

# How to Develop Effective *in vitro* Assays for Early Drug Discovery

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A comprehensive guide to assay  
design and development

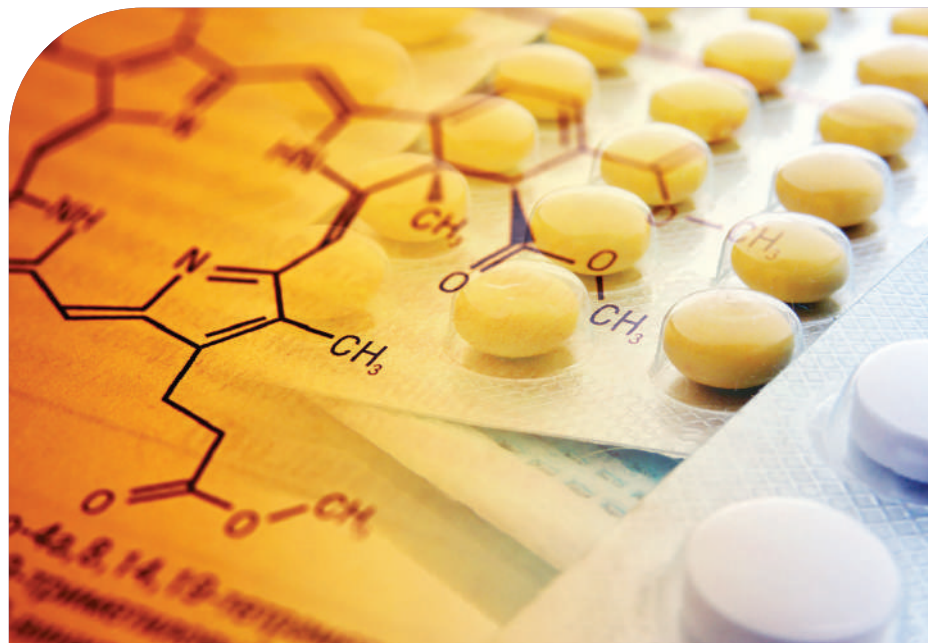
## It's an Exciting Time for Drug Discovery!

Novel technologies are opening research avenues that were not even possible just five years ago, and growing links between industry, CRO's and academia are helping to drive the translation of drug candidates into new medicines that can potentially transform many patients' lives.

Biological assays are one of the key tools driving this progress forward. In early drug discovery, bioassays not only reveal whether a compound acts on a biological target, but also how effective it is and if it has any potentially harmful toxic effects. In turn, this can influence decisions about whether the candidate warrants further study in human clinical trials, potentially leading to its release onto the market.

The importance of bioassays in early drug discovery may be clear, yet what is less clear is how bioassays should be developed to ensure they are optimized for their specific purpose. Assay design and development is a complex process, involving many considerations that can each determine the specificity, sensitivity, and reliability of a compound's action on a target. As such, if you develop your own assays, what you produce can mean the difference between valid and invalid results that help or hinder your drug discovery research.

This eBook aims to provide you with a comprehensive guide to the key considerations involved in assay design and development, which can help to ensure the success of your research programme.



## Contents

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|                  |                                                             |           |
|------------------|-------------------------------------------------------------|-----------|
| <b>Chapter 1</b> | The importance of bioassays in early drug discovery         | <b>4</b>  |
| <b>Chapter 2</b> | Key principles of assay design for project success          | <b>10</b> |
| <b>Chapter 3</b> | A practical guide to building a successful assay            | <b>15</b> |
| <b>Chapter 4</b> | Is your assay fit-for-purpose?                              | <b>19</b> |
| <b>Chapter 5</b> | Frequently Asked Questions                                  | <b>24</b> |
| <b>Chapter 6</b> | Looking ahead: Future trends in drug screening technologies | <b>28</b> |
| <b>Chapter 7</b> | Summary                                                     | <b>31</b> |



## 1

## The Importance of Bioassays in Early Drug Discovery

During the ten to fifteen year period of bringing a new drug to market, biological assays play a pivotal role in the process of early drug discovery. As one of the most important tools in pre-clinical research, bioassays identify whether a compound or biologic has the desired effect on the target protein involved in a disease pathway (see Box 1 for a definition of bioassays in the context of this eBook. Hereafter, the term 'assay' refers to this definition of a bioassay). Ultimately, it is hoped these compounds or biologics will have the potential to alleviate a patient's symptoms and so great care must be taken when designing and constructing a cascade of assays to drive the discovery process.

Assays allow researchers to measure the effect a compound or biologic has on a biological target and to what extent the molecule elicits these effects. This can be obtained in isolation using a biochemical assay, by expression of the target in a recombinant cellular background, or by using more physiologically relevant methods that measure a phenotypic response related to the bioactivity of the target.

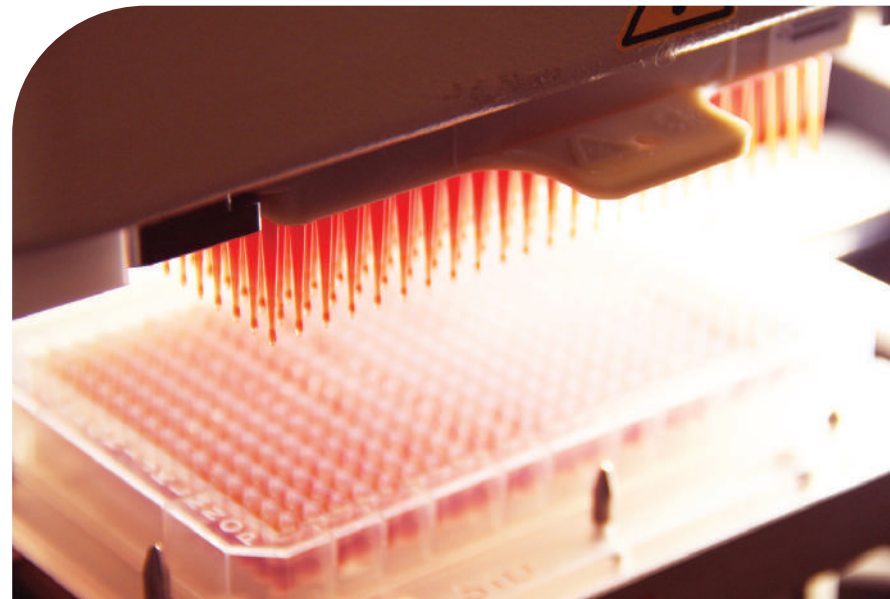
**Box 1.** In the context of this eBook, the term 'bioassay' is defined as an artificial biological system developed to measure the intervention of a compound or biologic within that system. The pharmacological intervention within the system can result in a stimulatory (agonist) or inhibitory (antagonist) response.



### Relevance

The closer the assay mimics the interplay of the target within the disease biology then the more likely the molecule will function as predicted when translated to clinical studies (without off-target and possible toxic side effects).

**VS**



### Throughput

Assays with greater physiological relevance are more complex, time-consuming and difficult to implement successfully. This results in lower throughput, with fewer compounds tested and so limits the information available about which molecules to progress to the next stage.

## Stages of Early Drug Discovery and Assay Considerations

Early drug discovery is often presented as a linear process (Figure 1), containing various biology and chemistry disciplines. Each discipline brings data to the table, however the process should be viewed as more cyclical than linear, involving iterations of both biology and chemistry to prosecute molecules with appropriate biological efficacy and physicochemical properties.

*“The early drug discovery process should be viewed as more cyclical than linear”*

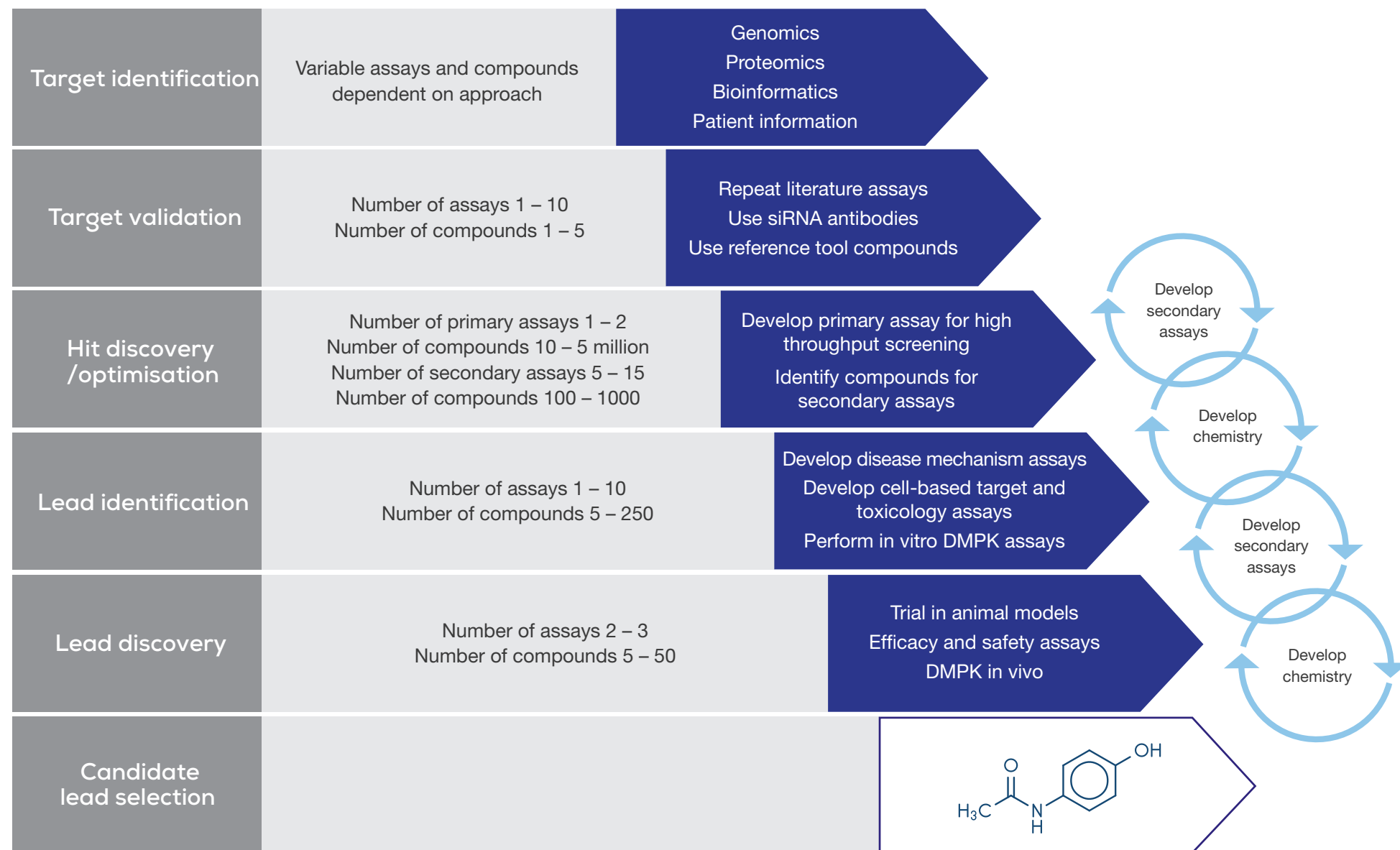
**1. Target selection/identification:** Define the relationship between target and disease; build a platform of evidence for target relevance within the disease; confirm that the mechanism of disease progression is relevant to the target and that pharmacological intervention has the potential to result in a positive effect on patient outcomes.

**2. Target validation:** Validate the target as a candidate for investigation; confirm the credibility and quality of the information and its relevance to disease progression prior to target nomination e.g. if the target has been identified through literature searches then repeat the assays to confirm published data, use siRNA, antibodies and tool compounds to modulate target activity.

**3. Hit discovery/hit optimisation:** Develop a primary assay for compound screening; consider developing secondary assays to implement in a screening cascade. By screening many compounds in the primary assay, promising pharmacophores are identified. Further exploration involves the input of medicinal chemistry with structural modification leading to improvements in potency. Further assays are developed to investigate selectivity and specificity against the target and related family members. Technology interference assays are also developed.

**4. In vitro lead identification/lead optimisation:** More complex, lower throughput, arguably more physiological plate-based assays are conducted, generally in cells relevant to the target mechanism of disease. Efficacy and safety profiling are important to de-risk potential off target negative effects or gain insight into potential toxicity. Furthermore, studies involving Drug Distribution, Metabolism and Pharmacokinetics (DMPK) of the lead compounds are used to determine if physicochemical properties pre-dispose lead compounds to undue metabolic activity and predicted distribution *in vivo*.

Figure 1: The different stages of the early drug discovery process and assay considerations at each stage.



**5. *In vivo* lead discovery/lead optimisation:** Use of animal models of disease to assess target mechanism of action, efficacy, safety profiling, unpredicted toxicity and further investigation of the DMPK properties *in vivo*. At this stage, cells and samples can be isolated from animals and biomarker assays developed.

**6. Candidate lead compound selection:** Only after achieving the desired profile of efficacy, distribution/metabolism and safety in pre-clinical studies will the lead molecule be nominated for progression to human clinical trials.



All of the above stages involve making informed decisions on which compounds to progress based on statistically sound and biologically relevant assays. Considerations when developing assays for compound progression will be discussed in detail in the following chapters, but initial examples include:

### **Sensitivity and specificity**

What is the specificity of the compound for the target and does it affect multiple targets? Are the compounds selective against the target and inactive against related family members

### **Cellular efficacy and toxicity**

Can you confirm the binding activity of the compound in a secondary (orthogonal) cell-based assay? Has the compound killed the cells in the assay?

### **Binding properties and kinetics**

What mechanistic studies can be performed to examine compound binding properties (reversible/irreversible, fast/ slow binding)?  
Can target mutation studies be undertaken to evaluate the structure of the binding site?

### **DMPK properties**

Does the compound have good solubility and permeability across cell membranes (Log D and Caco-2 permeability)? Does the compound show toxicity in hepatocyte cells or does it have high microsomal clearance?

Assays are essential in early drug discovery research. Their quality, reliability and consistency are entirely dependent on how well they are designed. Principle considerations and practical guidelines for successful plate-based assay development are examined in the subsequent chapters.

## 2 Key Principles of Assay Design for Project Success

Assay design is a major factor underpinning the success of any drug discovery programme. A well-designed assay should be one in which every step in the protocol has been evaluated. One analogy is that assays are like aeroplanes; if you need to travel 500, 5,000 or 50,000 miles, you want to fly in a well-designed aeroplane and get the same outcome every flight: you arrive safely!

### Target-Based Drug Discovery Versus Phenotype-Based Drug Discovery

From an assay perspective, there are two main approaches to address your target, namely target-based drug discovery (TDD) and phenotype-based drug discovery (PDD). TDD relies on your knowledge of the target protein and its mechanism of action in the context of the biology/disease. This enables the development of assays to study the activity of the target, either as a biochemical or recombinant cell-based readout. Alternatively, PDD makes no assumptions about the specific target, but considers the holistic output of the biological system and the intervention of compounds or biologics to influence this readout. TDD and PDD are both acceptable approaches, but at this stage, it is crucial that you consider which would be best suited to studying your target.



No matter if you're flying 500 miles or 5,000 miles, you still expect the same degree of engineering, build quality and safety assurance to be invested into the aeroplane you're travelling on. The same principle applies to assay design and development.

Whether you're testing 5, 5,000 or 5 million compounds, you should make sure your assay behaves in a similarly reproducible manner that is effective at getting to your desired endpoint. Otherwise, your experiment may crash and burn!

## Designing an Assay: What are the Key Considerations?

There are a number of key questions you should consider to help inform the construction of the appropriate assay for your requirements:

### 1. What is the target and how does it respond?

Understanding the biology of your target is important. Consider how the compound or biologic will interact with the target protein and what the effect of this will be on the disease mechanism. For example, will you be looking for inhibitors (antagonists), activators (agonists), positive or negative allosteric modulators, cyto-toxic or cyto-static agents? Consider whether the activity of the target requires other associated proteins.

### 2. Is there a literature precedent for the target and assay type?

Searching the literature may uncover clues about the assay formats that can be used for different target classes. [Table 1](#) outlines a number of biochemical and cell-based assays that could be used. This list is not exhaustive given the vast range of existing assays. Nonetheless, it offers some initial guidance on the literature precedent available.

### Are you looking for an agonist, antagonist or modulator of the biology?

You must define what type of biological response your assay will detect, which will be driven by the nature of the intervention by the compound or biologic. Typically, assays are developed to identify molecules that inhibit (antagonise) biological processes and therefore the assay is designed to detect the competition of a compound for the target-binding site versus the endogenous stimulus. Alternatively, assays can be developed to identify molecules that stimulate biological processes and drive responses. Here, assays are developed to detect molecules that compete with or interact with the binding site of the target (agonist). There are other examples in which the molecule binds to an alternative allosteric binding site and, in doing so, alters the conformation of the binding site on the target to either enhance or inhibit its activity indirectly. Each assay will generate different but complementary readouts.

In addition to considering the type of response, it is important to decide how many compounds will be screened through the assay (10s, 100s, 1000s, 100,000s) and whether automation will be available, such as plate-based liquid handling instruments, plate readers with stackers or robotic systems that use scheduling software. These not only enable higher-throughput, but can also enhance the consistency of your assays.

Table 1: Biochemical and cell-based assays used for different target classes

| Target type                              | Biochemical                                                                                                                       | Cell-based                                                                                                                                                                                                                                                                       |
|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| G protein coupled receptor (GPCR)        | Radioligand binding (but, this is not a functional assay, so it cannot discriminate between agonists and antagonists)             | Functional assay measuring second messengers<br>e.g. cAMP, Ca <sup>2+</sup> , inositol phosphate, GTPγS<br>Kinetic e.g. calcium flux<br>Accumulation/equilibrium e.g. cAMP, β-arrestin, inositol phosphate<br>Label-free (DMR) phenotypic measurement of the whole cell response |
| Nuclear hormone receptor                 | FP<br>Radioligand binding                                                                                                         | EFC<br>High content screening                                                                                                                                                                                                                                                    |
| Ion channel<br>(voltage or ligand gated) | hERG fluorescence polarisation<br>Ligand binding                                                                                  | Ion flux e.g. calcium sensing dyes<br>or cell-based potassium ion channel screening<br>Membrane potential dyes<br>Patch clamp (e.g. IonWorks™)<br>or electrophysiology rigs                                                                                                      |
| Transporters                             | No available assays – transporters are multi-meric proteins and require a gradient to work successfully                           | Uptake/efflux e.g. inhibition of fluorescence probe<br>ATPase stimulation<br>Bi-directional transport<br>Inside-out vesicles                                                                                                                                                     |
| Kinase                                   | Classic radioactive P33 incorporation,<br>FRET, HTRF®/LANCE®, TR/FRET, AlphaScreen™, EFC<br>Coupled assay for ATP/ADP measurement | HTRF®/LANCE®, AlphaScreen™<br>in cell lysates                                                                                                                                                                                                                                    |

Assay abbreviation definitions (in alphabetical order): BRET – Bioluminescence resonance energy transfer; CETSA – Cellular thermal shift assay; ChIP – Chromatin immunoprecipitation; DELFIA – Dissociation-enhanced lanthanide fluorescence immunoassay; DMR - Dynamic mass redistribution; EFC - Enzyme fragment complementation assay; FP – Fluorescence polarisation; FRET – Fluorescence resonance energy transfer; GTPγS - Guanosine 5'-O-(3-thiotriphosphate); hERG – Human Ether-à-go-go-related gene; HTRF® - Homogeneous time-resolved fluorescence; LANCE®- Lanthanide chelate excite; LDR – Long distance repeat; TR-FRET – Time-resolved fluorescence resonance energy transfer; UPLC-MS – Ultra performance liquid chromatography tandem mass spectrometry.

Table 1: Biochemical and cell-based assays used for different target classes

| Target identification       | Biochemical                                                                                          | Cell-based                                                                                                                                                                                                             |
|-----------------------------|------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Histone modifying enzymes   | Peptide substrates, Histone-specific or co-factor based detection                                    | Antibody-based e.g. ChIP (qPCR), epigenetic HTRF®, LanthaScreen, AlphaLISA, DELFIA®<br>Binding e.g. CETSA-AlphaScreen™<br>Mass spectrometry e.g. UPLC-MS<br>Gene expression e.g. LDR<br>Phenotypic e.g. cell viability |
| Bromodomains                | AlphaScreen™, TR-FRET, EFC                                                                           | Histone-binding<br>Chromatin-displacement<br>Fluorescence recovery after photobleaching (FRAP)                                                                                                                         |
| Transcription factor        | Transcription/translation in vitro                                                                   | Reporter gene assay with DNA response element<br>High content assay for translocation                                                                                                                                  |
| Protease                    | Quenched FRET substrate                                                                              | Fluorescence molecular beacon                                                                                                                                                                                          |
| Protein:Protein interaction | Biacore™<br>AlphaLISA<br>HTRF®/LANCE®, TR/FRET                                                       | Yeast two-hybrid<br>BRET-based (NanoBRET™)                                                                                                                                                                             |
| Nucleic acids               | Fluorescence dye-based assays<br>Hybridisation assays e.g. bDNA<br>Extraction/amplification e.g. PCR | Cell-impermeant or cell-permeant detection dyes                                                                                                                                                                        |

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#### 4. Are the reagents you require available commercially or will you have to make them?

You also need to determine if the reagents you need to build your assay are available commercially. For example, if you need a cell line expressing your target or a source of recombinant proteins, or endogenous (natural) ligand, enzyme substrate or antibody to the target, investigate whether they are commercially available in the format and quantity required. Consider the amount you will need for the lifetime of the project as variability in future batches could impact the consistency of your data. If reagents are not commercially available, consider expressing and purifying your own but be aware this will have an impact on time and cost.



#### 5. Availability of a pharmacological tool compound or standard?

The availability of a pharmacological tool compound or standard enables the assay to be developed within a defined activity range. A tool compound modulates the activity of the readout in a predictable dose-dependent manner. In the case of an agonist, the standard defines the magnitude of activation that can be achieved by the biological system and can act as a reference for the activity of test compounds. If the standard is an inhibitor, it can define the minimum signal. This can be different to the biological background signal. The standard can also be used to measure the consistency of the assay, testing the standard repeatedly in separate plates on separate days with the same reagents should result in reproducible  $IC_{50}$  (Inhibitor Concentration – antagonist) or  $EC_{50}$  (Effector Concentration – agonist) values (see Chapter 4 for more information).

Now that you have considered the key principles involved in designing the most appropriate assay for your requirements, the next steps are the practical aspects of building it. This involves applying a variety of important technical considerations, which are outlined in Chapter 3.

## 3 A Practical Guide to Building a Successful Assay

Once you have analysed your assay design and are confident that the readout is appropriate in the context of the biology, you are now ready to build your assay. When constructing any assay, there are several technical and practical steps needed to ensure it is robust, reliable, and fit-for-purpose. These are outlined below to help optimise your finished assay.

### Measuring Target Activity

For example, will your assay measure the turnover of a substrate or product formed during the time allowed for the reaction to occur (end-point assay), or will you use a kinetic readout over seconds, minutes or hours e.g. activation of intracellular calcium stores (seconds), translocation of a protein (minutes) or cell death (hours)? Will this be measured in a cellular background, where the target is over-expressed, using a biochemical approach or a phenotypic readout?

For a target-based drug discovery approach, your assay design should have already identified the type of target activity being measured. However, you also need to define how your assay will measure this activity.

***“You need to determine how your assay will measure the defined target activity”***

If you will be measuring the target activity in a biochemical assay, then there are some crucial questions you need to consider:

- If your protein has been expressed in a recombinant system, is it active and can this be maintained during isolation and purification?
- Does it have the necessary phosphorylation and glycosylation states to behave in a native manner in your solution-based assay?
- Do you need a sub-unit/domain or the full-length protein –does this make a difference?

Alternatively, if you have adopted a phenotype-based approach, then you will need to consider the following questions:

- Is the biological readout directly related to target engagement or is the readout examining the activity of the pathway on which your target is one of the key components?
- Do you need additional orthogonal assays to build confidence that modulations in readout due to pharmacological intervention are target-related?
- Is the assay you are building susceptible to interference by other factors, either biology or technology related? Can you develop other assays to rule this out and improve the robustness of, and confidence in, your data?

## Practical Considerations when Developing your Assay

The following steps highlight important considerations when developing an assay. The process is iterative and involves balancing a number of variables to generate the most appropriate conditions for success (Figure 2):

### 1. Plate format

Choosing which density of multi-well plate to use (i.e. 6/24/48/96/384/1536), will depend on how many compounds you will be testing and how expensive your assay reagents are. For example, using a 384-well plate for your assay development is a good approach, as this will decrease the well volume thereby decreasing the cost per test, making it more cost-effective. However, this needs to be balanced with the availability of both liquid handling and reader technology required to work with these plate formats.

### 2. Plate type

You can use a variety of different plate types, such as white/black solid or transparent bottom plates. It is important to bear in mind that different plate types are better suited to different assay types. For example, white solid plates work well for luminescence assays as they channel photons to the detector, whilst black solid plates are better for fluorescence assays as they generate less background fluorescence. For cell-based assays, it can be more appropriate to use clear bottom plates so that you can observe the cells under a phase contrast microscope.

### 3. Final assay volume

This must be considered in the context of your assay design in relation to various parameters including:

- i. The concentration of your commercially available reagents
- ii. The stock concentration of your compounds and solvent
- iii. The solubility of your reagents in various buffers and acidic, basic and neutral pH
- iv. The final assay volume in relation to the components and the available liquid handling technology

### 4. Assay homogeneity

Heterogeneous assays involve multiple steps, including washing and adding reagents at various stages. Homogeneous assays use more simple mix and read procedures and avoid the necessity of processing samples by separation or washing steps. As such, homogeneous assays are faster and easier to perform making them more amenable to high-throughput screening and automation. They are also typically more reproducible due to fewer steps being required. Therefore, homogeneous assays should be used whenever possible.

### 5. Reagent addition and combination

When adding components to your assay, it is important to define the best order to add these to the well and the most appropriate liquid handling equipment to do so. Variables such as volume and concentration of reagent to add, the order in which different reagents should be added, the dispensing method, and whether you can combine non-reactive components before you add them to the well should be considered.

### 6. Reagent stability

You should ensure that your reagents are stable in the context of your assay development. Key considerations include:

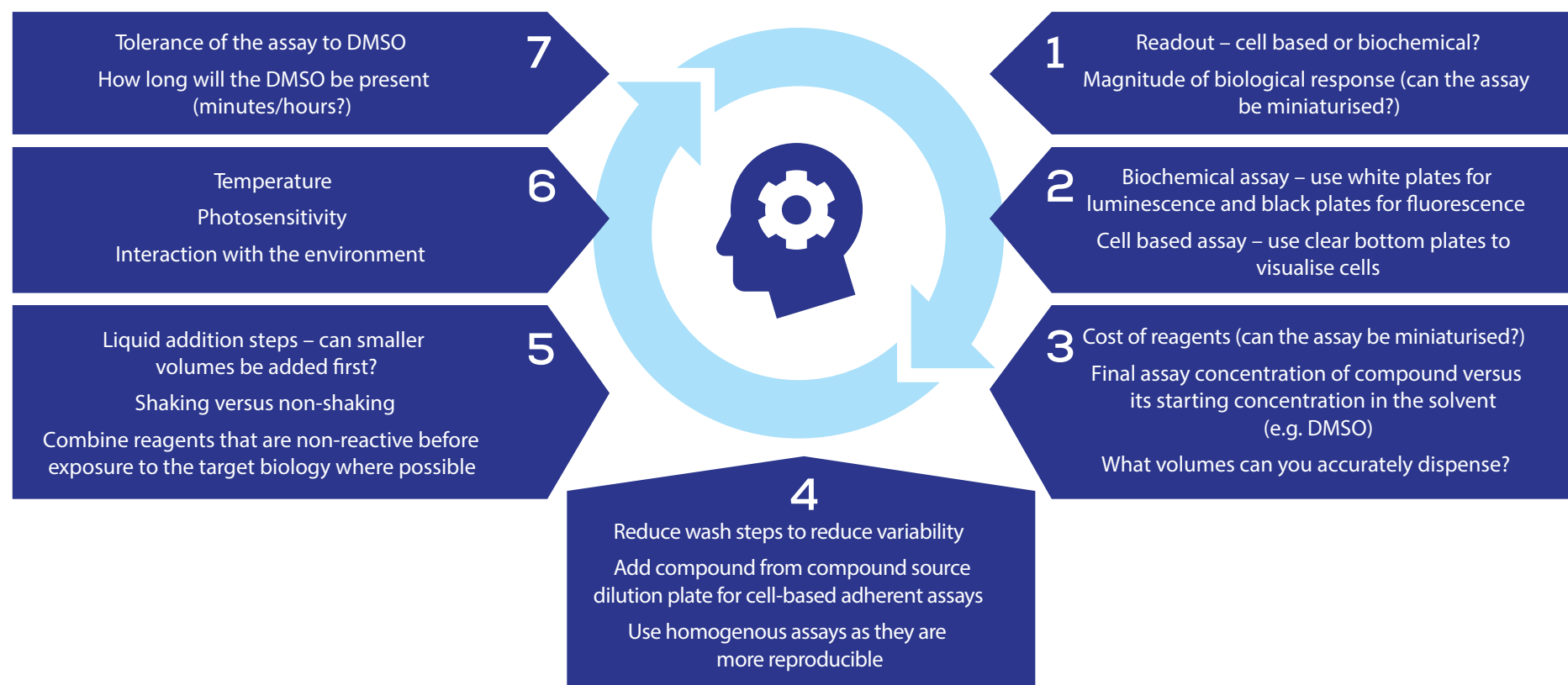
- i. Temperature stability – do the reagents need to be kept on ice or at room temperature, and how will this affect your assay?
- ii. Photosensitivity – do the reagents, or the plate to which the reagents have been added, need to be protected from ambient light?
- iii. Environmental interactivity – could your reagents adhere non-specifically to the plastic ware, or to the plates, affecting their actual concentration in the final assay volume?

### 7. Compound addition

Traditionally, compounds have been dissolved in dimethyl sulfoxide (DMSO) and typically to concentrations between 2mM and 10mM. However, unless you are screening small molecular weight fragments, it is unlikely that you will be able to increase the concentration above 10mM as the compound may be insoluble. Consider that solubilising compounds at less than 2mM in DMSO means that the final concentration of DMSO in the assay will be dependent on the tolerance threshold of your assay to DMSO. Remember to maintain equal concentrations of DMSO in all wells of your assay.

It is equally important to consider at what stage you should add your compound to the assay. Consider how your compound interacts with your target protein (i.e. does it associate quickly, or will it require time to bind?). This will allow you to determine how long to incubate the compound with the target protein before adding the other assay components.

Figure 2: Key considerations for assay development



Taking this critical look at the steps taken to build your assay is essential in ensuring its reliability and consistency. But, how do you quantitate the consistency and reliability of your assay? This is the focus of Chapter 4.

## 4 Is your Assay Fit-For-Purpose?

Identifying whether your assay is fit-for-purpose is an extremely important step in assay development. By interrogating your assay using statistics, you can generate confidence in its reproducibility and consistency to make data driven decisions about which compounds to investigate further. This chapter highlights the guidelines and statistical rules you should follow to ensure both your assay's quality and reliability.

### Quality Control in Assay Design

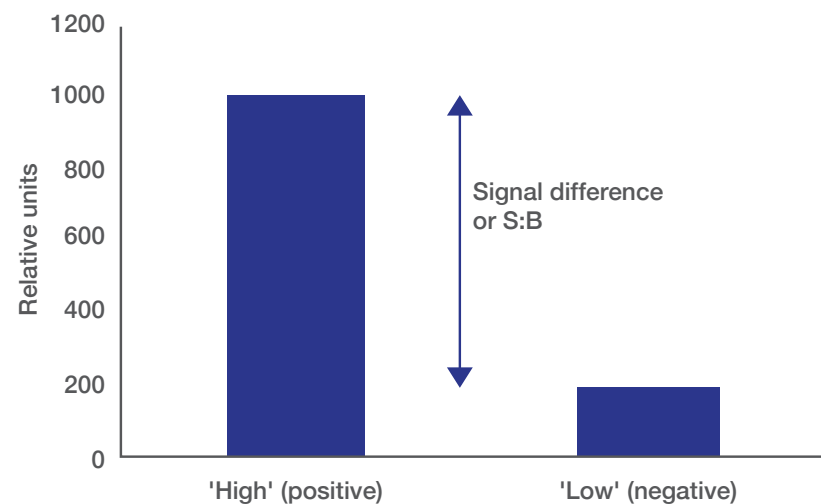
#### 1. Setting up sufficient control wells

Pragmatically, you should design your plate layout to include complete columns or rows to keep dispensing as simple as possible, whilst having sufficient controls to determine the reproducibility of the signal (positive biological response) and noise (biological background) for your assay (Figure 3).

Controls differ depending on your assay type. For a cell-based assay, you could represent the 'low' (negative) control by treating wells with DMSO. For a biochemical assay, you could use either an inhibitor to block the activity of the target protein or a chelator to terminate the reaction. The 'high' (positive) control can be generated by a stimulus of the biological response in the absence of test compounds. Any plate should contain an equal number of 'high' and 'low' controls so as not to weight the statistics in any particular direction. Consider the physical location of your control wells in your plate layout. If these are on the

edge of the plate, then they will be exposed to greater extremes of temperature. However, if you locate them in the centre of the plate, conditions will be more optimal, but this will be more difficult to set up from a liquid handling perspective.

Figure 3. The 'high' control (positive) in a cell-based assay is generated in the presence of a stimulus whilst in a biochemical assay only DMSO or buffer is added. The 'low' control (negative) in a cell-based assay is the background response when just DMSO or buffer is added, whilst in a biochemical assay it is the signal in the presence of an inhibitor.



## 2. Use an appropriate pharmacological reference or standard compound

If available, use a pharmacological tool or reference compound as a standard to enhance your assay's robustness. This allows you to measure assay reproducibility or inter-assay variation independently of the controls. Screening the tool compound in a dose-response manner will allow you to generate an IC<sub>50</sub> or EC<sub>50</sub> for the compound.

## 3. Use the following statistical parameters:

### a. Calculate signal-to-background (S:B) or signal-to-noise (S:N).

S:B is a measure of the ratio of the mean of the 'high' (stimulated/positive) controls to the mean of the 'low' (background) controls. S:N is calculated as the ratio of the maximum mean signal to the minimum mean signal divided by the standard deviation of the assay. If possible, the assay window should be at least 2-fold. However, remember that a large assay window is secondary to the variability associated with the assay. A small assay window that is consistently reproducible from well to well is just as good.

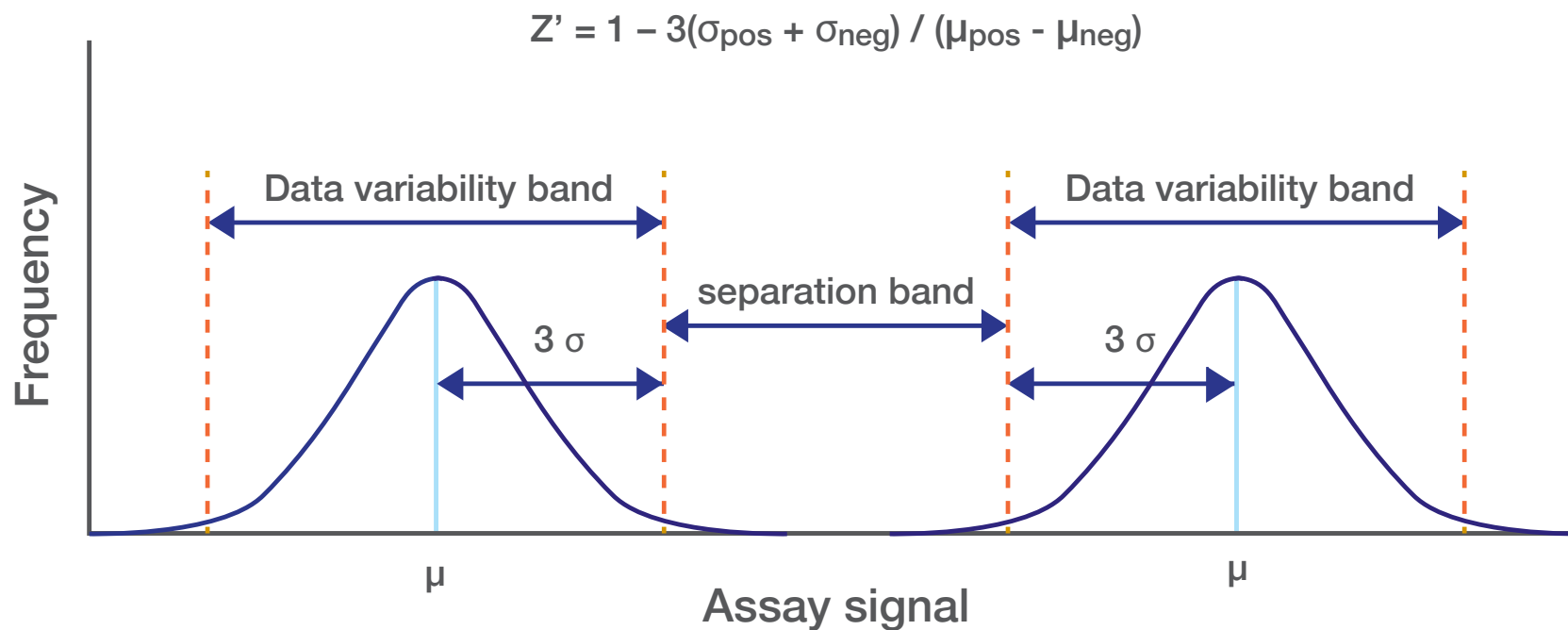
### b. Measure assay variability

This is measured using the standard deviation of the control wells, the 'coefficient of variation' (CV). Ideally, aim for a low CV of less than 10%. Also, monitor the number of steps that you use to set up the assay (fewer steps will reduce variability, or use a homogeneous assay).

### c. Compute the Z prime, Z-factor, and Z-score

Z prime is used as a measure of reproducibility of the assay controls and is often used when testing small and large compound sets in high-throughput screening assays or iterative screening assays that are run for many months (Figure 4). The Z-factor takes into consideration the standard deviation of the positive and negative controls along with the distance (assay window) observed between the mean values for the positive and negative signals. A reproducible assay will have a value of between 0.5 and 0.99, representing extremely reproducible control samples. Lastly, the Z-score is calculated using the data from all the sample wells on a plate. The Z-score is dependent on a mostly homogeneous null effect across all samples on the plate thus having a data distribution and standard deviation representative of this null effect. Any compound with activity outside of this null effect of distribution can be considered a 'hit'.

Figure 4. Z prime is defined in terms of the mean and standard deviation of the positive and negative controls. It assumes that data is normally distributed, with 99% of the data within three standard deviations of the mean.  $\mu$  equals the mean of the distribution of either the positive or negative controls (left and right in the diagram).  $3\sigma$  equals three times the standard deviation of the positive and negative controls which equals 99% of the negative and positive data distribution.



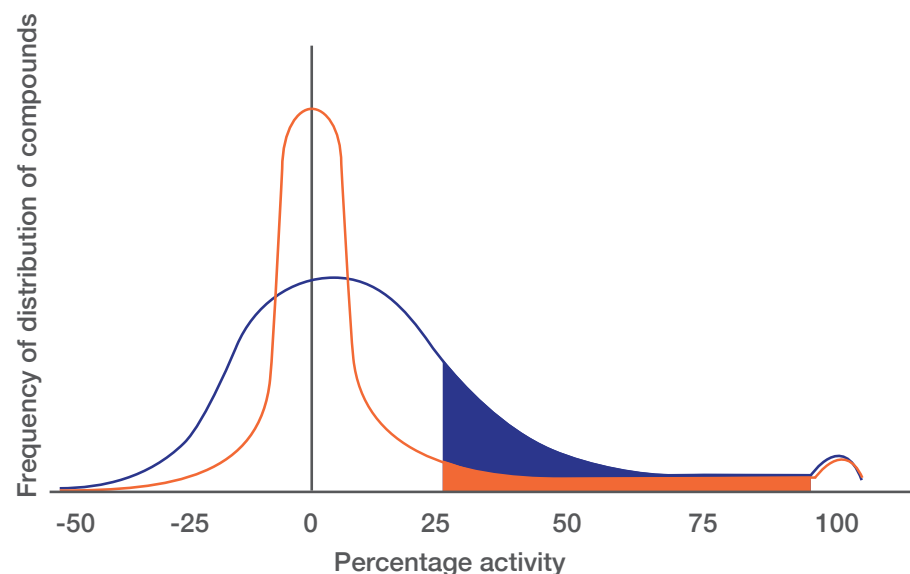
Zhang et al. 1999

## Definition of an Active or 'Hit' Compound

All compounds screened in an assay will have some activity, many will generally have activity centred at approximately zero percent (or the null effect). The largest assay variability in the 'no effect' control also occurs at this zero point and therefore in any compound set screened there will be a spread of compounds with activity around this zero point. For some assays, the spread of activity drops off sharply beyond this 'noise' and therefore active compounds are more easily defined by having activity greater than the noise. In other assays, the noise can be a broader spread of data and therefore discriminating active from inactive compounds becomes more of a pragmatic art than a science based on rationale and statistics.

An active compound is defined as a compound having activity greater than the ambient noise of the assay. A 'hit' is a compound that, when tested in the same assay many times, consistently reproduces its activity with variability that is consistent with the statistics of the controls. For example, if a compound is identified in the assay with an activity of say 40% and when repeated in triplicate in the same assay, it is 35, 45 and 15 then it is a 'hit'. If its activity is 45, 15 and 15 then it is not a 'hit' (Figure 5).

Figure 5. Percentage activity of each compound versus the frequency distribution of the compounds. The gold line depicts an 'excellent' assay i.e. almost all the compounds are inactive and a low cut-off (25%) is used to identify compounds for retesting (solid gold area). The purple line depicts a 'poor' assay i.e. it is not as clear cut in terms of cut-off. If 25% is used, significantly more 'noisy' compounds will be retested (solid purple area plus solid gold area). Note any activity observed at 90 – 100% potentially represents technology interference.

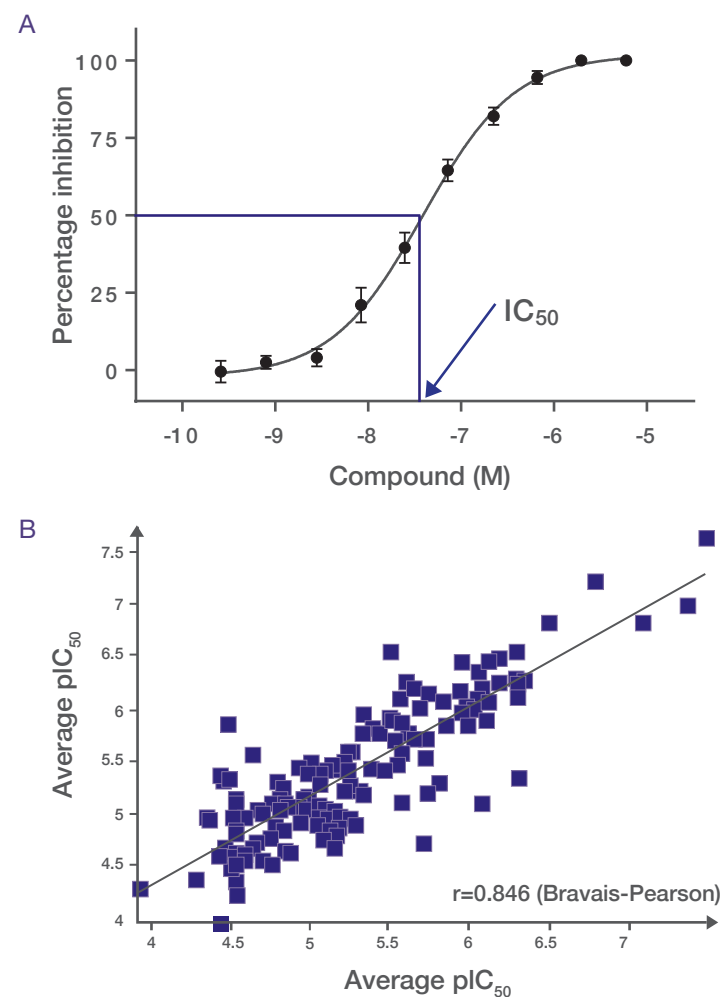


## Pharmacological Validation of your Assay

You must ensure that your assay delivers statistically acceptable, reproducible results. You can test this by screening a primary pharmacology plate containing compounds with known activity and ideally a range of potencies, along with plates containing control wells. These pharmacology plates should be screened both on the same day and between days, and the data correlated to determine both inter- and intra-assay variability and also to generate a level of confidence that the assay detects active compounds consistently (Figure 6).

Quality control and assay validation are undoubtedly essential in developing consistent and reproducible assays, informing decisions about which compounds or biologics should be taken forward to the next stage of the process. Given the complexity of the assay design process, it is not surprising that things can go wrong. In Chapter 5 we take a look at some of the most common issues you might experience and how to address them.

Figure 6. Graph A. Typical dose response inhibition plot (on a log scale) showing interpolation of the  $IC_{50}$  value. Graph B. Correlation of the  $pIC_{50}$  values for a primary pharmacology plate, containing compounds with a range of potencies, tested on separate occasions.



## 5 Frequently Asked Questions

Issues and complications can often arise during the assay development process and these need to be addressed to ensure the assay is built to the highest quality. As a guide to overcoming these potential obstacles, this chapter considers some of the most common questions asked during the assay design process.

### Q. What should I define as a 'hit'?

**A.** A 'hit' is defined as a consistently 'active' compound, but how do you define 'active'? If you screen 1,000 compounds in your assay, you need to rank them based on their activity by expressing the change observed as a percentage of your controls (DMSO and stimulus). You then plot this as a distribution of percentage activity (X-axis) versus frequency (Y-axis) (Chapter 4 Figure 5). Based on this distribution, you need to pragmatically select a reasonable number of compounds that you can retest. If you select say a 30% cut-off, anything with activity greater than 30% is defined as an active compound and is taken forward for retesting in your assay. A 'hit' is defined as a compound that in the assay, gives the same level of activity when tested a number of times.

### Q. Can I miniaturise my assay from 96- to 384-wells?

**A.** Yes, but it's not as easy as reducing all the reagents by four-fold. Using the parameters from a 96-well assay is a good starting point, but typically it is best to begin with lower densities of cells and lower concentrations of reagents and work upwards to find the optimum concentrations and signal-to-noise ratio.

### Q. What is an ideal compound screening concentration?

**A.** The ideal compound screening concentration for hit discovery should be identified to avoid interference from solvents (e.g. DMSO), as well as to ensure a sufficient compound concentration is obtained to achieve a 'hit' in your assay. For single compound screening assays a compound concentration of 10µM is recommended. However, for fragment screening, a compound concentration of 100µM is more suitable to ensure greater binding site occupancy.

### Q. Does compound solubility matter?

**A.** Yes – 10mM is the recommended concentration when making stock solutions in DMSO. In general, this should allow a final assay concentration of at least 10µM resulting in minimal DMSO present in the final assay. The use of stock solutions higher than 10mM is likely to cause compound solubility issues.

### Q. Why do I need to pre-incubate my compounds with my target before adding the stimulus/inhibitor?

**A.** Compounds can often have different binding kinetics. Some associate with the target quickly while others require time to bind, some dissociate quickly while others do not. As such, you should routinely incubate targets with the compound (in both biochemical and cell-based assays) for a minimum of 15 to 20 minutes prior to adding the modulating agent.

### Q. How do I deal with plate patterns and control layouts?

**A.** In either 96- or 384-well format, the best place for the controls is in the centre of the plate to avoid edge effects. It is also best to use a column or row for each of the controls. For cell-based assays, the edge of the plate often creates issues associated with evaporation problems.

### Q. Can I screen with an assay if the Z prime is less than 0.5?

**A.** Possibly. Z prime will inform you of how good (or bad) your assay will be at reproducing the effect of a compound when retested. If your Z prime is negative (and it can be) then your assay is fundamentally not reproducible. If your Z prime is positive, then the higher this number the better chance you have of repeatedly identifying the same compound in your assay with a statistically reproducible level of effect.

### Q. What can I do if I don't have a pharmacological tool or reference compound?

**A.** It is very difficult to develop assays in the absence of a pharmacological tool or reference compound. You need to identify a means of modulating the biology that your assay will detect. For example, in a biochemical assay, you can ablate the signal by adding a chelator to terminate the reaction. In a cell-based assay, you can modulate the readout with compounds that act up or downstream of the target protein. Nonetheless, having a compound that behaves in a consistent pharmacological manner is best.

**Q. If I perform a dose-response experiment on a standard, how many dilutions and what concentrations should I use?**

**A.** Pragmatically, you should count the number of wells in a column or row and take this as the first consideration. Next, you should consider dilutions of compound of either one in two dilutions or half log (1 in 3.16) dilutions. Then think about your maximum assay concentration, whilst taking into account the tolerance of the assay to the solvent (DMSO), this calculation is based on the stock concentration and the final assay volume. If you know nothing about the compound's activity, try starting at 50 $\mu$ M and performing half log dilutions.

Although common obstacles can occur in assay design and development, it is possible to overcome these by following the above general guidance. However, following the key principles and practical advice outlined in this eBook, largely depends on the technologies available to you. Remember that this is an iterative not a linear process.

As technological innovations continue to proliferate, Chapter 6 examines the future trends in drug screening technologies and how these can enhance the assay development process and, ultimately, the search for new drugs.

## 6 Looking Ahead: Future Trends in Drug Screening

Progress in the field of genomics, gene editing and stem cell re-programming, coupled with innovation in both material sciences and engineering, along with advances in microscopy and imaging software, have brought significant advances to the drug discovery arena. As technologies continue to advance, the potential for developing more sophisticated assays continues to grow, creating exciting times for pre-clinical drug discovery.

### Innovations in Liquid Handling – Sub-Microlitre Dispensing and Microsonic Mixing

Recent advances using high frequency sound to move small volumes of liquid (2.5 nanolitre drops) have resulted in the ability to 'fire' small quantities of compounds dissolved in DMSO from source to destination plate, with ten millimetres or more between well bottoms. The technology is contactless (no tips are required) so there are no cross-contamination issues, and by using consistent 2.5 nanolitre droplets, dispenses can be tuned to defined volumes whilst maintaining very low final DMSO concentrations to avoid solvent interference in assays.

***"If you always do what you have always done, you'll always get what you always got!"***

Similarly, there are now dispensers that use inkjet dispensing technology to dispense compounds dissolved in DMSO at volumes of 10 picolitres, providing a wider dynamic range when setting up a concentration-response curve.

Typically, once liquids are added to a 96-, 384- or 1536-well plate these cannot be mixed any further. However, using ultrasonic sound waves, solutions can be mixed in the wells to overcome this problem. This approach allows liquids with high specific gravity to be mixed in each well of the plate, improving compound solubility and assay reagent mixing.

## Acoustic Mass Spectrometry

Mass spectrometry (or mass spec) is an analytical chemistry technique that measures the mass-to-charge ratio and abundance of gas-phase ions in a sample, in order to identify what type and amount of chemicals are present. This technique is being used to study the output of biological responses by analysing the molecules that remain in the solution after the reaction has finished. Acoustic mass spec differs from standard mass spec (which uses a needle to aspirate and spray the samples) by sending a sonic pulse through the liquid, creating a 'mountain of liquid on the surface'. A second pulse shatters the 2.5 nanolitre 'mountain of liquid' into hundreds of femtolitre droplets. This effectively sends a tornado of droplets through a charged field to generate a stream of ionised particles in the mass spectrometer.

This combination of high speed and low sample volume meets the requirements of ultra-high-throughput screening, and potentially provides higher quality screening results at a reduced cost compared to standard mass spec.

## Three-Dimensional Bioprinting

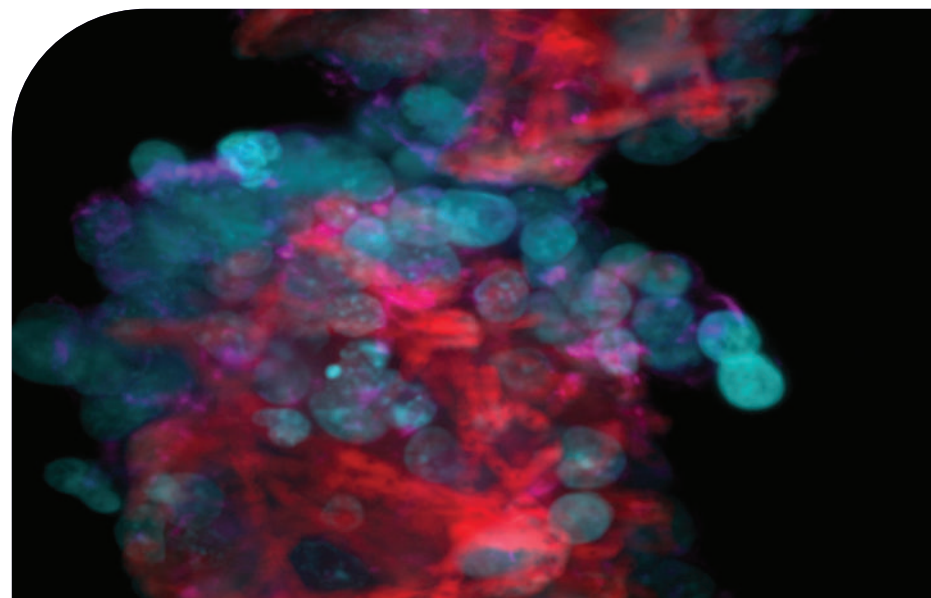
Three-dimensional (3D) printing technologies are gaining momentum in their application to biology. 3D bioprinting involves printing cell patterns onto a surface, usually contained within a matrix material, where cell function and viability are preserved within the printed construct. This allows the cells, plus the matrix, to be dispensed and polymerised to form 3D structures. Since organs are composed of tissues, and the differentiation between tissues tends to be formed of boundary layers between different cell types, it is therefore possible to print layers of different cell types to form tissues. By doing so, scientists can recapitulate the physiology of the tissue in vitro. However, the one disadvantage of this technology in its current form, is the inability to repeat the process many hundreds of times, making it difficult to screen many compounds through the same micro-fabricated tissue.

## Three-Dimensional Cell Culture Technologies

Since the beginning of cell culture, cells have been grown in two-dimensions on plastic surfaces. However, humans are 3D organisms composed of cells living in a 3D environment, with multicellular interactions. Increasingly, scientists are recognising that growing cells in 3D will influence their behaviour along with the biological readout, and so technologies designed to maintain cells in three-dimensions have been increasing in use over the last few years.

The most frequently used approach is spheroids, formed either in hanging drop plates or in ultra-low attachment plates. When cells are added to these formats, they combine at the apex of the drop to form a tissue. Alternatively, cells can be grown in substrate matrices (e.g. Matrigel™) or can adhere to man-made matrices such as 'honeycomb' polystyrene or scaffolds made from poly-L-lactide (Figure 7). When cultured in 3D, the cells exhibit a different phenotype to that in two-dimensions, including more complex cellular behaviour that can better mimic that seen *in vivo*. This represents a more physiologically-relevant microenvironment and possibly identify compounds active in 3D cell-based assays but not in 2D.

Figure 7. Confocal image of HEK-293T cells cultured for 48 hours on an electrospun random fibre lattice. Blue shows nuclei, purple shows laminin protein, red shows electrospun material manufactured containing rhodamine.



The continued emergence of such innovative technologies can transform the way that screening assays are designed, developed and implemented in the future, thus generating more reliable, reproducible, and fit-for-purpose assays to drive the discovery of new compounds and their eventual translation into novel medicines.

## 7 Summary

This eBook has explored the key considerations involved in developing effective assays for early drug discovery. These exclusive insights are based on the decades of experience we have in pre-clinical discovery research gained at several leading pharmaceutical companies, across a wide range of therapy areas and using a variety of technologies. Inspired by the support we give to our clients as they navigate the complexities of designing and constructing an optimal assay for their research, we wanted to provide an introduction to the essential information crucial to the success of your programme.

We hope that by highlighting the key principles of designing reproducible and consistent assays, we can help you overcome some of the common problems that can arise during assay development, providing a great platform from which to start building the best assay for your needs.

Designing assays for early drug discovery is extremely complex and so we're always happy to help with any challenges or answer any questions you may have along the way.



[charnwooddiscovery.com](http://charnwooddiscovery.com)



[info@charnwooddiscovery.com](mailto:info@charnwooddiscovery.com)