Introduction to Assay Development

A poorly designed assay can derail a drug discovery program before it gets off the ground. While traditional bench-top assays are suitable for basic research and target discovery, high throughput screening (HTS) assays need to meet stringent requirements. Careful consideration should go into the design, optimization, and validation of any novel assay developed to screen compounds to reliably identify hits. Assays should (1) be **relevant** to the disease biology; (2) produce **reproducible** results within the assay (using replicates) and between days and operators; (3) perform in a **robust** manner, which refers to the capacity of an assay to be unaffected by slight, but deliberate variations in parameters such as pH, incubation time, and different lots of reagents; and (4) have sufficient **sensitivity** to distinguish relatively weak hits from background with enough **specificity** to only detect compounds with the desired activity.

This guide provides a high level overview of some of the most important factors to consider when designing an assay, with an emphasis on small molecule, high throughput screening programs.

Before you begin

Before embarking on a potentially long and difficult assay development process, consider the questions below.

- Has your target (molecule, pathway, or phenotype) been prosecuted previously?
  - Identifying a readily available assay can save a large amount of time, money and effort.
- Will a target-based or phenotypic approach better suit your program?
  - This depends on what is known about the target or pathway of interest and the goals of the drug discovery program.
- Can the assay be developed with the expertise currently on hand?
  - Academic centers with screening core facilities and contract research organizations (CROs) can work with investigators to design and develop the appropriate assay.

HTS Requirements

Compound libraries can contain hundreds of thousands, or even millions of compounds. With miniaturization, robotic platforms, and automated liquid handling, HTS can test more than 10,000 compounds per day. However, not all screens are amenable to HTS. In some cases, it may be appropriate to use a less high throughput assay to preserve biological relevance of the system. Below is a checklist to determine whether your assay can be scaled up to an HTS format.

- The assay can be performed in 10 or less automated steps and ideally without wash or reagent removal operations. If wash/removal is required it should be kept to a minimum, if possible.

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- The total assay volume is less than 200µl for 96-well, 25µl for 384-well, or 10µl for 1536-well plate formats.
- All reagents are stable at room temperature for at least the time required for the assay.
- Assay reactions are stable for long enough to measure the final readout for the entire plate or series of plates to minimize intra- and inter-plate variation.
- Assay reagents are stable in storage for long periods of time and available in large batches of consistent quality.

Fundamentals for Successful Assays

- **Vehicles**: Different vehicles, like dimethyl sulfoxide (DMSO), are often used in chemical libraries to dissolve compounds with poor solubility in water. The vehicles themselves can affect assay performance, especially in cell-based assays, and should be titrated using different vehicle concentrations to determine the sensitivity of the assay to a particular vehicle. Generally less than or equal to 0.1% DMSO is acceptable. The vehicle concentration needs to be the same in all samples and controls.
- **Controls**: Within-plate and assay-wide controls are used to evaluate quality, variability, and detect sources of error throughout the assay system. The most appropriate negative and positive controls will depend on your target, desired activity of compounds, and other factors specific to your assay. The best positive control is often a compound with the activity that you are targeting.
- **Statistical Analysis Plan**: As with all experiments, the statistical analysis plan should be carefully designed before validating the assay and performing the screen.
- **Dynamic Range**: This is the range of activity of compounds that can be accurately measured by the assay. It is defined as the range between the means of the negative (or background) and positive controls, and will be affected by nearly every component of the assay. If the dynamic range is too narrow, potential active compounds may not be detected, thus reducing the chance of finding novel, chemical modulators for your target.
- **Final Format**: If you intend to scale up an assay, it is imperative to test assay performance under the final conditions that will be used in the large-scale screen. Assay reagents and cells can react differently in miniaturized plate formats and liquid handling systems. For longer, multi-plate runs, the effect of timing and order of reagent addition should be determined.

Assay Design Considerations

Here we list some of the most important factors to consider when designing your screen. These points highlight some of the key considerations for the most common types of assays. Comprehensive guidance for a wide variety of assays and targets can

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be found in the Assay Guidance Manual [1].

Biochemical/cell-free assays

Consider how your drug target performs under physiological conditions. Biological targets often act with native binding partners or are part of multimeric complexes, and normally function in specialized cellular microenvironments (at a specific pH, salt concentration, etc). Replicating these conditions *in vitro* can be challenging, but will yield more biologically relevant hits. In some cases, specialized and alternate forms of the target, such as truncated forms of large proteins, may need to be used. However, the more a target is altered from its natural form, the less relevant identified hits may be to the *in vivo* target.

- Only use highly pure, high-quality preparations of peptides, proteins, cofactors, and enzyme substrates that produce consistent activity. If a single lot is not available, make sure you test each new lot for activity.
- The concentrations of targets, cofactors, and substrates need to be carefully titrated. Balancing the concentrations of these factors and the test compounds with the kinetic properties of the target is critical to identifying legitimate hits. For enzymatic targets, different proportions of enzyme versus substrate concentrations can result in the identification of modulators with different modes of action.

Cell-based assays

Select the type of cells for your assay based on disease relevance, availability and ease of handling.

- **Immortalized cell** lines are relatively inexpensive, widely available, and if properly maintained, highly reproducible between lots. However, they may not accurately reflect the *in vivo* behavior of their progenitors due to changes involved during immortalization and passaging.
- **Primary cells** are isolated directly from animal or human tissue and have similar molecular, physiological, and morphological characteristics as cells *in vivo*. Primary cells are more difficult to obtain in large amounts on a consistent basis, may suffer from donor-to-donor variability, and can be more difficult to maintain. This is offset with the advantage of a more physiologically relevant system and avoiding overexpression of your target.
- **Induced pluripotent stem cells (iPSCs)** are cells isolated from human donors that have been reprogrammed to a pluripotent state and can be differentiated into a wide variety of cell types. iPSCs are a valuable tool for testing compounds on human cells that are difficult to isolate, such as neurons, but protocols for differentiation and maintenance vary widely, and the process can be costly and time consuming. Conversely, disease phenotypes in these cells are likely to better mimic the human condition.

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Assay formats and detection systems

- Determine whether your assay will measure activity continuously, at multiple time-points, or at a single pre-determined endpoint. Establish the desired outcome of the assay (ligand binding, kinase activity, ion channel opening, nucleic acid degradation, etc.) based on the specific drug target, reporter and detection systems available.
- Select an appropriate reporter (if necessary) and detection system. The type of detection system can greatly impact critical assay parameters, including sensitivity, potential for compound interference, and dynamic range.
    - Indirect detection methods (i.e. absorbance, fluorescence, bioluminescence, etc.) use reporter systems and are ideal for larger scale screens due to their ease of use.
    - Direct detection methods, which use label-free systems, are increasingly being used. Label-free systems utilize biophysical and other measurements to determine compound activity in screens, avoiding many confounding factors that can occur with the use of reporter/indirect systems.
    - More quantitative and sensitive analytical methods, such as liquid chromatography mass spectrometry (LC-MS), electrophysiology, and RNAseq can be used if screens are to be run on a smaller-scale.

Assay Optimization

After establishing the basic format and protocol, it is important to experimentally optimize your assay. Although extensive optimization can be costly and time consuming, it is an essential step in ensuring the assay has sufficient sensitivity and specificity.

- Nearly every component of the assay can be adjusted individually. As outlined above for biochemical assays, target, substrate, cofactor, and test compound concentrations can all be adjusted to optimize the performance of the assay. In cell-based assays, factors including cell density, media composition, and others should be monitored to determine how they affect assay performance. For example, the presence of serum can effectively reduce your actual compound concentration due to small molecule binding to serum components.
- The more variables an assay has, the less efficient it becomes to optimize each one by one. Therefore, you should identify the most impactful factors to adjust.
- **Statistical Design of Experiments** is an iterative process that uses measurements of variation between replicate experiments to quickly and efficiently identify the most crucial assay components to optimize. It can also be used to identify interactions between adjustments to different components, while minimizing the number of experiments that need to be performed. [2]

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Assay Validation

Screening assays for drug discovery need to be rigorously validated. Experimental validation can determine the robustness and reproducibility of your assay. These essential parameters will provide confidence around identifying valid actives and hits during your full-scale screening campaign.

Running validation experiments

- Validation efforts should be performed in replicate experiments across multiple days
- The same equipment, reagents, and experimental conditions that will be used for the full-scale screening campaign should be used during final validation testing.
- Use measures of statistical effect size to predict assay performance. The gold standards are the Z and Z' scores, which are unit-less scales ranging from 0 to 1. Generally, Z scores of 0.5 or greater are indicative of high quality assays.
    - The Z' score allows you to perform initial validation using only controls and vehicles. If available, use compounds with known activities against your target, as they may give further information on compound performance in the assay.
    - The Z score provides further validation using a representative subset of your compound library. It takes into account the dynamic range (the range between the means of the negative (or background) and positive controls) and the amount of variation present in both samples and noise (the standard deviations).

Z score calculations

Note: σ=standard deviation | μ=mean | s=signal | c=control

- To calculate the **Z' score** the means and standard deviations of the positive (c+) and negative (c-) controls are used.
- The **Z score** is calculated using the signal and background (or negative control) means and the standard deviations of both samples and background (or negative control).

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

$$Z = 1 - \frac{(3\sigma_{s} + 3\sigma_{c})}{|\mu_{s} - \mu_{c}|}$$

Assay validation is an iterative process. If validation testing yields a low Z-score, further assay optimization may be warranted. One or more of the assay conditions may need to be adjusted in order to improve assay performance in subsequent validation tests.

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