should be noted that this*

*"standard error" is a measure of "goodness of fit" of the data to the curve fitting equation and not the "standard error" of aggregate data values*).

## Normalizing Data using a Positive Control Curve

In some cases, it is preferable to use a reference curve to define the dynamic range of the assay. In those cases, the fitted Top of the reference curve is substituted for the Max while the fitted Bottom of the reference curve is substituted for the Min in normalization calculations. This may be particularly useful in Agonist assays where the reference agonist curve is strongly recommended. It is still preferable to define the dynamic range on each plate so that individual plate drift is assessed and single plates can pass/fail. Additionally, the upper and lower asymptote of the reference curve should be established by the data in order to use them for dynamic range determination.

## Application of a Standard Curve

Use of a standard curve is required wherever possible when the raw data is not a linear function of the biological response. For example, optical densities, fluorescence units and luminescence units often cannot be directly used for calculations of activity as they are often non-linear functions of the concentration of the relevant biological product. This standard curve is used to convert the raw data to concentration of biological substance. The calculated concentrations are then used to calculate the Normalized Result, as discussed in each assay section. The standard curve data should be generated with an appropriate number and spacing of points, fit by an appropriate dose response model so that bias and precision are within acceptable limits, and all raw data within the scope of the assay can be converted to the biological response.

## B. DATA TYPES AND ASSOCIATED RULES FOR RADIOLIGAND BINDING ASSAYS: INHIBITION MODE

### Normalized Results
For radioligand binding methods, the use of Inhibition is recommended to quantify the ability of individual concentrations of a substance to inhibit the total specific binding of radioligand. The use of % bound for normalization is discouraged, but it's recommended that biologists calculate and track changes to % bound as a measure of assay performance.

Calculation:

$$\text{Inhibition (\%)} = 100 - \left[ \frac{(\text{Measured Binding - Min})}{(\text{Max - Min})} \right] x100$$

Max = maximum binding
Min = non-specific binding

## Derived Results:  Absolute IC50 and Relative IC50

Absolute IC50 = the molar concentration of a substance that reduces the specific binding of a radioligand to 50% of the maximum specific binding.

Relative IC50 = the molar concentration of a substance that reduces the specific binding of a radioligand to 50% of the range of the binding curve (Top – Bottom) for that particular substance.

Notes:

- For incomplete curves, the response data should span 50% for an IC50 to be used for the determination of a Ki.
- The Top and Bottom parameters should be within +/- 20% of the Top and Bottom dynamic range control values.

## Derived Results:  Ki

The equilibrium dissociation constant of a test compound (Ki) should be calculated using the standard Cheng-Prusoff equation:

$$ Ki = \frac{IC50}{1 + \frac{[R]}{Kd}} $$

[R] = concentration of radioligand used in the assay
Kd = the equilibrium dissociation constant of the radioligand in the assay

## Notes:

- Ki carries the same prefix as the IC50 from which it is derived.
- For competitive binding mechanisms, a Ki is recommended to be reported for radioligand binding assays, which produce IC50's by 3 or 4-parameter curve fitting methods.
- For uncompetitive or complex (ill-defined) binding mechanisms, an IC50 is preferred, since one of the main assumptions for the use of the Cheng-Prusoff equation is based on a competitive, bimolecular interaction**.**

## C. DATA TYPES AND ASSOCIATED RULES FOR ENZYMATIC ASSAYS: INHIBITION MODE

### Normalized Results

Inhibition with a UOM of % based on complete enzyme inhibition (dynamic range of the assay)

Calculation:

$$\text{Inhibition (\%)} = 100 - \left[ \frac{\left( \text{Activity of Enzyme with Test Cmpd \& Substrate - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Max = The observed enzyme activity measured in the presence of enzyme, substrate(s) and cofactors utilized in the method

Min = The observed enzyme activity measured in the presence of substrate(s) and cofactors utilized in the method, and (a) in the absence of enzyme, or (b) in the presence of a fully inhibited enzyme

### Derived Results: Absolute IC50, Relative IC50

Relative IC50 = the molar concentration at which 50% of maximal inhibition for that substance is observed.

Absolute IC50 = the molar concentration of a substance that reduces the enzymatic activity to 50% of the total enzymatic activity.

## D. DATA TYPES AND ASSOCIATED RULES FOR IN VITRO FUNCTIONAL ASSAYS

### 1. Antagonists

### Normalized Results

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation

$$\text{Inhibition (\%)} = 100 - \left[ \frac{\left( \text{Response in presence of Test Cmpd \& Ref Agonist - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Max = (a) response in the presence of diluents and in the absence of test substance and agonist; or (b) response in the presence of maximally effective antagonist and challenge dose of agonist.
Min = response in presence of some concentration of a reference agonist challenge does.

## Derived Result: Rel IC50

Relative IC50 = the molar concentration of a substance (antagonist) that reduces the efficacy of the reference agonist or the constitutive activity of the biological target by 50% of the antagonist curve (Top-Bottom) for that particular test substance.

## Derived Result:  Kb

Calculation of Kb by Schild analysis isn't standard practice due to throughput and cost disadvantages.  Consequently, the Cheng-Prusoff equation is typically used to reduce the data and subsequently assigned the label of Kb.

Calculation: Use standard Cheng-Prusoff equation for functional assays.

$$Kb = \frac{IC_{50}}{1 + \dfrac{[A]}{EC_{50}}}$$

[A] = the concentration of the reference agonist that is being inhibited
$EC_{50}$ = the Relative $EC_{50}$ of the reference agonist determined in the same run of the
assay.

If the slope of the curve for the reference agonist deviates significantly from 1, the use of the modified Cheng-Prusoff equation (see section VI) is recommended.

## Other Derived Results:
## Schild Kb

Schild Kb is measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC50 concentrations are used) in the absence and presence of one or more concentrations of the antagonist. Schild Kb offers a true evaluation of a test compound's ability to mechanistically perform as an antagonist. This process exposes toxic effects and compound precipitation as false positive activity, and therefore, should be used when time and cost are not limitations.
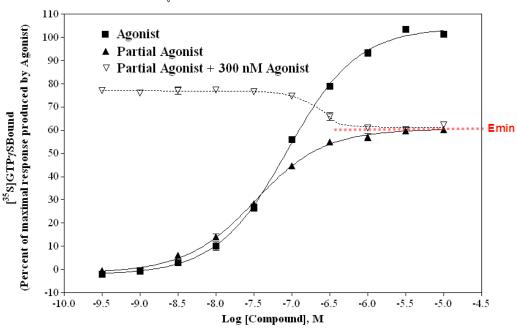
## Emin

The maximum activity of an antagonist test substance relative to a reference agonist. This is obtained by first generating a fitted top from a %Inhibition curve and then converting that to the corresponding %Stimulation of the reference agonist curve. The Emin value for antagonist mode should equal the relative efficacy for agonist mode for competitive

inhibitors.  In order to make use of Emin, the selected agonist concentration (i.e. EC80)
should produce an activity above the expected Emin value.



**Partial Agonist Stimulation of, or Inhibition of 300 nM Agonist-Stimulated, [$^{35}$S]GTPγS binding**

## Notes:

- Kb carries the same prefix as the IC$_{50}$ from which it is derived.
- The use of Abs IC50 is discouraged.
- Since partial antagonists exist, a full response curve with defined Top & Bottom can be achieved even if the %Inh doesn't exceed 50%.
- A concentration response curve for the reference agonist should be determined in each experimental run if a Kb is to be determined.  The frequency within the run depends on assay variability. A statistician should be consulted concerning this frequency during the assay validation process.

## 2. Agonists

## Normalized Data

Stimulation with a UOM of % should be calculated for responses to individual
concentrations of test substances.

Calculation:

$$\text{Stim (\%)} = \left[ \frac{\left( \text{Response in presence of Test Cmpd - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Min = the fitted Bottom of a 4 parameter logistic curve fitting equation applied to data generated from the positive control.

Max = (a) the maximum activity of a positive control agonist determined by the fitted Top of a 4 parameter logistic curve fitting equation applied to a concentration response curve from the positive control; or (b) the maximum activity of a positive control in Max wells, which should represent the empirically-derived saturating concentration of the positive control.

## Derived Results:  Relative EC50 and Relative Efficacy

Relative EC50= the molar concentration of a substance that produces 50% of that test substance's maximum stimulation.

Relative Efficacy = the maximum activity of a test substance relative agonist. The unit of measure (UOM) for Relative efficacy is %.

Calculation:

$$\text{Rel Eff (\%)} = \left[ \frac{\text{Fitted Top of Test Cmpd}}{\text{Fitted Top of Reference Agonist}} \right] x100$$

## Other Derived Results:
### Fold Activity and Fold Activity Max

The fold activity (or fold activity max) result is useful when comparing test compounds evaluated across multiple functional assays because varying levels of efficacy can be observed amongst the different or same reference agonists.  The intended use of this calculation is to provide additional information to reduce or define differences between assays so that differences between compounds can be further quantified.  For example, a compound run in an assay normalized to a reference agonist with low efficacy would appear to be more efficacious when compared to another compound run in a separate assay normalized to a reference agonist with high efficacy.  Comparing folds activities, which looks at the magnitude of compound-induced activity relative to baseline, enables a scientist to make a conclusion that is not influenced by differences in reference agonist responsiveness.  Also, the fold activity result of a control compound can be useful to quality control chart, tracking changes in assay responsiveness over time.

Calculation:

$$\text{Fold Act \& Fold Act Max} = \frac{\text{Raw data response in presence of Test Cmpd}}{\text{Min}}$$
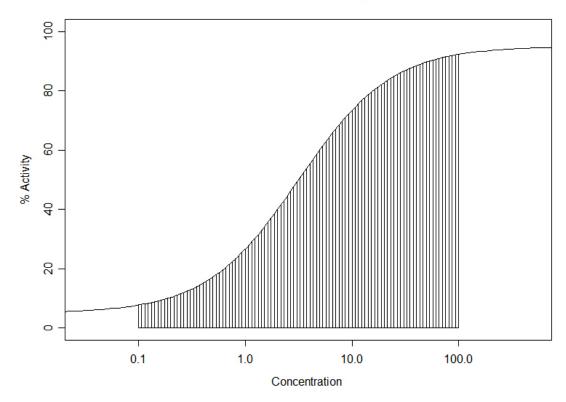
Min = Raw basal activity of constitutive receptor

Relative AUC

Relative AUC (Area Under the Curve) is defined as the ratio of the area under the fitted concentration-response curve for the test compound to the area under the fitted concentration-response curve for the reference compound.  Specifically, areas are calculated as the area under the curve that lies above the horizontal line $y = 0\%$.  The area calculation corresponds to the shaded region in the figure below, where the contribution to the area as one moves along the concentration axis is proportional to the log of the concentration distance covered, not the linear concentration distance covered.  One should calculate the area using an exact formula when it is available, as is the case for the 4PL and 3PL models.  Otherwise, one may use an approximation method, such as the trapezoid rule.  In either case, for the calculated value of relative AUC to be meaningful, the areas for both the test and reference compounds should be computed with respect to the same concentration range.  Likewise, the comparison between two relative AUCs is only meaningful when each is computed with respect to the same concentration range.  If the same concentration range was not used for assaying the test and reference compounds, the equations for the fitted curves may be used for extrapolation in order to compute the components of the relative AUC over the same concentration range.

Rel AUC is useful with functional assays in which compounds are measured with varying efficacies (agonists and partial agonists) and potencies.  Since Rel AUC measures the area of activity, both efficacy and potency data are essentially combined, generating a value that provides an overall assessment of activity and selectivity between tested compounds.  However, Rel AUC should not be a substitute but rather a supplement to individual efficacy and potency data during the analysis process.

## AUC over concentration range 0.1 to 100



This figure illustrates the "area of activity" that is used in the calculation as Rel AUC.

Calculation:

$$\text{Rel AUC} = \left[ \frac{\text{AUC of Test compound}}{\text{AUC of Reference compound}} \right]$$

## Notes:

- A four-parameter curve fit should be used for the Ref Agonist.
- The maximum and minimum asymptotes should be defined by the data for the Ref Agonist
- Calculation of Rel Eff assumes that both the test compound and positive control each have a defined Top asymptote.

## Orphan receptors – Stimulation Mode

Assays exist for which there are no identified positive control or reference agonist compounds. An example of this situation is an assay that utilizes an "orphan" target as a bio-entity. An "orphan" target is a bio-entity that has a primary sequence suggesting it is

a member of one of the super families of biological targets; however, no ligand for this "receptor" has been identified. Generally, it is the aim of the research effort to identify ligands for this "orphan" so that a protocol for a validated assay can be created.  Until at least enough data is gathered to identify a ligand for these types of bio-entities, assays utilizing them will be considered "validated" at only the hit to lead level.  During this period, responses to individual concentrations of test substances can be normalized by one of the following formulae, which either make use of a known nonspecific activator or simply use basal activity of the constitutive receptor.

## I. Orphan Receptors Normalized to Nonspecific Activator

Stimulation with a UOM of  % should be calculated for responses to individual concentrations of test substances.

$$\text{Stim (\%)} = \left[ \frac{\left(\text{Response in presence of Test Cmpd - Min}\right)}{\left(\text{Max - Min}\right)} \right] x100$$

Max = fully activated by nonspecific activator
Min= constitutive receptor (no activation)

## II. Orphan Receptors Normalized to Constitutive Receptor

Responses to individual concentrations of test substances that increase the measured activity of the orphan target are normalized to the basal level of activity of the target measured in the absence of the test substance.  These responses can be expressed as either a percent of the basal activity or as a fold of the basal activity using one of the following

Calculation:

$$\text{Fold Act \& Fold Act Max} = \frac{\text{Raw data response in presence of Test Cmpd}}{\text{Min}}$$

Min = Raw basal activity of constitutive receptor

## Notes:

- Results from this equation can generate percents much greater than 100.
- Expression of Fold Act or Fold Act Max should only be determined until either a nonspecific activator or ligand is identified; and should only be used to rank order compounds tested in the same assay.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist.  If the calculated value is less than 1, the test compound could be an inverse agonist.

## 3. Potentiators

Potentiation assays measure the ability of an inactive test substance to augment the response produced by a relatively low concentration of an active substance in some biological system. Currently, these assays are run in one of two modes. The following paragraphs address the most frequently used mode.

The first mode involves the addition of one or more concentrations of a test substance in the presence of a fixed concentration of the known active substance called the "Reference Agonist". In this mode, potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone. But, how does one normalize this response?

It is recognized that potentiation assays might be executed when no known potentiator exists. However, no potentiation assay should be run without the existence of a known Reference Agonist. Therefore, the response to the specific concentration of the Reference Agonist plus the test substance (potentiation) should be normalized to the fitted Top of a concentration response curve of the Reference Agonist determined at least once in every run of the assay. The frequency of the determination of the concentration response curve of the Reference Agonist for the purpose of normalizing other responses in any potentiation assay would be dependent upon other factors such as plate variability and run-to-run reproducibility.

## Normalized Data

Stimulation with a UOM of % should be calculated for responses to the Reference Agonist.

Potentiation with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Pot (\%)} = \left[ \frac{\left( \text{Response in presence of Test Cmpd \& Challenge Dose - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Min = Response in the presence of challenge dose
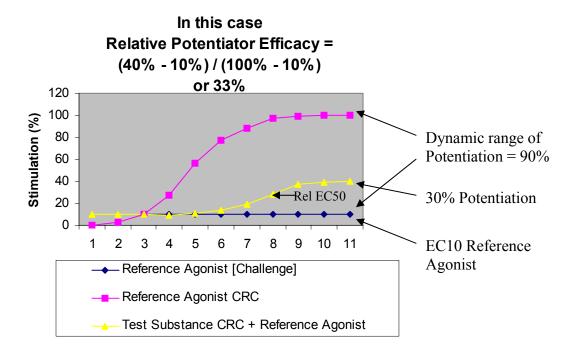Max = Response in the presence of full agonist dose

## Notes:

- This provides for a Potentiation equal to 0% when the response to the combination of test substance and Reference Agonist is equal to the response to the Reference Agonist alone (e.g. a test substance that is not a potentiator).

## Derived Data

Relative EC50= the molar concentration of a substance that produces 50% of that test substance's maximum stimulation.

Relative Potentiator Efficacy:  There is little if any discussion in the scientific literature addressing a standard term or calculation of efficacy of a potentiator.  It is suggested that this result type be termed Relative Potentiator Efficacy (or Rel Pot Eff) to distinguish it from the Relative Efficacy of an agonist.  It is equal to the fitted Top of the potentiation curve minus the <u>normalized</u> response to the specific concentration of Reference Agonist alone divided by 100 minus the <u>normalized</u> response to the specific concentration of Reference Agonist alone.  The following cartoon illustrates the above decisions.



**In this case**
**Relative Potentiator Efficacy =**
**(40% - 10%) / (100% - 10%)**
**or 33%**

Dynamic range of Potentiation = 90%

30% Potentiation

EC10 Reference Agonist

Legend:
- Reference Agonist [Challenge]
- Reference Agonist CRC
- Test Substance CRC + Reference Agonist

## 4. Inverse Agonists

According to multiple models of drug-receptor interaction, receptors have been demonstrated to exist in equilibrium between two states.  These two states are R*, the active form of the receptor, and R, the inactive form.

Agonists exhibit higher affinity for the active form of the receptor. When an agonist binds to a receptor, it stabilizes the active form of the receptor, shifts the equilibrium toward the active state and produces a response in the biological system under investigation. Substances that produce this kind of effect possess positive intrinsic activity.

Antagonists exhibit equal affinity for both forms of the receptor. When an antagonist binds to a receptor, it stabilizes the initial equilibrium between the active and inactive

forms of the receptor. Therefore, no observable change in the activity of the biological system occurs. Substances of this type possess zero intrinsic activity.

Inverse agonists exhibit higher affinity for the inactive state of the receptor. When an inverse agonist binds to a receptor, it stabilizes the inactive form of the receptor, shifts the equilibrium toward that state and produces an opposite response in the biological system. These substances possess negative intrinsic activity.

Receptors have been demonstrated to exist in a constitutively active state both *in vitro* and *in vivo*. *In vitro*, the constitutive activity observed in assays utilizing transfected cell lines is generally attributed to the over expression of the receptor at levels hundreds to thousands of times higher than occur *in vivo*. Under these conditions, the total number of receptors in the active state is sufficiently high to produce a measurable response even when no exogenous substance has been added to the system. The addition of an inverse agonist to the system produces a decrease in the measured response. The magnitude of the decrease is related to the amount of negative intrinsic efficacy of the inverse agonist.

The possibility for confusion exists when one desires to quantify results for potential drug candidates that are inverse agonists. Some of the questions that arise are:

1. Since the measured response is a decreased activity produced by an inverse agonist, is the normalized result type Inhibition or Stimulation?
2. What is the algorithm for normalized results?
3. What is the algorithm for fitting concentration response curves?
4. Is the result type describing potency of a test substance a Relative EC50 or a Relative IC50 or something else?
5. How is the result type describing potency differentiated from the potency result type for an agonist?
6. Is Relative Efficacy a negative number?

There are no absolute answers to these questions provided by the current literature; however, there is a consistent theme.

1. The most frequently used normalized result type is Inhibition with a unit of measure of %.
2. The dynamic range for inverse agonists is the difference between activity in the absence of, or fully inhibited, biological target and the constitutive activity. Use of the "absence" method is preferable in early development of inverse agonist assays because it eliminates the dependency on a pre-existent known inverse agonist to compare responses of test substances to. However, as with other functional assays, as soon as an appropriate inverse agonist has been found, it should be utilized as a positive control in the assay for the purpose of calculating relative efficacies.

## I. Assays Normalizing Data to an Inverse Agonist Control
## Normalized Data

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Inh}\,(\%) = \left[ \frac{\left(\text{Response in presence of Test Cmpd - Min}\right)}{\left(\text{Max - Min}\right)} \right] x100$$

Min = Response activity in presence of constitutively active receptor alone
Max = Response activity in presence of positive control and receptor

## Derived Data

Relative EC50 Inverse = the molar concentration of a substance that produces 50% of the range of inverse agonist curve (Top – Bottom) for that particular test substance.

Rel Efficacy Inverse = 100 x (Fitted Top of the test substance expressed as %/Fitted Top of the Positive Control Reference Inverse Agonist expressed as %)

Calculation:

$$\text{Rel Eff Inv}\,(\%) = \left[ \frac{\text{Fitted Top of Test Cmpd}}{\text{Fitted Top of Reference Inverse Agonist}} \right] x100$$

## Notes:

- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- Hill Coefficient and Rel Eff Inv values are positive.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

## II. Assays Normalizing Data to No Receptor Control (Orphan Receptor)
## Normalized Data

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Inh (\%)} = \left[ \frac{\left( \text{Response in presence of Test Cmpd \& Receptor - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Min =  Response activity in the presence of the constitutively active receptor alone
Max = Response activity in the absence of the receptor

## Derived Data

Relative EC50 Inverse = the molar concentration of a substance that produces 50% of the range of inverse agonist curve (Top – Bottom) for that particular test substance.

## Notes:

- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- Hill Coefficient and Rel Eff Inv values are positive.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist.  If the calculated value is less than 1, the test compound could be an inverse agonist.


## III. Assays Normalizing Data to Reference Agonist:
## Normalized Data

Stimulation with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Stim (\%)} = \left[ \frac{\left( \text{Response in presence of Test Cmpd - Min} \right)}{\left( \text{Max - Min} \right)} \right] x100$$

Min = the fitted Bottom of a 4 parameter logistic curve fitting equation applied to data generated from the Reference Agonist

Max = the maximum activity of a Reference Agonist determined by the fitted Top of a 4 parameter logistic curve fitting equation applied to a concentration response curve from the positive control.

## Notes:

- %Stimulation values will be negative for inverse agonist test compounds.

## Derived Data

Relative EC50 Inverse = the molar concentration of a substance that produces 50% of that test substance's inverse agonism.

Relative Efficacy = the maximum activity of a test substance relative to a Reference Agonist. The unit of measure (UOM) for Relative efficacy is %.

Calculation:

$$\text{Rel Eff Inv}(\%) = \left[\frac{\text{Fitted Bottom of Test Cmpd}}{\text{Fitted Top of Reference Agonist}}\right] x100$$

## Notes:

- Rel Eff and Hill Coeff values for inverse agonists will be negative.
- Calculation of Rel Eff assumes the test compound have a defined Bottom asymptote and Reference Agonist have a defined Top asymptote.
- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist.  If the calculated value is less than 1, the test compound could be an inverse agonist.

## E. GLOSSARY

**Abs IC50**:  Absolute IC50; the molar concentration of a substance that inhibits 50% of the dynamic range of the assay.  In contrast to Rel IC50, Abs IC50 is not the inflection point of the curve.  It's determined to be the concentration at which 50% inhibition is realized.

**Bottom**: The lower asymptote of a logarithmically derived curve.  The Bottom value can be determined with real values or predicted using the logarithm applied to the result data set.

**CRC**:  Concentration-response curve mode.  The mode to describe an assay performed with multiple concentrations of a given test substance, which might then render a logarithmically-derived graph curve.

**Emin**: The maximum activity of an antagonist test substance relative to a reference agonist. This is obtained by first generating a fitted top from a %Inhibition curve and then converting that to the corresponding %Stimulation of the reference agonist curve. The E-min value for antagonist mode should equal the relative efficacy for agonist mode for competitive inhibitors.

**Fold Activity**: The ratio of biological activity in the presence of an exogenous substance to that in its absence. It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells. This result type is used exclusively with single point assays. If the value is greater than 1, the test compound is likely an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

**Fold Activity Max**: The maximum observed Fold Activity in a concentration response curve whether it was excluded or not. It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells. If the value is greater than 1, the test compound is likely an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

**Fold Activity Max (FA)**: The maximum observed Fold Activity in a concentration response curve whether it was excluded or not. The (FA) indicates that his result type is summarized. Since activity can be detected at different test substance concentrations, the summarized value must be viewed with this knowledge.
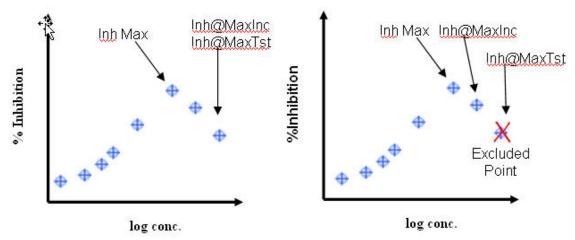
**Hill Coeff**: Derived slope a three or four parameter logistic curve fit. Should not be fixed to any given value without consultation with a statistician. It should not be a negative value except for inverse agonist assays.

**Inh**: Activity determined for a single point inhibition assay. Unit of Measure is always %.

**Inh @ Max Inc**: Inhibition observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (see Illustration below)

**Inh @ Max Tst**: Inhibition observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (see Illustration below)

**Inh Max:** Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm (see Illustration below)

**Inh Max (FA):** Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm.  This result type differs from Inh Max by allowing summarization to occur; the FA is defined as 'for averaging'.  Since this result type could yield an average value from multiple test substance concentrations, the value should be used with this knowledge and therefore with caution.

**Ki**:  Result from the Cheng-Prusoff equation or from a slightly modified derivation.  This label is used primarily with binding assays (see QB manual for formula) and represents the affinity of a compound for a receptor.  Documentation of the formula and any changes to the Cheng-Prusoff should be noted in the assay protocol.

**Kb**:  Result from the Cheng-Prusoff equation or from a slightly modified derivation.  This label is used primarily with functional antagonist assays (see QB manual for formula) and represents the affinity of a compound for a receptor.  This label doesn't represent results mechanistically determined via the Schild analysis; rather the label Schild Kb is used in those calculations.

**Pot**:  Potentiation result type for single point mode. Many potentiation assays involve the addition of one or more concentrations of a test substance in the presence of a fixed concentration of the known active substance called the Reference Agonist.  In this mode, potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone.

**Pot @ Max Inc**: Potentiation observed at the highest included concentration of a substance from an analysis of a concentration response curve.

**Pot @ Max Tst**: Potentiation observed at the maximum concentration of a single substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results.

**Pot Max**: The maximum potentiation observed for a substance in a single run of a potentiation concentration response mode method regardless of whether it was included in the parametric fit to produce derived results.
Pot:  to be added Activity determined for a single point potentiation assay.

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**Rel AUC**:  Defined as the ratio of the area under the fitted concentration-response curve for the test compound to the area under the fitted concentration-response curve for the reference compound.

**Rel EC50**:  Relative EC50; the molar concentration of a substance that stimulates 50% of the curve (Top – Bottom) for that particular substance.  It can also be described as the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.

**Rel EC50 Inv**: The Relative EC50 of an inverse agonist.

**Rel Eff**: The maximum activity of a test substance relative to a standard positive control agonist.  The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of an Agonist control.  The agonist control should have a four parameter curve fit with defined lower and upper asymptotes but can have the Bottom fixed to zero in certain cases.  The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

**Rel Eff Inv**: The maximum activity of a test substance relative to a standard positive control inverse agonist.  The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of the Inverse Agonist control.  The inverse agonist control should have a four parameter curve fit with defined lower and upper asymptotes but can have the Bottom fixed to zero in certain cases.  The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

**Rel IC50**:  Relative IC50; the molar concentration of a substance that inhibits 50% of the curve (Top – Bottom) for that particular substance.  It can also be described as the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.
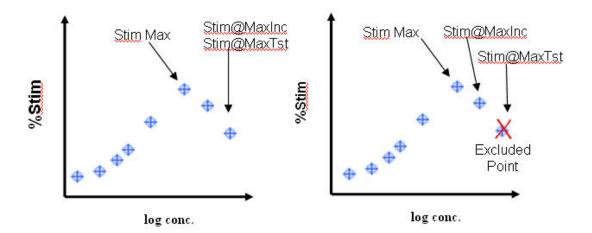
**Rel Pot Eff**: The fitted top of the potentiation curve minus the normalized response to the specific concentration of Reference Agonist alone divided by 100 minus the normalized response to the specific concentration of Reference Agonist alone.

**Stim**: Activity determined for a single point stimulation assay.  Unit of Measure is always %.

**Stim @ Max Inc**:  Stimulation observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

**Stim @ Max Tst**: Stimulation observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

**Stim Max:** Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm   (See illustration below)



**Stim Max (FA):** Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm.  This result type differs from Stim Max by allowing summarization to occur; the FA is defined as 'for averaging'.  Since this result type could yield an average value from multiple test substance concentrations, the value should be used with this knowledge and therefore with caution.

**Schild Kb**: A measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC50 concentrations are used) in the absence and presence of one or more concentrations of the antagonist.  See pp. 335-339, Pharmacologic Analysis of Drug-Receptor Intercation, 3rd Ed. by Terry Kenakin.

**SP**:  Single point mode.  Assay performed with once concentration of test substance.  Common result types used include Inh and Stim.  Result values should always include the concentration of the test substance used to determine the activity.

**Stephenson's Kp**: A measure of affinity for a partial agonist that is calculated through the comparison of equi-active concentrations of a full agonist in the absence and presence of a single concentration of the partial agonist.  See pp. 284-286, Pharmacologic Analysis of Drug-Receptor Intercation, 3rd Ed. by Terry Kenakin.

**Top**: The upper asymptote of a logarithmically derived curve.  The Top value can be determined with real values or predicted using the logarithm applied to the result data set.

# SECTION XII

# MECHANISM OF ACTION ASSAYS FOR ENZYMES

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## SECTION XII:  MECHANISM OF ACTION ASSAYS FOR ENZYMES

## A. GLOSSARY OF MOA TERMS

The definitions for these terms were gathered from references

**Active site** – the specific and precise location on the target responsible for substrate binding and catalysis.

**Allosteric activators** – an allosteric effector that operates to enhance active site substrate affinity and/or catalysis. (Copeland, Enzymes, pg368)

**Allosteric effector** – small molecule that can bind to sites other than the enzyme active site and, as a result of binding, induce a conformational change in the enzyme that regulates the affinity and/or catalysis of the active site for its substrate (or other ligands). (Copeland, Enzymes, pg368)

**Allosteric repressors** – an allosteric effector that operates to diminish active site substrate affinity and/or catalysis. (Copeland, Enzymes, pg368)

**Allosteric site** – a site on the target, distinct from the active site, where binding events produce an effect on activity through a protein conformational change. (Kenakin, A Pharmacology Primer, p195).

**Alpha** – typically noted as the ratio, $K_I'/K_I$. It reflects the effect of an inhibitor on the affinity of the enzyme for its substrate, and likewise the effect of the substrate on the affinity of the enzyme for the inhibitor. (Copeland, Enzyme, pg268)

**Biochemical assay** – the in vitro based mechanism used to measure the activity of a biological macromolecule (enzyme).

**Cofactor** – nonprotein chemical groups required for an enzyme reaction.

**Enzyme** – protein that acts as a catalyst for specific biochemical reaction, converting specific substrates into chemically distinct products.

**Multivariate fitting**

> Fitting a more than 2 variable model (Example: Response, [Inhibitor], [Substrate]) to all of the data from an MoA experiment using nonlinear regression.

**Inhibitor** – any compound that reduces the velocity of an enzyme-catalyzed reaction measured in a biochemical assay, as represented by percent inhibition or $IC_{50}$.

**Initial velocity** – the initial linear portion of the enzyme reaction when less than 10% of the substrate has been depleted or 10% of the product has formed. (QB Manual, Section IV, pg5)

**In vitro** – (to be defined later)

**Ligand** – a molecule that binds to the target. (Kenakin, A Pharmacology Primer, pg 198)

**Linearity** – A relationship between two variables that is best described by a straight line. In MoA experiments, the amount of product formed should be linear with respect to time.

**Substrate** – a molecule that binds to the active site of an enzyme target and is chemically modified by the enzyme target to produce a new chemical molecule (product).

**Target** – a macromolecule or macromolecular complex in a biochemical pathway that is responsible for the disease pathology.  (QB manual, Section XII, pg3)

**$k_{cat}$** – turnover number representing the maximum number of substrate molecules converted to products per active site per unit time.  (Fehrst, Str Mech Prot Sci, pg109)

**$K_I$** – the affinity of the inhibitor for free enzyme.

**$K_I$'** – the affinity of the inhibitor for the enzyme-substrate complex.

**$K_M$** – the concentration of substrate at ½ Vmax, according to the Henri-Michaelis-Menten kinetic model (QB manual, Section IV, pg9)

**$k_{off}$** – the off-rate associated with the release of inhibitor from an enzyme-inhibitor complex.

**$k_{on}$** – the on-rate associated with the formation of an enzyme-inhibitor complex.


# B.  OVERVIEW OF MOA IN DRUG DISCOVERY

The purpose of a mode of action study is to characterize the interaction of a compound with its target to understand how the compound interacts with the target and how natural substrates at physiologic concentrations will modulate this activity.  These compounds are often inhibitors of enzymes but only rarely become drugs due to the requirement of a drug to not only inhibit the target but to have acceptable solubility, permeability, protein binding, selectivity, metabolism and toxicity profiles.  This potential of the compound to become a drug is slowly revealed through the analysis and tracking of these characteristics, as chemistry elaborates the structure activity relationship (SAR).   As described in the body of this document, certain types of biochemical behavior are associated with good drug-like properties both *in vitro* and *in vivo*.

Most biochemical screens are designed to provide a chemical starting point based upon the most robust, simple and inexpensive modality for screening.   This is due to the required reproducibility in the screening process and the potentially large number of molecules to be run through the screen.  Most enzymatic screens are designed to identify inhibitors regardless of their mode of action.  Thus, screens are usually run at or below the $K_m$ for the substrate(s).  In the case of an enzyme with two substrates, the screen is often designed to run under pseudo-first order kinetics by running the assay under conditions where one substrate is at saturation, well above its $K_m$, and the second is at or below its $K_m$ for the enzyme.  One can therefore identify inhibitors that have competitive, noncompetitive and uncompetitive behavior with regard to the substrate at or below $K_m$ and noncompetitive or uncompetitive behavior with regard to the other substrate at well above its $K_m$ for the enzyme.

In the drug discovery process, the screening phase casts a wide net and the ability to further analyze compounds in more detail is limited, therefore the number of actives

isolated from a screen for follow-up are determined by the overall hit rate, the repeat rate upon retesting and determination of the $IC_{50}$ in a concentration response curve (CRC) test. In general, activities range from mid-micromolar to sub-micromolar for enzyme inhibitors right out of the screen. It is this piece of information (the $IC_{50}$), along with an analysis of the structural classes of active molecules by a medicinal chemist, which defines the initial SAR, if there is one in the data. It is after this initial analysis that MoA studies can prove valuable by further defining the nature of the inhibitor from a biochemical point of view. Mechanism of action studies at this point in the drug discovery process define the nature of the SAR by elucidating the type of inhibition by which the discovered molecules operate. Thus, one can define if the discovered inhibitor is competitive with substrate, for example, and as described below, potentially suffers from certain liabilities associated with this mechanism.

Cell based assays of biochemical actives are usually utilized to identify promising molecules in a second round of low to medium throughput screening. If a molecule shows significant activity in a cell based assay, then it continues through the flow scheme. The lack of cell based activity of biochemically potent actives is usually attributed to lack of cellular permeability, with a wave of the hand; however, an understanding the MoA of a compound at this stage can add depth to the interpretation of cellular activity or its absence. Knowing a compound is competitive with a substrate helps establish the binding pocket and in combination with structural and SAR information provide an immediate direction for further chemical synthesis. However, these competitive compounds with promising structure and potent biochemical activity might compete with a cellular substrate present at high intracellular concentration thus show no significant cell based activity. Alternatively, more potent cell based activity than is biochemically predicted from $IC_{50}$ curves might correlate with unusual kinetic behaviors such as slow binding behavior and/or slow off rates (tight binding). As there is no single unique answer, biochemical MoA studies help in interpretation of cell based activities, and provide further support for molecules with desirable characteristics to move forward in the flow scheme. Traditionally, as MoA studies were slow, laborious efforts, only a few selected molecules could be readily analyzed. With the advent of laboratory automation and enhanced data processing, it is now possible to assess a larger number of compounds rapidly. Therefore, it is feasible (and desirable) to examine the results of a screening campaign, in addition to standard cell based assays in the second tier, by an analysis of MoA.

## C. TYPES OF INHIBITION

There are 3 main types of inhibition (competitive, noncompetitive, and uncompetitive) that are most commonly used to describe the binding of an inhibitor to a target enzyme. However, a complete analysis of the mechanism of action requires the scientist to also evaluate other potential inhibition events, including allosteric, partial, tight-binding, and time-dependent inhibition. A review of these types of inhibition is provided.

## Competitive Inhibition

A competitive inhibitor binds only to free enzyme.  Often this binding event occurs on the active site of the target, precisely where substrate also binds.  Although this is the case for a majority of competitive inhibitors, it is a misleading oversimplification.  It is more appropriate to state that the binding of a competitive inhibitor and the binding of substrate are mutually exclusive events.  Figure 1 below provides illustrations of some possible mutually exclusive binding events.
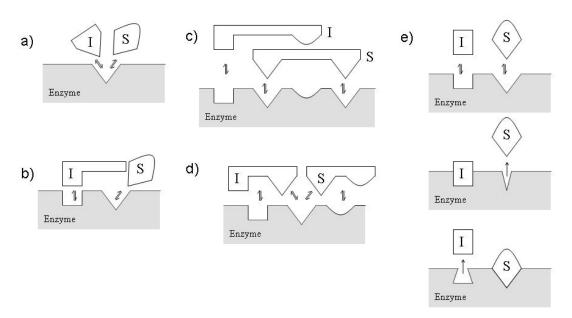


**Figure 1** – Examples of Competitive Inhibition where Substrate (S) and Inhibitor (I) binding events are mutually exclusive.  (a) Classical model for competitive inhibition where S and I compete for the same precise region of the active site.  (b) I does not bind to the active site, but sterically hinders S binding.  (c) S and I binding sites are overlapping.  (d) S and I share a common binding pocket on the enzyme.  (e) I binding can result in a conformational change that prevents S binding (and vice versa).  This was adapted from Segal, Enzyme Kinetics.

Despite the differences in binding to the free enzyme illustrated in Figure 1, all competitive inhibitors have the same effects on substrate binding and catalysis.  A competitive inhibitor will raise the apparent $K_M$ value for its substrate with no change in the apparent Vmax value.  As a result, it is often stated that competitive inhibition can be overcome, observed by an increase in the apparent $K_I$ value, at higher concentrations of substrate.  This characteristic will have physiological consequences on the observed efficacy of drugs.  As an enzyme's reaction is inhibited by a competitive inhibitor, there is an increase in the local concentration of substrate.  Without a mechanism to clear the substrate, a competitive inhibitor will lose potency.  This is not the case for a noncompetitive inhibitor.

**Noncompetitive Inhibition**

A noncompetitive inhibitor binds equally well to both free enzyme and the enzyme-substrate complex.  These binding events occur exclusively at a site distinct from the precise active site occupied by substrate.  Figure 2 provides some illustrations of the more common noncompetitive binding events.
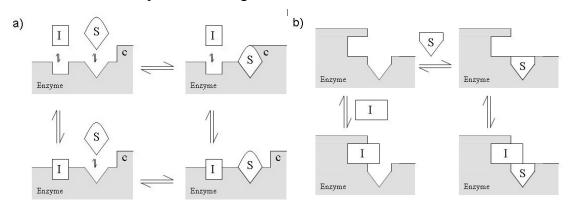


**Figure 2** – Examples of Noncompetitive Inhibition where Inhibitor (I) binding occurs at a site distinct from the Substrate (S) binding site and the Catalytic center (c) of the active site.  (a) In this model, the binding of S induces a conformational change to align the catalytic center near S for catalysis.  However, when I binds at a separate site, the conformational change does not occur and enzyme activity is inhibited.  (b) In this model, I can sterically hinder S binding and release.  However, unlike Figure 1-B, I and S can occupy the enzyme at the same time. This was adapted from Segal, Enzyme Kinetics.

In contrast to a competitive inhibitor, a noncompetitive inhibitor will lower the apparent Vmax value, yet there is no effect on the apparent $K_M$ value for its substrate.  Essentially, the $K_I$ of the inhibitor does not change as a function of the substrate concentration.

In some circumstances, a compound may have unequal affinity for both free enzyme and the enzyme-substrate complex.  This mixture of competitive and noncompetitive phenotypes is called mixed inhibition.

**Uncompetitive Inhibition**

An uncompetitive inhibitor binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor complex.  When encountered, the apparent Vmax value and the apparent $K_M$ value should both decrease.  Despite their rarity in drug discovery programs, uncompetitive inhibitors could have dramatic physiological consequences.  As the inhibitor decreases the enzyme activity, there is an increase in the local concentration of substrate.  Without a mechanism to clear the buildup of substrate, the potency of the uncompetitive inhibitor will increase.
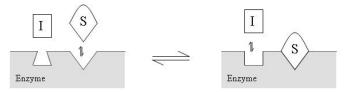
**Figure 3** – An example of Uncompetitive Inhibition where Inhibitor (I) only binds in the presence of Substrate (S).
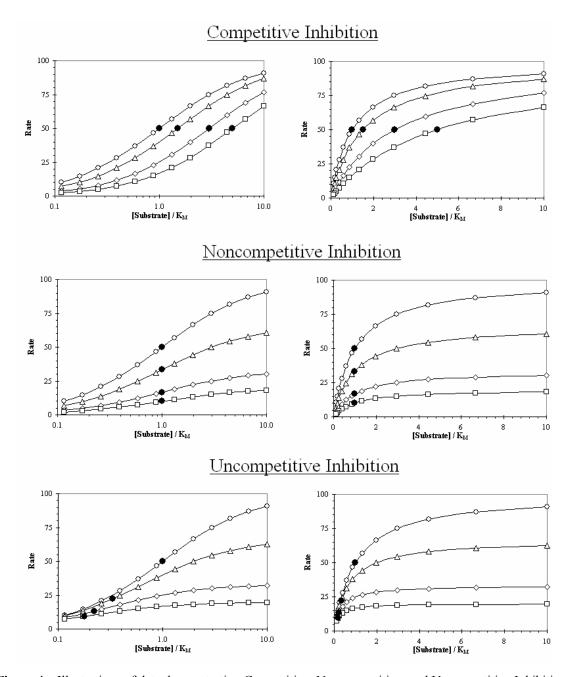


**Figure 4** – Illustrations of data demonstrating Competitive, Noncompetitive, and Uncompetitive Inhibition. The circles represent those rates obtained without the addition of inhibitor. The triangles contained $0.5 \times K_I$ of inhibitor, the diamonds contained $2.0 \times K_I$ of inhibitor, and the squares contained $4.0 \times K_I$ of inhibitor. The black circles depict the shifts in the apparent $K_M$ for each binding modality.

## Allosteric Inhibition

An allosteric inhibitor decreases activity by binding to an allosteric site, other than or in addition to the active site on the target. This interaction is characterized by a

conformational change in the target enzyme that is required for inhibition. These conformational changes can affect the formation of the usual enzyme-substrate active site complex, stabilization of the transition state, or reduce the ability to lower the activation energy of catalysis. Figure 1e and Figure 2a are classical examples of allosteric inhibition. As such, an allosteric inhibitor may display a competitive, noncompetitive, or uncompetitive phenotype with respect to substrate binding.

**Partial Inhibition**

Partial inhibition results from the formation of an enzyme-substrate-inhibitor complex that can generate product with less facility than the enzyme-substrate complex. This can be illustrated in Figure 2a. When "I" is a partial inhibitor bound in the enzyme-substrate-inhibitor complex, the catalytic center may retain some ability to align near the substrate and facilitate catalysis. As a consequence of these structural changes, partial inhibitors can also be allosteric inhibitors of enzyme activity. In direct contrast, full inhibition results in an enzyme-substrate-inhibitor complex where the catalytic center is not capable of aligning near the substrate for catalysis.

**Tight-Binding Inhibition**

In this type of inhibition, the population of free, soluble inhibitor is significantly depleted by the formation of the enzyme-inhibitor or enzyme-substrate-inhibitor complex. While tight-binding inhibitors can bind to the target enzyme in a competitive, noncompetitive, or uncompetitive manner with respect to substrate binding, they can display noncompetitive phenotypes. However, a tight-binding inhibitor typically binds with an apparent affinity ($K_I$) near the concentration of enzyme (active sites) present in the biochemical assay.

**Time-Dependent Inhibition**

Time-dependent inhibitors bind slowly to the enzyme on the time scale of enzymatic turnover, and thus display a change in initial velocity with time. This has the effect of slowing the observed onset of inhibition. Time-dependent inhibitors also impede the observed recovery of enzyme activity following inhibition, resulting in slow $k_{off}$ values. As illustrated in Figure 5, these inhibitors typically yield nonlinear initial velocities and nonlinear recoveries of enzyme activity.
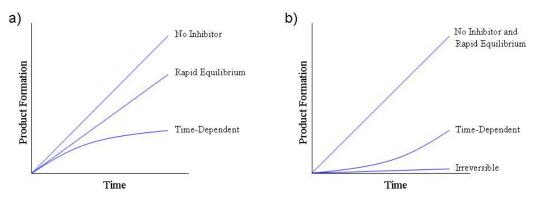
**Figure 5** – Illustrations of time-dependent inhibition.  (a) This graph depicts the decrease in the initial velocity (product formed vs time) observed for classical, rapid equilibrium inhibitor and a time-dependent inhibitor.  The latter yields a nonlinear progress curve consistent with a slow $k_{on}$ value. (b) This graph depicts the recovery of enzyme activity (product formed vs time) following dilution of the enzyme-inhibitor complex with substrate.  Dilutions of classical, rapid equilibrium inhibitor complexes recover full activity immediately after dilution.  Dilutions of time-dependent inhibitor complexes recover enzyme activity more slowly, indicative of a compound with a slow $k_{off}$ value.  Dilutions of irreversible inhibitor complexes maintain the enzyme-inhibitor complex after dilution.

Some time-dependent inhibitors covalently attach to the target enzyme.  For those inhibitors, the $k_{off}$ value is zero and the inhibition is said to be irreversible.  These are typically less attractive molecules, unless the formation of the covalent species is specific to the reaction mechanism of the enzyme.  Some inhibitors are for all practical purposes irreversible, with very low $k_{off}$ values, despite their inability to covalently attach to the enzyme.  This stands in direct contrast to rapid equilibrium, reversible inhibitors that bind to and release from the enzyme at rates that are rapid in comparison to the rate of enzyme turnover.

Interestingly, many successful therapeutic drugs are time-dependent inhibitors.  For these inhibitors with slow $k_{off}$ values, the rate of release of inhibitor from the enzyme-inhibitor complex (recovery of enzyme activity) proceeds independent of the substrate concentration and the physiological mechanism to remove inhibitor.  This makes time-dependent inhibition a very attractive and proven strategy for the discovery and development of drugs.

## D. PERFORMING MOA STUDIES

When performing classical steady-state mechanism of action studies, the scientist should carefully consider and incorporate the proper biochemical and statistical guidelines provided in this section.  These guidelines should assist in the initial characterization of the enzyme-inhibitor complex.  However, in some cases the classical steady-state experiment is not sufficient and additional characterizations are required.  Examples include compounds that display tight-binding inhibition, time-dependent inhibition, covalent modification, or nonspecific inhibition of the enzyme.  Therefore, we also provide guidelines to identify these additional types of inhibitors are plan the appropriate follow-up analysis.

## Classical Steady-State Experiments

These types of studies involve measurements of the $V_{max}$ and $K_M$ of a substrate at a range of inhibitor concentrations. The scientist should refer to Section IVG of the QB Manual for a description of how to perform measurements of the $V_{max}$ and $K_M$ for a substrate. As mentioned previously, changes in the apparent $V_{max}$ and $K_M$ give the scientist a view of the binding modality (competitive, noncompetitive, or uncompetitive) and the potency ($K_I$ and $K_I$'). Figure 6 illustrates the classical steady-state experiment used to determine the binding modality and potency.
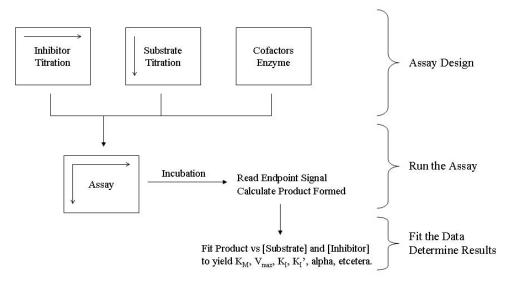


**Figure 6** – Classical Steady-State analysis of the mechanism of action. The inhibitor and substrates are serially diluted to achieve concentrations in the assay that span their respective binding constants ($K_I$ and $K_M$). The addition of enzyme and cofactors will initiate the enzymatic reaction. The order of addition typically depends on the assay in question and may be altered for time-dependent inhibitors (discussed later). The assay incubates for some period of time, the signal is read, the data is fit, and the results are analyzed.

The methodology proposed here to determine the binding potency and modality of an inhibitor is derived from a steady-state model of enzyme kinetics. The term steady-state refers to a constant concentration of the enzyme-substrate complex present during the reaction. As summarized by Copeland (Enzymes 2ed) and Segal (Enzyme Kinetics), there are several assumptions that simplify the mathematical treatment of the kinetics. When these assumptions fail, the steady-state MoA model proposed here is not valid.

1) The enzyme is acting catalytically and the concentration of substrate is much greater than the concentration of enzyme.
2) During the initial phase of the reaction (initial velocity), there is no buildup of any intermediate other than the enzyme-substrate complex.
3) There is very little product formed over the course of the reaction so that the depletion of substrate is minimal and the reverse reaction is insignificant.
4) The concentration of inhibitor is much greater than the concentration of enzyme so that the depletion of free inhibitor resulting from the formation of the enzyme-inhibitor complex is minimal.

The scientist should utilize the following guidelines in the design, execution, and analysis of a classical MoA experiment.

## *Guidelines for Assay Design*

- As described in Section IVD, it is essential to ensure that the enzyme, substrate, co-factors, and buffer conditions have been fully evaluated and characterized. Wherever possible, the scientist should strive to achieve in-vitro conditions that will best represent the physiological conditions in a robust, reproducible manner. The selection of these factors can have a large impact on the binding modality and potency observed.

- An enzyme titration should be performed to determine the concentration of active sites in the assay. Consult Copeland, Enzyme 2ed, pg313 or an experienced enzymologist for more information.

- There should be at least 5 concentrations of substrate tested, spanning a range of at least ½x$K_M$ to 5x$K_M$, for each concentration of inhibitor tested. As illustrated in Figure 7, the ability to distinguish a competitive inhibitor from a noncompetitive or uncompetitive inhibitor is increasingly enhanced at concentrations of substrate above its $K_M$ value. The ability to distinguish noncompetitive inhibition from uncompetitive inhibition is more challenging and can be improved with very accurate determinations of the apparent $K_M$. Therefore, the scientist should strive to judiciously increase the range and number of concentrations of substrate tested.
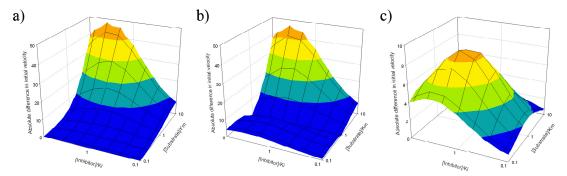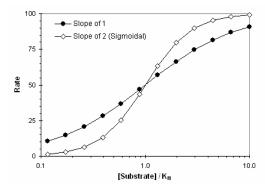


**Figure 7** – Residual plots demonstrating the difference in observed rate of enzyme activity (z-axis) at each concentration of substrate (y-axis) and inhibitor (x-axis) for 2 binding modalities. (a) Competitive Inhibition vs Noncompetitive inhibition. (b) Competitive inhibition vs Uncompetitive inhibition. (c) Noncompetitive vs Uncompetitive inhibition. Taken together, competitive inhibitors are best distinguished from noncompetitive and uncompetitive inhibitors at both high [substrate] and high [inhibitor]. Noncompetitive and uncompetitive inhibitors are best distinguished from each other at [substrate] and [inhibitor] near their binding constants ($K_M$ and $K_I$). Therefore, the range and density of concentrations tested are both important.

- The plot of the [substrate] vs initial velocity should not display sigmoidal kinetics, unless it is a mechanistic feature of substrate binding and catalysis for that enzyme. The impact of sigmoidal kinetics on the $K_M$ curve is illustrated in Figure 8. Sigmoidal kinetics may be a sign of an impure enzyme or the presence of

multiple isoforms of the enzyme (ex. multiple phosphorylation states of the same kinase). Refer to Copeland, Enzyme 2ed, pg382 or an enzymologist experienced with sigmoidal kinetics.
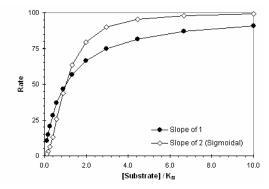


**Figure 8** – Comparison on enzyme data for a system with a proper slope of 1 and another displaying a sigmoidal relationship (ex. slope of 2) between the substrate concentration tested and the rate observed.

- The initial velocity should be measured. In order for the steady-state assumptions to hold, it is recommended that less than 10% of the substrate be converted to product. Section IVC describes this guideline in more detail. However, initial velocity conditions do not infer linearity and the user should refer to the guideline directly below.

- The formation of product should be linear with respect to time. This is best achieved by measuring the rate of product formation at the chosen concentrations of substrate using the assay conditions, detection system, and instruments that will be used for the final assay. Linearity should be assessed visually from plots of the raw data.

- There should be at least 8 concentrations of inhibitor tested at each concentration of substrate. The range of inhibitor concentrations tested should span the $K_I$ or $K_I$', depending on the binding modality. Reporting of binding constants outside of the range of concentrations tested should be avoided. It is also recommended to include inhibitor concentrations at or above ~10x$K_I$ to ensure maximum inhibition and the identification of any potential Partial Inhibitors. It should be noted that any observation of Partial inhibition could instead be a consequence of a compound's poor solubility.

- Where available, a control inhibitor should be evaluated under the exact conditions that will be used for the final assay.

- In addition to the experimental wells containing a matrix of substrate and inhibitor dilutions, the final assay should include both high and low controls. The high control should contain the substrate titration without inhibitor to reflect the maximum enzyme activity at each substrate concentration. The low control should contain the substrate titration without enzyme or substrate and without inhibitor. The low controls should reflect the signal expected for no enzyme activity at each substrate concentration. Depending on the composition of the

inhibitor stocks, DMSO might be needed in the control wells to assure consistency across all the experiments.

- The concentration of DMSO should be kept constant in MoA experiments for a particular target. DMSO can have a significant impact on enzyme activity and the concentration of DMSO in the wells containing compound should be identical to the concentration of DMSO in the control wells (described directly above). DMSO can also impact the solubility of a compound and its observed potency. Therefore, the concentration of DMSO should be consistent in replicate MoA experiments (or in comparison to $IC_{50}$ experiments).

- It is recommended to evaluate, in the standard assay conditions, dependence of [enzyme] on the $IC_{50}$ of the compounds to be tested. Shifts in the $IC_{50}$ as a function of the [enzyme] is an indication of tight-binding inhibition and/or solubility issues. When this is encountered, the scientist should consult with an enzymologist experienced with tight-binding inhibition.

- If detergents are required for enzyme activity or automation, the scientist should strive to maintain their concentrations well below the critical micelle concentration (CMC). The formation of micelles, at high concentrations of detergents, can interfere with the determinations of the binding modality and potency. An exception to this rule would include assays requiring detergents as part of the mechanistic evaluation. If the assay can only be run above the CMC, the scientist should consult with an enzymologist experienced with lipids, micelles, and surface dilution kinetics.

- The reaction should be measured under steady-state conditions. As described by Copeland in Enzymes 2ed, this includes the following … 1) there should not be any appreciable buildup of any enzyme intermediates other than the ES complex, 2) the [substrate] should be >> [enzyme], and 3) the initial phase of the reaction is measured so that the [product] ~ 0, the depletion of substrate is minimal, and the reverse reaction is insignificant.
- The concentration of a required cofactor should be >> [enzyme].


### *Statistical Validation of the Designed Assay*

The requirements for statistical validation of an MoA assay can be divided into two situations:  (1) high-throughput assays using automation that can test many compounds, and (2) low-throughput assays in which only one or a few compounds are tested. In the first case, a replicate-experiment study should be performed as described in Section III of the QB manual. Briefly, 20-30 compounds should be tested in two independent runs. Then the MSD or MSR and limits of agreement are determined for each of the key results, including $V_{max}$, $K_m$, $K_i$, $K_i$', and $\alpha$ or $\alpha_{inv}$. Specific acceptance criteria have not been determined. The reproducibility should be judged as suitable or not for each situation. For low-throughput assays, a replicate-experiment study is not required. At a minimum, key results from the MoA experiment, such as $V_{max}$, $K_m$, and $K_i$, should be compared to previous/preliminary experiments to ensure consistency. The data from the MoA experiment should be

examined graphically for outliers, goodness of fit of the model to the data, and consistency with the assumptions and guidelines for designing and running the assay (see Guidelines for Assay Design above and Guidelines for Running the Assay below).

### Guidelines for Running the Assay

- The assay should be run under the exact same conditions as developed using the guidelines above. In addition, the assay should be run within the timeframe where the reagents are known to be stable.

- When a control inhibitor is included, then the $K_I$ (and/or $K_I$') value should be compared with legacy data to ensure robust, quality results. It is also recommended to include additional inhibitors with alternative binding modalities, if available.

- The $K_M$ and $V_{max}$ values from the high controls and the signal from the low controls should be compared with the legacy values determined in identical conditions, as described above.

- A standard curve should be included for detection systems yielding signals that are nonlinear with respect to the amount of product formed. This nonlinearity is a common feature in fluorescent-based assays. The standard curve should be used to covert the signal produced to the amount of product formed. The resulting amount of product formed over the course of the assay time should be used in the data fitting methodologies. Please refer to the QB Manuel titled 'Immunoassay Methods'.

### Guidelines for Data Fitting and Interpretation

- The multivariate dataset (v, [I],[S]) should be fit using a non-linear regression analysis with the appropriate models described below. Linear transformations of the data should be avoided as they will distort the error of the experiment and were historically used only before the introduction of computer algorithms.

- The scientist should perform any necessary background corrections, before the multivariant fitting, so that a signal or rate of 0 represents that expected for conditions lacking enzyme activity. Depending on the assay design, this may include a single background correction applied to the entire experiment or several different corrections. The latter should be used when the background signal varies with the [substrate] tested. Here there should be a background correction for each [substrate] tested.

- The traditional model of general mixed inhibition is

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{K_i'}\right)} \qquad \text{(P1)}$$

where v is the speed of the reaction (slope of product formed vs. time), $V_{max}$ is the upper asymptote, [S] is the substrate concentration, and [I] is the inhibitor concentration. See the glossary for definitions of $K_m$, $K_i$, and $K_i$'. This model can also be written as

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{\alpha K_i}\right)} \quad \text{or} \quad \text{(P2)}$$

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{\alpha_{inv}[I]}{K_i}\right)} \quad \text{(P3)}$$

where $\alpha = 1/\alpha_{inv} = K_i'/K_i$. This model reduces to specific models for competitive, non-competitive, and un-competitive inhibition as described in this table:

| Inhibition | Description | $K_i$ | $K_i$' | $K_i'/K_i$ |
|---|---|---|---|---|
| Competitive | The inhibitor binds only to free enzyme. This binding most often occurs in the active site at the precise location where substrate or cofactor (being evaluated in the MoA study) also binds. | finite | Infinite | infinite |
| Mixed | These inhibitors display properties of both competitive and noncompetitive inhibition. | finite | Finite | > 1 |
| Noncompetitive | The inhibitor binds equally well to both free enzyme and the enzyme-substrate complex. Consequently, these binding events occur outside the active site. | finite | Finite | = 1 |
| Uncompetitive | The inhibitor binds only to the enzyme-substrate complex at a location outside the active site. | infinite | Finite | = 0 |

Another form of this model that has better statistical properties, in terms of parameter estimation and error determination, is:

$$v = \frac{[S]}{\theta_1 + \theta_2[I] + \theta_3[S] + \theta_4[I][S]} \quad \text{(P4)}$$

where

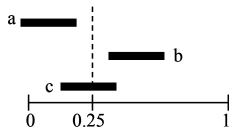$$V_{max} = \frac{1}{\theta_3}$$

$$K_M = \frac{\theta_1}{\theta_3}$$

$$K_I = \frac{\theta_1}{\theta_2}$$

$$K_I' = \frac{\theta_3}{\theta_4}$$

$$\alpha = \frac{\theta_2\theta_3}{\theta_1\theta_4} = \frac{1}{\alpha_{inv}} = \frac{K_I'}{K_I}$$
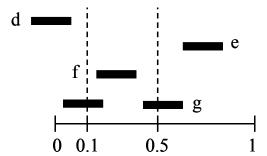
More details on these models can be found in a paper written by the primary authors of this chapter (to be submitted to JBS).

- The following data analysis steps are encoded into a web tool that is available at this link:  MoA Web Tool

    1. Fit a robust multiple linear regression of 1/v vs. 1/[S], [I]/[S], and [I].  This provides starting values of the $\theta$ parameters for the non-linear regression in the next step.

    2. Fit model P4 to the data (v, [I], [S]).

    3. Calculate the parameters of interest from the $\theta$ values..

    4. Calculate confidence limits for each key parameter value using Monte Carlo simulation.

    5. Make decisions of mechanism based on the value of $\alpha$ or $\alpha_{inv}$ and the associated confidence limits.

- Alpha or alpha inverse should be used to assign the binding modality.  If alpha is less than 1, the mechanism is:

    a.  Uncompetitive if the upper confidence limit of alpha is less than 0.25

    b.  Noncompetitive if the lower confidence limit of alpha is more than 0.25

    c.  Not competitive, otherwise



if alpha inverse is less than 1, then the mechanism is:

    d.  Competitive if the upper confidence limit of alpha is less than 0.1

    e.  Noncompetitive if the lower confidence limit of alpha is more than 0.5

    f.  Mixed, if both confidence limits are within [0.1, 0.5]

    g.  Not declarable, otherwise

The details of how these cutoffs were chosen are in the JBS paper.

- When the signal measured at $10xK_I$ (representing full enzyme inhibition by the compound) is >>0 (baseline corrected), the compound is displaying Partial (and/or Allosteric) Inhibition.  This difference might also be observed when the incorrect conditions were chosen for the low control to represent no enzyme activity, if there was not enough inhibitor (relative to the $K_I$ or $K_I$') to achieve maximum inhibition, and/or if the compound tested is poorly soluble.

- When the $K_I$ or $K_I$' resulting from the fit is within 10-fold of the concentration of active sites in the assay, the compound will start to display tight-binding inhibition.  Inaccuracies in the binding modality and potency will result.  In some cases where the inhibitor is not soluble, tight binding inhibition may exist at much higher $K_I$ or $K_I$' values.  As recommended previously, the dependency of the enzyme concentration on the inhibitor's potency is the best method to identify tight-binding inhibition. The scientist should consult with an expert in tight-binding inhibition to further characterize the inhibitor.

- Data suggesting that a compound is noncompetitive (and in some cases mixed) should be handled with caution.  Compounds that are time-dependent, irreversible, poorly soluble, nonspecific, and/or tight-binding will display a noncompetitive/mixed phenotype in this type of classical steady-state experiment.  As such, it is critical to evaluate these additional potential mechanisms of action, described herein.

- Additional recommendations for data analysis can be found in the next section.

## When the Steady-State Assumptions Fail

The steady-state MoA model proposed for here for data fitting requires several important assumptions hold true.  While a majority of these assumptions are covered in the previous sections, the invalidation of a few key assumptions should prompt the scientist to perform additional mechanistic characterizations.  These key assumptions, a mechanism to flag their breakdown in the steady-state MoA model, and a recommended plan of action are presented.

### Tight Binding Inhibition

The [inhibitor] in solution should be much greater than the [enzyme] in the assay.  This assumption fails most frequently in 2 circumstances.  First, some compounds bind to their target with such high affinity ($_{app}K_I$ values within 10 fold of the [enzyme]) that the population of free inhibitor molecules is significantly depleted by formation of the EI complex.  Second, some compounds are both very potent and poorly soluble.  The poor solubility of the inhibitor will increase the observed $_{app}K_I$ value (relative to the [enzyme]).  In both cases, the compounds are called tight binding inhibitors.
- How can tight binding inhibitors be flagged in the steady-state MoA model?

- o Regardless of their true binding modality, they display a noncompetitive phenotype.
- o They have observed $_{app}K_I$ values between ½ and 10-fold of the [enzyme] in the assay.
- o Poorly soluble compounds may also display tight binding inhibition. This is often masked by an inflated observed $_{app}K_I$ value.
- What is the recommended plan for an appropriate characterization?
  - o Calculate the dependence of the $IC_{50}$ values on the [enzyme]. Using a fixed concentration of substrate at $K_M$, the $IC_{50}$ of the inhibitor should be measured at ≥5 concentrations of enzyme. If the $IC_{50}$ changes significantly as a function of the [enzyme], the inhibitor is displaying tight binding properties and requires further characterization. If the $IC_{50}$ does not change significantly, the compound is not tight binding, this key assumption ([Inhibitor]>>[Enzyme]) is true, and the steady-state MoA model is valid. These 2 phenotypes are illustrated in Figure 9.
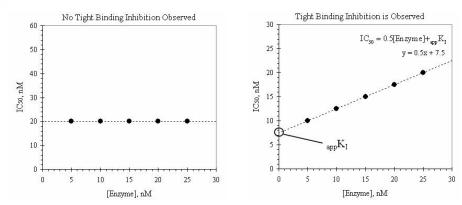


**Figure 9** – Plotting the $IC_{50}$ vs [Enzyme] will reveal whether a compound is tight binding. As depicted on the left, no change in the $IC_{50}$ suggests that the compound is not tight binding and the assumption ([I] >> [E]) holds true. As depicted on the right, a change in the $IC_{50}$ (with a slope of 0.5) suggests that the compound is tight binding and requires additional characterization.

  - o Calculate the dependence of the $IC_{50}$ values on the [substrate]. Using a fixed concentration of enzyme, the $IC_{50}$ of the inhibitor should be measured at >5 concentrations of substrate. The range of concentrations of substrate should span the $K_M$ (as recommended previously). As illustrated in Figure 10, the change in the $IC_{50}$ vs [substrate] is described by the equation listed below and yields the true binding potency ($K_I$ and $K_I$'). The ratio of $K_I$'/$K_I$ (termed alpha, α) reflects the binding modality. Inhibitors with alpha values statistically equal to 1.0 are noncompetitive, values statistically less than 1.0 are uncompetitive, and values statistically greater than 1.0 are competitive.
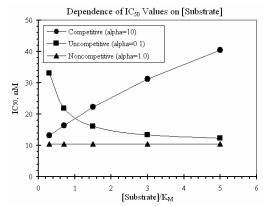
**Figure 10** – A plot of the $IC_{50}$ vs [substrate] will reveal the binding modality for a tight binding inhibitor. The quality of this assessment is predicated on the choice of a range of substrate concentrations that span the $K_M$. The graph illustrates that competitive inhibition is best identified at substrate concentrations above $K_M$. In contrast, uncompetitive inhibition is best identified at substrate concentrations below $K_M$. The true $K_I$ and/or $K_I$' values can be obtained from a fit using the model below.

Model to Determine Tight Binding MoA

$$IC_{50} = \frac{[S] + K_M}{\dfrac{K_M}{K_I} + \dfrac{[S]}{K_I'}} + \frac{[E]}{2}$$

- o These methodologies are described in more detail in Chapter 9 of Enzymes 2ed by Copeland. We also recommend consulting with a statistician and an enzymologist experienced with tight binding inhibition.

### Time-Dependent Inhibition

When the reaction is started with enzyme, there should be a linear relationship between the enzyme reaction time and the amount of the product formed from that reaction. This linearity should be preserved for all enzyme reactions lacking inhibitor or having rapid equilibrium binding events outside of the time window measured. However, the addition of inhibitor may result in a nonlinear progress curve (Figure 11) with an initial burst of enzyme activity ($v_i$) followed by a final, slower steady-state rate ($v_s$). Although the steady-state MoA model may still apply under some circumstances, additional characterizations are required.

- How can time dependent inhibitors be flagged in the steady-state MoA model?
  - o For kinetic enzyme assays, the progress curve showing product formation over time is nonlinear (Figure 11).
  - o For endpoint enzyme assays, time dependent inhibitors can display a noncompetitive phenotype regardless of their true binding modality. Otherwise, they can be identified by observing a shift in inhibitor potency with either a change in the enzyme reaction time and/or a change in the enzyme/inhibitor pre-incubation time.
- What is the recommended plan for an appropriate characterization?

- More appropriately characterize and model the nonlinear progress curves (product formed vs time) observed. Illustrations of these progress curves and the appropriate models to use are found below in Figure 11. The resulting fit of the data to the nonlinear model should produce the $v_i$, $v_s$, and $k_{obs}$ for all the [substrate] and [inhibitor] tested.



Model describing the linearity:

$$[P] = vt$$

Model describing the nonlinearity:

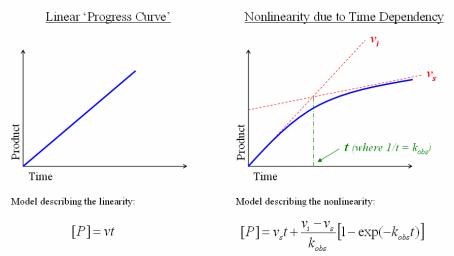$$[P] = v_s t + \frac{v_i - v_s}{k_{obs}}\left[1 - \exp(-k_{obs}t)\right]$$

**Figure 11** – Progress curves for linear, rapid equilibrium inhibition (left) and nonlinear, time dependent inhibition (right). Nonlinear progress curves resulting from time dependent inhibition can be fit to the model shown above. The resulting fit will yield the initial velocity ($v_i$), steady-state velocity ($v_s$), and the rate constant for the interconversion between $v_i$ and $v_s$ ($k_{obs}$), under the conditions tested. These values can be used to assess the true binding potency and modality.

  During this evaluation, $k_{obs}$ values reflecting timepoints (t) outside of the window tested should be avoided. For example, valid $k_{obs}$ values from a kinetic run starting at 2min and ending at 60min should range between 0.5min$^{-1}$ to 0.08min$^{-1}$. As a general rule, the total time of the reaction should be 5 times greater than 1/$k_{obs}$. As a result, the scientist may need to choose a smaller range of [substrate] spanning $K_M$ and [inhibitor] spanning $_{app}K_I$.
- In most cases, the initial ($v_i$) and steady-state ($v_s$) velocities can be fit separately to the steady-state MoA model (presented in the previous section) to yield the binding potency ($K_I$ and/or $K_I$' value) and modality for each phase of inhibition.
- A more traditional approach to determine the apparent potency of the inhibitor requires the scientist to plot the $k_{obs}$ values as a function of the [inhibitor] at a fixed [substrate]. This can yield 2 main types of plots illustrated in Figure 12 below. 1) If there is a linear relationship between the $k_{obs}$ and the [inhibitor] tested, the one-step model shown should be used to determine the $_{app}K_I$ (potency at the steady-state velocity, $v_s$). 2) If there is a hyperbolic relationship between the $k_{obs}$ and the [inhibitor] tested, the two-step model shown should be used to determine the $_{app}K_I$ (potency at the initial velocity, $v_i$) and the $_{app}K_I^*$ (potency at the steady-state velocity, $v_s$).

One Step Model:

$$k_{obs} = k_{off}\left(1 + \frac{[I]}{_{app}K_I}\right)$$

Two Step Model:

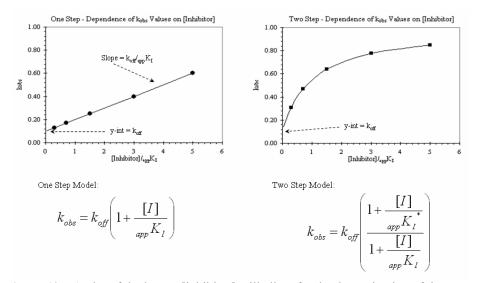$$k_{obs} = k_{off}\left(\frac{1 + \dfrac{[I]}{_{app}K_I{}^*}}{1 + \dfrac{[I]}{_{app}K_I}}\right)$$

**Figure 12** – A plot of the $k_{obs}$ vs [inhibitor] will allow for the determination of the $_{app}K_I$ value for a time dependent inhibitor. If the relationship between $k_{obs}$ and the [inhibitor] is linear, the one-step model shown above should be used. If the relationship is nonlinear, the two-step model should be used.

o   A more traditional approach to determine the binding modality of a time dependent inhibitor requires a determination of the $_{app}K_I$, from the previous $k_{obs}$ vs [inhibitor] plot, at each [substrate] spanning the $K_M$. The $_{app}K_I$ (and $_{app}K_I{}^*$) can then be graphed as a function of [substrate] and fit to the model shown below (Figure 13). The ratio of $K_I{}'/K_I$ (termed alpha, α) determined from the model below will reflect the binding modality. Inhibitors with alpha values statistically equal to 1.0 are noncompetitive, values statistically less than 1.0 are uncompetitive, and values statistically greater than 1.0 are competitive.
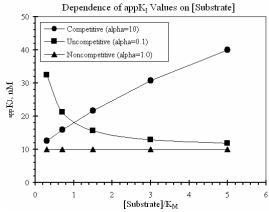


**Figure 13** – A plot of the $_{app}K_I$ (and $_{app}K_I{}^*$) vs [substrate] will allow for the determination of the true binding potency and modality. The modeled lines above are generated using the equation shown directly below where alpha = $K_I{}'/K_I$.

Model to Determine Time Dependent MoA

$$_{app}K_I = \frac{[S] + K_M}{\dfrac{K_M}{K_I} + \dfrac{[S]}{K_I'}}$$

Where possible, we recommend avoiding the iterative fitting into the one-step or two-step models and the model directly above.  The scientist should consult with a statistician and enzymologist to perform a global fit of the data to an equation where the one-step or two-step models are solved for the $_{app}K_I$ shown directly above.

o   A parallel approach to determine the binding modality requires the scientist evaluate the $k_{obs}$ values as a function of the [substrate] at a fixed [inhibitor].  The $k_{obs}$ of a competitive inhibitor will decrease with increasing [substrate] relative to $K_M$.  The $k_{obs}$ of an uncompetitive inhibitor will increase with increasing [substrate] relative to $K_M$.  The $k_{obs}$ of a noncompetitive inhibitor will not change with increasing [substrate] relative to $K_M$).  These trends are illustrated in Figure 14.
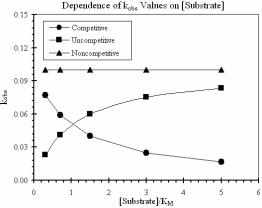


**Figure 14** – A plot of the $k_{obs}$ vs [substrate] will reveal the binding modality for a time dependent inhibitor.  It is important to choose [substrate] well above and below the $K_M$ to improve the ability to best distinguish the true binding modality.

o   These methodologies are described in more detail in Chapter 10 of Enzymes 2ed by Copeland.  Also be aware that a compound can display both time dependent and tight binding properties.  This would require a combination of experiments described above that may require the assistance of a statistician or an experienced enzymologist.

*Covalent Modification*

During the initial phase of the reaction (initial velocity), there is no buildup of any intermediate other than the enzyme-substrate complex.  This assumption most often fails when a compound is an irreversible inhibitor of the enzyme.  This type of inhibition can be the result of an immeasurably slow $k_{off}$ value and/or covalent modification of the enzyme.

•   How can irreversible inhibitors be flagged in the steady-state MoA model?

- o Regardless of their true binding modality, they display a noncompetitive phenotype.
- o Irreversible inhibitors are time dependent with $v_s$ values that approach zero. In contrast, reversible time dependent inhibitors have finite, non-zero $v_s$ values. The quality of this observation can be limited by the timepoints measured and the [inhibitor] evaluated.
- o The observed $k_{off}$ value is zero. This can be observed in a plot of the kobs as a function of the [inhibitor], shown in Figure 12. Irreversible inhibitors will yield a y-int ($k_{off}$) of zero.
- What is the recommended plan for an appropriate characterization?
  - o In addition to the characterizations described in the sections above, the scientist can measure the release of inhibitor from the enzyme-inhibitor complex. This is often performed by pre-incubating the enzyme with inhibitor at 10x$K_I$ to achieve 100% inhibition (all enzyme is in the EI complex reflecting $v_s$), then diluting the assay 30 fold with substrate, and continuously (kinetically) measuring product formation. As illustrated in Figure 15, reversible inhibitors will regain enzyme activity while irreversible inhibitors remain inactive. This experiment can be properly interpreted when 3 controls are included containing 1) no inhibitor throughout to reflect full enzyme activity at the amount of DMSO tested, 2) 10x$K_I$ throughout to achieve 100% inhibition, and 3) 0.3x$K_I$ throughout to reflect the expected amount of inhibition remaining after substrate dilution. Assuming the 10x$K_I$ control is inactive, the final rate ($v_s$) for the experiment can be divided by the final rate of the 0.3x$K_I$ control to yield the fraction of recovered activity.
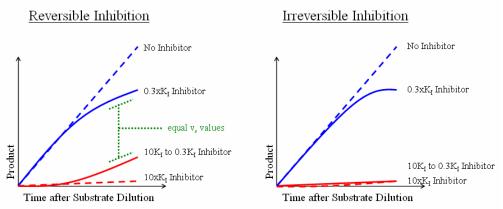


**Figure 15** – The recovery of enzyme activity following dilution of the EI complex can be an indication of the reversibility of the inhibitor. Irreversible inhibitors (right) will not recover any enzyme activity following dilution of the EI complex with substrate. In contrast, a reversible inhibitor (left) will recover enzyme activity equivalent to the 0.3x$K_I$ control.

  - o It is important to remember the there is no clear distinction between reversible and irreversible time dependent inhibition. The quality of the determination can often reflect the range and density of timepoints measured, [inhibitor] chosen, and other limitations specific to the assay.

Therefore, it would be wise for the scientist to consult an analytical chemist to perform a MS-based strategy to confirm irreversible inhibition resulting from covalent modification of the enzyme.

### Nonspecific Inhibition

Some compounds may form large colloid-like aggregates that inhibit activity by sequestering the enzyme.  These types of compounds can display enzyme dependency, time-dependent inhibition, poor selectivity against unrelated enzymes, and binding modalities that are not competitive.  This can be especially problematic when an enzyme is screened against a large diversity of compounds in a screening campaign.  Although these compounds do not formally violate the steady-state assumptions, they can generate misleading results which produce inaccurate characterizations of the inhibitor-enzyme complex.  The scientist is encouraged to read the Shoichet Review published in Drug Discovery Today (2006).  The chart below was taken from that reference and provides an introduction to the considerations that should be made for evaluating these types of inhibitors.

**BOX 1**

**Evaluating candidate hits for aggregation-based inhibition:**

1. Is inhibition significantly attenuated by small amounts of nonionic detergent?
   (i) If so, the compound is very likely acting through aggregation.
   (ii) In cases where you cannot use detergent, e.g. cell-based assays, it may be possible to use high (1 mg/ml) concentrations of serum albumin instead.

2. Is inhibition significantly attenuated by increasing enzyme concentration?
   (i) If so, the compound is very likely an aggregator. Except when the enzyme concentration to $K_i$ ratio is high, increasing enzyme concentration should not affect percent inhibition.

3. Is inhibition competitive? If so, the compound is unlikely to be an aggregator.
4. Does the inhibitor retain activity after spinning for several minutes in a microfuge? If not, particle formation is likely (see point 5).
5. Can you directly observe particles in the 50 to 1000 nm size range? We have typically used dynamic light scattering for this. Formation of particles does not guarantee promiscuous inhibition, but it is a worrying sign.
6. Is the dose–response curve unusually steep? There are classical reasons for curves, but they too are a worrying sign.

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# SECTION XIII

# GLOSSARY OF QUANTITATIVE BIOLOGY TERMS

# SECTION XIII:
# GLOSSARY OF QUANTITATIVE BIOLOGY TERMS

**Absolute EC50:** The molar concentration of a substance that increases the measured activity in an agonist assay to 50% of the range of activity expressed relative to maximum and minimum controls.

**Absolute IC50:** The molar concentration of an inhibitor required to block a given response by 50%, half way between the maximum and minimum controls if the response is expressed relative to the maximum and minimum controls.

**Accuracy:** A measure of the closeness of agreement of a measured test result obtained by the analytical method to its theoretical true (or accepted reference) value. This is relevant only for calibration-curve based applications where a purified or relative reference standard material is available to quantify the analyte levels in test samples.

**Accuracy Profile:** A plot of the mean percent recovery (or percent relative error) and its confidence interval versus the concentration of the spiked standards (validation or QC samples). It is used to judge the quality of a calibration-curve based assay in terms of total error (bias plus variance).

**Assays/Methods:**

   **Assay-Biochemical:** Biological measurements performed with purified or crude biochemical reagents.
   **Assay-Cell-based:** Biological measurements performed in which at least one of the reagents consists of a population of live cells.
   **Assay Design:** see Multivariate (Factorial Experiments), aka, Experimental Design.
   **Assay-In vitro:** From the Latin meaning 'in glass'. Any assay (biochemical or cell-based) conducted in a synthetic container (*e.g.* microtiter plate, microfluidic cell).
   **Assay-In vivo:** From the Latin meaning 'in life'. Typically used for assays conducted in living animals (*e.g.* mice, rats, etc.) with the exception of microorganisms (*e.g.* yeast, bacteria or *C. elegans*).
   **Assay Platform:** Technology used to measure response or output. (e.g. Fluorescence polarization or Radiometric counting).
   **Assay-Phenotypic:** An assay where the measured signal corresponds to a complex response such as cell survival, proliferation, localization of a protein, nuclear translocation etc. The molecular target is not assumed.
   **Assay-Primary:** The first assay performed in a testing scheme to identify biologically active chemical entities in a screening mode.
   **Assay-Secondary:** Assays that follow the primary assays to confirm the biological activity of chemical entities identified in the primary assays. This can also include selectivity and specificity assays.
   **Assay-Selectivity/Specificity:** Assays employed to elucidate the specificity of biologically active chemical entities towards a set of closely related disease targets.
   **Assay Validation**: see Validation
   **Assay-Separation :** Prior to detection a physical separation of at least one component from the assay is performed. (Standard ELISA, filtration, HPLC etc.)
   **Assay- non-separation:** Any assay where a physical separation is not required prior to detection.

**Assay-Target based**: An assay where the measured response can be linked to a known set of biological reagents such as a purified enzyme, domain or a reporter gene.

**Biological Target:**  A macromolecule or a set of macromolecules in a biochemical pathway that is responsible for the disease pathology.

**Bottom:**  The lower asymptote of a logarithmically derived curve.  The Bottom value can be determined with real values or predicted using the logarithm applied to the result data set.

**Calibration Curve:**  Also called "standard curve", a calibration curve is a regression of the assay response on the known concentrations in "standard" samples. It is a model that fits data from standards and is used for calculating (calibrating) values of unknown test samples.  For example, measurement of protein/biomarker expression levels of various compounds from in-vitro and in-vivo samples.

**Central Composite Designs:**  A type of multi-factor experiment that is used to optimize the most important factors in an assay (usually 3 to 5 factors).

**Classification & Regression Tree Models:**  A set of statistical methods in which observations are classified into groups based on a set of predictor variables and their relationship with a response variable.  These models can be used for multivariate correlation analysis.

**Cluster Analysis:**  A set of statistical methods in which objects (e.g., compounds) are divided into groups such that objects within a group are similar across a set of 2 or more variables.

**Concordance Correlation Coefficient:**  A measure of agreement between two variables, i.e., how closely the paired values match.  That is, it measures the degree of closeness of the data to the agreement line (Y=X line).  Since the Pearson's correlation measures the degree of departure of the data from the best straight line, which can be considerably different from the agreement line, the concordance correlation coefficient is more stringent than the Pearson's correlation.

**Control Compound:**  A compound that is routinely run in the same manner as the test compounds in every run of the assay.  This term does not refer to the plate controls used to define the maximum and minimum responses, and they may or may not be a "literature standard" or "reference" compound.

**CRC**:  Concentration-response curve mode.  The mode to describe an assay performed with multiple concentrations of a given test substance, which might then render a logarithmically-derived graph curve.

**Dynamic Range:**  It is the interval between the upper and lower concentration of the analyte in the sample for which the assay has been demonstrated to have acceptable level of accuracy, precision, linearity, etc.

**EC50:** The effective concentration of an agonist, which *produces* 50% of the maximum possible response for that agonist.

**Emin**: The maximum activity of an antagonist test substance relative to a reference agonist. This is obtained by first generating a fitted top from a %Inhibition curve and then converting that to the corresponding %Stimulation of the reference agonist curve. The E-min value for antagonist mode should equal the relative efficacy for agonist mode for competitive inhibitors.

**Factor:**  An assay variable that can be changed by the user.  Examples include the amount of a reagent, incubation time, buffer type, etc.

**False Positive**: A hit where the signal modulation is not related to the targeted activity. The sources of false positives include, random or systematic errors in liquid handling, spectrophotometric or fluorescence interference of the assay signal by chemical compounds, reagent instability etc. It is important to note that false positives can be reproducible when they are not related to random errors (as in the case of compound interference).

**Fold Activity**:  The ratio of biological activity in the presence of an exogenous substance to that in its absence.  It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells.  This result type is used exclusively with single point assays.  If the value is greater than 1, the test compound is likely an agonist.  If the calculated value is less than 1, the test compound could be an inverse agonist.

**Fold Activity Max**:  The maximum observed Fold Activity among the concentrations included in a concentration response curve.  It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells.  If the value is greater than 1, the test compound is likely an agonist.  If the calculated value is less than 1, the test compound could be an inverse agonist.

**Four Parameter Logistic Model:**  A non-linear regression model commonly used for fitting dose-response and concentration-response data.  The four parameters are Minimum (response at zero dose), Maximum (response at infinite dose), Relative EC50 (or IC50, ED50, etc.) and Slope.  The 4PL model can be written in several mathematically equivalent versions.  Two popular versions are given below.

$$Y = bottom + \frac{top - bottom}{1 + \left(\dfrac{x}{EC50}\right)^{slope}}$$

$$Y = bottom + \frac{top - bottom}{1 + 10^{(\log(x) - \log(EC50))*slope}}$$

**Fractional Factorial Experiments:**  A type of multi-factor experiment in which only a subset of factor level combinations is tested.  These experiments are very efficient for screening a large number of factors prior to optimizing the most important factors.

**Generalized Additive Models:**  Statistical models in which more general (e.g., nonlinear) relationships between variables can be examined.  These models can be used for multivariate correlation analysis.

**High Throughput Screening (HTS):**  Greater than 100,000 compounds screened per screen.

**Homogeneous Assay:** All assay components exist in solution phase at the time of detection (e.g. none of the components are in beads or cells). Technically no component scatters light.

**Heterogeneous Assay:** One or more assay components are present in solid phase at time detection. (e.g.: SPA, cells or IMAP).
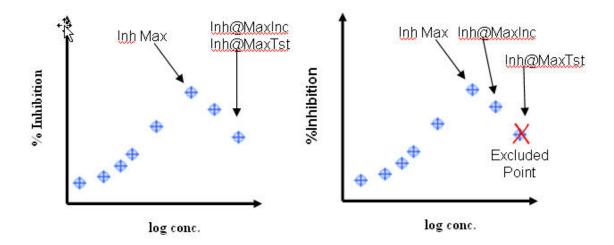
**Hill Coefficient**:  Derived slope a three or four parameter logistic curve fit.  Should not be fixed to any given value without consultation with a statistician.  It should not be a negative value except for inverse agonist assays.

**Inhibition**: Reduction of a predefined stimulus.  Unit of Measure is always % when normalized to the dynamic range of the assay.

**Inhibition at Max Included Concentration**:  Inhibition observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (see Illustration below)

**Inhibition at Max Tested Concentration**: Inhibition observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (see Illustration below)

**Inhibition Max:** Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm (see Illustration below)

**Inverse Agonist:**  When an inverse agonist binds to a receptor, it stabilizes the inactive form of the receptor, shifts the equilibrium toward that state and produces a response opposite to that produced by an agonist in the biological system under investigation. These substances possess negative intrinsic activity.

**Least Squares (Pearson's) Correlation Coefficient**:  A measure of linear correlation between two variables.

**LsA (Limits of Agreement):**  These are statistical limits that define the region that contains 95% of all potency ratios.

**Mean Ratio (MR):**  The average ratio of potencies between the two runs.

**Multivariate (Factorial) Experiments (Experimental Design):**  A system of experimentation for optimizing assays in which multiple factors are varied simultaneously in such a way that the effect of each factor can still is determined.  In addition, one can also measure interactions between factors and use this information to more efficiently optimize an assay.

**Multiple Linear Regression:**  A statistical method where the response variable is a linear function of several predictor variables.  This can be used for multivariate correlation analysis.

**Multivariate Correlation Analysis:**  A statistical analysis method where correlative relationships between 3 or more variables are examined.
Nonlinear Regression:  Statistical methodology for fitting models that is nonlinear in their parameters, for example, the four-parameter logistic model.

**One Factor at a Time Experiments:**  A series of experiments in which one factor is changed at a time.  Once the "best" condition for one factor is found, it is fixed at that setting for subsequent experiments.  This approach to assay optimization will not find the optimum conditions if at least one factor interacts with another, i.e., the best level of one factor depends on the levels of another factor.

**Overall MSD (Minimum Significant Difference):**  The minimum difference in efficacies of two compounds evaluated in different runs that is statistically significant, i.e. that should be considered a real change in efficacy.  The Overall MSD is defined for a single run of each compound.

**Overall MSR (Minimum Significant Ratio):**  The minimum ratio in potencies of two compounds evaluated in different runs that is statistically significant, i.e. that should be considered real change in potency.  The Overall MSR is defined for a single run of each compound.

**Optimization:**  The process of developing an assay (prior to validation) wherein the variables affecting the assay are elucidated (e.g., Antibody concentration, incubation time, wash cycles, etc.).  This process is ideally carried out using a multi-variate factorial approach where the inter-dependence between multiple variables/parameters can be taken into account.

**Orphan Receptor:**  A biological target that has a primary sequence suggesting it is a member of one of the super families of biological targets; however, no ligand for this

"receptor" has been identified. Generally, it is the aim of the research effort to identify ligands for this "orphan" so that a protocol for a validated assay can be created.

**Percent Recovery:**  The calibrated value of a standard or validation sample divided by its expected value (known concentration), expressed as a percentage.

**Plate Format:** Microtiter plate well density (e.g., 96-, 384- or 1536-well) and plate composition (e.g., clear bottom black or clear bottom polystyrene, etc.)

**Potentiation**:  Many assays involve the addition of one or more concentrations of a test substance in the presence of a fixed concentration of the known active substance called the Reference Agonist.  In this mode, if an increased stimulus is observed the test compound is deemed a potentiator.  Potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone.

**Precision:** A quantitative measure (usually expressed as standard deviation,  coefficient of variation, MSR) of the random variation between a series of measurements from multiple sampling of the same homogenous sample under the prescribed conditions.

**Precision Profile:**  A plot of the variability of calibrated values (expressed as a CV) versus concentration of standard.  It is used to judge the quality of a calibration curve in terms of the variability in the calibrated values.  It also determines the working range of a calibration curve.

**Production MSD (Minimum Significant Difference):**  The minimum difference in efficacies of two compounds evaluated in different runs that is statistically significant, taking into account the number of runs routinely applied to all compounds in the assay.  For example, if all compounds are routinely tested twice on separate days then the average of both runs will have greater precision than each individual run, and the Production MSD reflects this increased precision.

**Production MSR (Minimum Significant Ratio):**  The minimum ratio in potencies of two compounds evaluated in different runs that is statistically significant, taking into account the number of runs routinely applied to all compounds in the assay.  For example, if all compounds are routinely tested twice on separate days then the average of both runs will have greater precision than each individual run, and the Production MSR reflects this increased precision.

**Quantitative Biology:**  A set of skills that is essential for the design, optimization and validation of reproducible and robust assays/methods to establish the pharmacological profiles of biologically active chemical entities.  The practice of quantitative biology requires the understanding of how cellular, biochemical and pharmacological principles can be integrated with analytical and automation technologies, employing appropriate statistical data analysis and information technology tools.

**Relative AUC**:  Defined as the ratio of the area under the fitted concentration-response curve for the test compound to the area under the fitted concentration-response curve for the reference compound.

**Relative EC50**:  Relative EC50; the molar concentration of a substance that stimulates 50% of the curve (Top – Bottom) for that particular substance.  It can also be described as

the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.

**Relative EC50 Inv**: The Relative EC50 of an inverse agonist.

**Relative Efficacy:** The maximum activity of a test substance relative to a standard positive control agonist.  The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of an Agonist control.  The agonist control should have a four parameter curve fit with defined lower and upper asymptotes but can have the Bottom fixed to zero in certain cases.  The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

**Relative Efficacy Inv**:  The maximum activity of a test substance relative to a standard positive control inverse agonist.  The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of the Inverse Agonist control.   The inverse agonist control should have a four parameter curve fit with defined lower and upper asymptotes but can have the Bottom fixed to zero in certain cases.  The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

**Relative IC50**:  Relative IC50; the molar concentration of a substance that inhibits 50% of the curve (Top – Bottom) for that particular substance.  It can also be described as the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.

**Relative Potentiator Efficacy**: The fitted top of the potentiation curve minus the normalized response to the specific concentration of Reference Agonist alone divided by 100 minus the normalized response to the specific concentration of Reference Agonist alone.

**Response Surface Analysis:**  A statistical analysis method that is used for central composite designs.  A quadratic polynomial model is fit to the data in order to find the optimum conditions for an assay.

**Repeatability:**  Repeatability is the *precision* of repeated measurements within the same analytical run under the same operating conditions over a short interval of time. It is also termed intra-assay or intra-batch precision.

**Reproducibility (Run to Run):**  A general term to describe the *precision* of results generated from multiple runs of a compound (or any homogenous test sample) in an assay.  An assay may lack reproducibility because of either high within-run or across-run variability, or because of systematic trend (drift) over time in the response. An assay that is reproducible across runs is one that has variation within acceptable limits and has no material systematic trends.

**Reproducibility (Lab to Lab):**  Reproducibility across labs expresses the precision between laboratories.  It is useful for assessing the "transferability" of an assay and/or the validity of comparing results from samples that are run in two or more laboratories.

**Robustness/Ruggedness of the Assay:**  Robustness is a measure of the capacity of the assay to remain unaffected by small, but deliberate changes in method parameters and provides an indication of its reliability during normal run conditions.

**Schild Kb**: A measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC50 concentrations are used) in the absence and presence of one or more concentrations of the antagonist. See pp. 335-339, Pharmacologic Analysis of Drug-Receptor Intercation, 3rd Ed. by Terry Kenakin.

**Signal to Noise Ratio:**  We define this as the signal (max minus min) divided by an estimate of variability (or noise) such as the standard deviation of the max controls or the standard deviation of max minus min.  There is some disagreement over the meaning of this term.

**Signal Window:**  A measure of separation between max. and min. controls in an assay that accounts for the amount of variability in the assay.  The formula is:

$$SW = \frac{mean_{max} - mean_{min} - 3(SD_{max} + SD_{min})}{SD_{max}}$$

**Simple Linear Regression:**  A statistical method for fitting a straight line to paired (X, Y) data.

**Spearman's Correlation Coefficient:**  A nonparametric measure of correlation between two variables.  It is applied to ranked values of the data and is therefore robust to outliers in the data.

**Specificity:**  The ability of the assay to determine unequivocally the analyte in the presence of components that may be expected to be present in the sample.

**Spike:**  Addition of a known quantity of a specific reference material or positive control to a sample matrix for recovery studies.
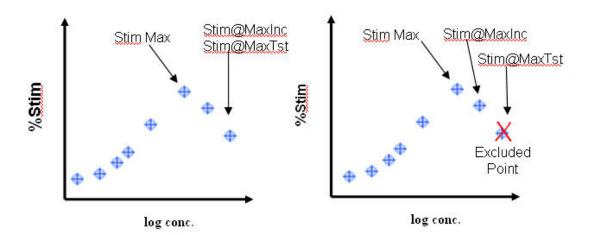
**Stephenson's Kp**: A measure of affinity for a partial agonist that is calculated through the comparison of equi-active concentrations of a full agonist in the absence and presence of a single concentration of the partial agonist.  See pp. 284-286, Pharmacologic Analysis of Drug-Receptor Intercation, 3rd Ed. by Terry Kenakin.

**Stimulation**: Increase of a measured output.  Unit of Measure is always % when normalized to the dynamic range of the assay (Min and Max control wells). Note that this calculation can generate percents much higher than 100.

**Stimulation at Max Included**:  Stimulation observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

**Stimulation at Max Tested**: Stimulation observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

**Stimulation Max:** Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm   (See illustration below)



**Target Platform:**  A set of biochemically and biologically related targets implicated in disease pathologies.  Examples include G-protein coupled receptors (GPCRs), nuclear hormone receptors (NHRs), Kinases, Proteases, Transporters, Ion channels and Chemokines.

**Testing Flow Scheme:**  Stages of testing NCEs as it progresses from active to hit to lead to a clinical candidate.  Tests include in-vitro assays, animal model tests, ADME assays biopharmaceutical and toxicological tests.

**Test-retest experiment:**  An experiment in which a set of (usually) 20-30 compounds is tested in two independent runs of an assay.  Its purpose is to estimate the MSR (for dose-response assays) or the MSD (for single-point assays or an efficacy measure in dose-response assays).  This experiment will provide a reliable estimate of only the within-run MSR.

**Top**: The upper asymptote of a logarithmically derived curve.  The Top value can be determined with real values or predicted using the logarithm applied to the result data set. The Unit of Measure is always %.

**Ultra High Throughput Screening (uHTS):** Greater than 500,000 compounds screened per screen.

**Validation:**  Validation includes all the laboratory investigations that demonstrate that the performance characteristics of an assay are suitable and reliable for its intended analytical use.  It describes in mathematical and quantifiable terms the performance characteristics of an assay.

**Validation/QC Samples:**  Samples of standard material that are prepared independently of the standards used for a calibration curve.  They are not used to fit the calibration curve, but they are calibrated against it to determine percent recovery.

**Within-Run MSD (Minimum Significant Difference):**  The minimum difference in efficacies of two compounds evaluated in the same run that is statistically significant, i.e. that should be considered a real change in efficacy.

**Within-Run MSR (Minimum Significant Ratio):**  The minimum ratio in potencies of two compounds evaluated in the same run that is statistically significant, i.e. that should be considered real change in potency.

**Z'-Factor:**  Another measure of separation between maximum and minimum controls in an assay that accounts for the amount of variability in the assay.  The formula is:

$$Z' = \frac{\text{Mean}_{max} - \text{Mean}_{min} - 3(\text{SD}_{max} - \text{SD}_{min})}{\text{Mean}_{max} - \text{Mean}_{min}}$$