Chapter 1

Design and Implementation of High-Throughput Screening Assays

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Abstract

HTS is at the core of the drug discovery process, and so it is critical to design and implement HTS assays in a comprehensive fashion involving scientists from the disciplines of biology, chemistry, engineering, and informatics. This requires careful analysis of many variables, starting with the choice of assay target and ending with the discovery of lead compounds. At every step in this process, there are decisions to be made that can greatly impact the outcome of the HTS effort, to the point of making it a success or a failure. Although specific guidelines should be established to ensure that the screening assay reaches an acceptable level of quality, many choices require pragmatism and the ability to compromise opposing forces.

Keywords: HTS process, Assay technology, Biochemical assays, Cellular assays, HTS quality, HTS validation.

1. Introduction to the HTS Process

In most pharmaceutical and biotechnology companies, high-throughput screening (HTS) is a central function in the drug discovery process. This has resulted from the fact that there are increasing numbers of validated therapeutic targets being discovered through advances in human genomics, and increasing numbers of chemical compounds being produced through high-throughput chemistry initiatives. Many large companies have over 100 targets in their pipeline at any given time, and lead compounds must be found to progress these targets. In some cases we know enough about the target and can apply knowledge-based approaches to hit discovery such as focused screening and structure-based design. However in many cases, particularly for more novel targets, there is limited knowledge about the types

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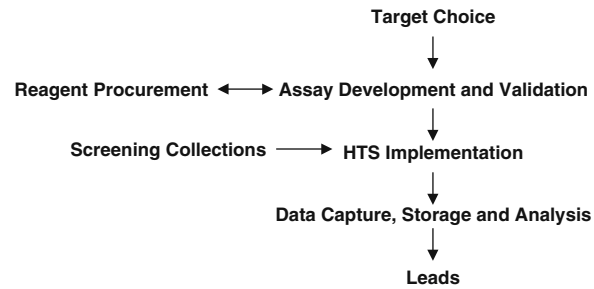
of compounds that may interact with the protein. As such, pharmaceutical companies often rely on HTS as the primary engine driving lead discovery.

The HTS process is a subset of the drug discovery process and can be described as the phase from Target to Lead. This phase can be broken down in the following steps:



It is critically important to align the target choice and assay method to ensure that a biologically relevant and robust screen is configured. The assay must be configured correctly so that compounds with the desired biological effect will be found if they exist in the screening collection. The assay must demonstrate low variability and high signal to background so that false negatives and false positives are minimized. The screen must have sufficient throughput and low cost to enable screening of large compound collections. To meet these requirements, organizations must ensure that communication between therapeutic departments, assay development groups, and screening scientists occurs early – as soon as the target is chosen – and throughout the assay development phase.

Reagent procurement is often a major bottleneck in the HTS process. This can delay the early phases of assay development – e.g., when active protein cannot be obtained – and also delay HTS implementation if scale-up of protein or cells fails to produce sufficient reagent to run the full screen. For efficient HTS operation, there must be sufficient reagent available to run the entire screening campaign before HTS can start. Otherwise, the campaign will need to stop halfway through and the screening robots will have to be reconfigured for other work. Careful scheduling between reagent procurement departments and HTS functions is critical to ensure optimum use of robotics and personnel. Modern HTS laboratories have borrowed concepts from the manufacturing industry to smooth the flow of targets through the hit discovery process (e.g., supply chain management, constrained work-in-progress, and statistical quality control) and these ideas have begun to pay off with higher productivity and shorter lead times.

Successful HTS implementation is multidisciplinary and requires close alignment of computational chemists directing the synthesis or the acquisition of compound collections, sample

management specialists maintaining and distributing screening decks, technology specialists responsible for setting up and supporting HTS automation, biologists and biochemists with knowledge of assay methodology, IT personnel capable of collecting and analyzing large datasets, and medicinal chemists capable of examining screening hits to look for patterns that define lead series. Through the marriage of these diverse specialties, therapeutic targets can be put through the lead discovery engine called HTS and lead compounds will emerge.

2. Choice of Therapeutic Target

There are three major considerations for choosing a therapeutic target destined for HTS: target validity (i.e., disease relevance), chemical tractability, and screenability. Disease relevance is the most important consideration and also the most complex. Since there is an inverse relationship between target novelty and validity, organizations should choose a portfolio of targets, which span the risk spectrum. Some targets will have a high degree of validation but low novelty (fast follower targets) and others will be highly novel but poorly linked to disease. Target validity can be assessed with genetic approaches and/or compound-based experiments. Genetic approaches such as gene knockouts or RNAi can be time-consuming and sometimes lead to false conclusions but can be performed without the need for expensive screening. Compound-based target validation approaches require taking a risk with less-validated targets and spending money to screen for tool compounds, followed by cell-based or in vivo experiments. Both approaches have their advantages and disadvantages, and most organizations use a combination. However, many fail to fully analyze the economics of this equation. Efforts to reduce the cost and increase the success rate of HTS can shift the equation in favor of running screens for targets on the less-validated end of the spectrum.

While disease relevance should be the primary consideration when choosing a target, one should also consider technical factors important to the HTS process. Chemical tractability considerations relate to the probability that drug-like compounds capable of producing the therapeutically relevant effect against a specific target are present in the screening collection and can be found through screening. Years of experience in HTS within the industry have suggested that certain target classes are more chemically tractable than others, including G protein-coupled receptors (GPCRs), ion channels, nuclear hormone receptors, and kinases.

On the other side of the spectrum, targets that work via protein–protein interactions have a lower probability of being successful in HTS campaigns. One reason for this is the fact that compound libraries often do not contain compounds of sufficient

size and complexity to disrupt the large surface of protein–protein interaction that is encountered in these targets. Natural products are one avenue that may be fruitful against protein–protein targets, since these compounds are often larger and more complex than those in traditional chemical libraries. The challenge for these targets is finding compounds that have the desired inhibitory effect and also contain drug-like properties (e.g., are not too large in molecular weight). Recently, several groups have had success with protein–protein interactions by screening for small fragments that weakly inhibit the interaction and building them up to produce moderate-sized potent inhibitors.

Certain subsets of protein–protein interaction targets have been successful from an HTS point of view. For example, chemokine receptors are technically a protein–protein interaction (within the GPCR class) and there are several examples of successful lead compounds for targets in this class *(1)*. Similarly, certain integrin receptors that rely on small epitopes (i.e., RGD sequences) have also been successful at producing lead compounds *(2)*. There may be other classes of tractable protein–protein interactions that remain undiscovered due to limitations in compound libraries.

Based on the thinking that chemically tractable targets are easier to inhibit, most pharmaceutical companies have concentrated much of their effort on these targets and diminished work on more difficult targets. While this approach has some merits, one should be careful not to entirely eliminate target classes that would otherwise be extremely attractive from a biological point of view. Otherwise, the prophecy of chemical tractability will be self-fulfilled, since today's compound collections will not expand into new regions and we will never find leads for more difficult, biologically relevant targets. There is clearly an important need for enhancing collections by filling holes that chemical history has left open. The challenge is filling these holes with drug-like compounds that are different from the traditional pharmacophores of the past. This is critical if we are to increase HTS success rates (proportion of targets which give starting points for medicinal chemistry) from the current 60% *(3,4)* to 80% or higher.

A final factor to consider when choosing targets is screenability – the technical probability of developing a robust and high-quality screening assay. The impact of new assay technologies has made this less important, since there are now many good assay methods available for a wide variety of target types (*see* **Section 3**). Nevertheless, some targets are more technically difficult than others. Of the target types mentioned above, GPCRs, kinases, proteases, nuclear hormone receptors, and protein–protein interactions are often relatively easy to establish as screens. Ion channels are more difficult, although new technologies are being developed, which make these more approachable from an HTS point of view *(5)*. Enzymes other than kinases and proteases must be considered on a case-by-case basis depending on the nature of the substrates involved.

The reductionist approach in which a single target is hypothesized to be important for disease carries some risks. Have you chosen an irrelevant or intractable target? What if a combination of targets is required to elicit the desired biological effect? An alternative approach gaining favor is the use of phenotypic and/or pathway assays for hit discovery. Phenotypic assays, sometimes called "black box" assays, measure a cellular property in response to test compound. Examples include secretion of protein factors, chemotaxis, apoptosis, and cell shape change. Pathway assays are more precise in that protein properties within a cellular pathway are measured. Examples include intracellular protein phosphorylation and cellular trafficking. Often a combination of these approaches can be used to turn a "black box" into a "gray box." An advantage of phenotypic and pathway assays is the fact that multiple targets are screened at once, providing multiple chances for compounds to "find" the most tractable and biologically relevant target(s) in the cell. However, phenotypic assays are more difficult to configure and more expensive to run, and hit deconvolution to define the specific target(s) of your hits is time-consuming and complex. Furthermore, provision of relevant cells is difficult but recent advances in human stem cells are beginning to alleviate this problem.

All of these factors must be considered on a case-by-case basis and should be evaluated at the beginning of a Target-to-Lead effort before making a choice to go forward. Working on an expensive and technically difficult assay must be balanced against the degree of validation and biological relevance. While the perfect target is chemically tractable, technically easy, inexpensive, and biologically relevant, such targets are rare. The goal is to work on a portfolio that spreads the risk among these factors and balances the available resources.

3. Choice of Assay Method

There are usually several ways of looking for hits of any given target. The first and major choice to make is between a biochemical and a cell-based assay. By biochemical we understand an assay developed to look for compounds that interact with an isolated target in an artificial environment. This was the most popular approach in the early 1990 s, the decade in which HTS became a mature and central area of drug discovery. This bias toward biochemical assays for HTS was partly driven by the fact that cell-based assays were often more difficult to run in high throughput. However, advances in technology and instrumentation for cell-based assays that translated to commercial products around the early 2000 s, together with disappointments in the success rates of molecular-based hit discovery campaigns, changed the tilt toward cell-based HTS. Among these advances are the emergence of

HTS-compatible technology to measure G protein-coupled receptor (GPCR) *(6)* and ion channel function *(5)*, confocal imaging platforms for rapid cellular and subcellular imaging, and the continued development of reporter gene technology.

In a recent survey *(3)*, HTS labs reported a 50/50 split between biochemical and cell-based assays in 2006, with a projection to be 60% cell-based, 40% biochemical in 2008.

For most drug discovery programs, both types of assays are required for hit discovery and characterization and subsequent lead optimization. Everything being equal (technical feasibility, cost, and throughput), cell-based assays are often preferred for HTS because compounds tested will be interacting with a more realistic mix of protein target conformations in their physiological milieu, i.e., with the right companions (proteins, metabolites, etc.) at the right concentration. Additionally, cell-based assays tend to avoid some common artifacts in biochemical assays such as aggregators *(7)*. On the other hand, cell-based assays may identify hits that do not act on the target or the pathway of interest and may miss hits of interest that do not penetrate the cell membrane.

If a cell-based assay is chosen for primary screening, a biochemical assay will often be used as a secondary screen to characterize hits and guide lead optimization. A wide variety of assay formats is now available at relatively affordable prices to cope with most needs in the HTS labs. The following sections provide a very succinct summary of some of the most popular choices. A recent comprehensive review by Inglese and coworkers is recommended for further reading *(8)*.

3.1. Biochemical Assay Methods

While laborious separation-based assay formats such as radiofiltration and ELISAs were common in the early 1990 s, most biochemical screens today use simple homogeneous "mix-and-read" formats. This is particularly true for HTS assays run in industrial labs that are conducted on high-density microtiter plates (384 or 1536 wells).

The most common assay readouts used in biochemical assay methods for HTS are optical, including absorbance, fluorescence, luminescence, and scintillation. Among these, fluorescence-based techniques are amongst the most important detection approaches used for HTS *(9)*. Fluorescence techniques give very high sensitivity, which allows assay miniaturization, and are amenable to homogeneous formats. One factor to consider when developing fluorescence assays for screening compound collections is wavelength; in general, short excitation wavelengths (especially those below 400 nm) should be avoided to minimize interference produced by test compounds.

Although fluorescence intensity measurements have been successfully applied in HTS, this format is mostly applied to a narrow range of enzyme targets for which fluorogenic substrates are available. A more widely used fluorescence readout is time-resolved fluorescence resonance energy transfer (TR-FRET) *(10)*. This is a

dual-labeling approach based upon long-range energy transfer between fluorescent Ln^{3+} complexes and a suitable resonance energy acceptor. These approaches give high sensitivity by reducing background and a large number of HTS assays have now been configured using TR-FRET. This technique is highly suited to measurements of protein–protein interactions and has also been tailored to detect important metabolites such as cAMP. Another versatile fluorescence technique is florescence polarization (FP), which can be used to measure bimolecular association events *(10)*. Immobilized metal-ion affinity-based FP (IMAP, Molecular Devices) *(11)* is a variation of FP that can be applied to test activity of kinases and other enzymes.

Radiometric techniques such as scintillation proximity assay (SPA, GE) *(12)* used to be very common in the 1990 s. Despite advances in imaging and bead technology that enabled faster readouts and reduced the occurrence of optical interferences, radiometric assays have several disadvantages including safety and limited reagent stability. In recent years, these techniques have been displaced by fluorescence assay technologies; current estimates from various surveys of HTS laboratories indicate that radiometric assays presently constitute around 5% of all screens performed.

Other technologies able to circumvent technical hurdles for niche difficult assays are amplified luminescence proximity (AlphaScreen, Perkin Elmer) *(13)*, electrochemiluminescence (ECL, Meso Scale Discovery) *(14)*, fluorescence correlation spectroscopy (FCS), and other confocal techniques *(9)*.

Label-free assays are a diverse set of techniques of growing interest and demand. Many of the methods are modern adaptation to the high-throughput environment of well-established technologies such as mass spectroscopy or calorimetry. An overview of the commercial solutions in place and their principles has been recently published by Rich and Myszka *(15)*.

3.2. Cell-Based Assay Methods

As recently as the mid-1990s, most cell-based assay formats were not consistent with HTS requirements. However, as recent technological advances have facilitated higher throughput functional assays, cell-based formats now make up a reasonable proportion of screens performed today. One of the most important advances in cell-based assay methodology is the development of the FLIPR® (MDS Analytical Technologies), a fluorescence imaging plate reader with integrated liquid handling that facilitates the simultaneous fluorescence imaging of 384 samples to measure intracellular calcium mobilization in real time *(6)*. This format is now commonly used for GPCR and ion channel targets. Based on the success of the FLIPR®, several additional cell-based assays for GPCRs were developed. One useful technology uses the photoprotein aequorin to measure intracellular calcium levels. When aequorin binds to calcium, it oxidizes coelenterazine with the

emission of light, which can be easily measured on a suitable plate reader. Another important cell-based assay method involves the measurement of intracellular cAMP levels, which allows the screening of Gi- and Gs-coupled GPCRs. Technologies for cAMP measurement include the older RIA, ELISA, and SPA methods as well as recent techniques such as TR-FRET, amplified luminescence proximity (AlphaScreen®), and enzyme fragment complementation (EFC, HitHunter™), which are less expensive and have higher throughput *(16)*.

Significant advances in ion channel screening have occurred over the past decade *(17)*. Calcium-sensing dyes read on FLIPR® are commonly used to measure channels that conduct calcium, while voltage-sensing dyes are used to track changes in membrane potential. An important advance in high-throughput ion channel assays was the development of FRET-based voltage-sensing dyes, where a pair of molecules exhibit FRET, which is disrupted when the membrane is depolarized. Ion flux assays using nonradioactive tracers analyzed by atomic absorbance spectroscopy (AAS) can now be run in HTS format using recently available instrumentation. And the standard for measuring ion channel activity, patch clamp measurements, has been facilitated by the development of automated instrumentation such as Ionworks Quattro, PatchXpress, and QPatch *(18)*. While these technologies are remarkable, further improvements are necessary before patch clamp measurements can be used for primary HTS.

Cellular phenotypes and pathways are now routinely measured using a variety of techniques amenable to HTS. The reporter gene assay is the oldest and most well-studied method, which allows the discovery of compounds that modulate a pathway resulting in changes in gene expression *(19)*. This method offers certain advantages relative to other cell-based assays, in that it requires fewer cells, is easier to automate, and can be performed in 1536 well plates. Descriptions of miniaturized reporter gene readouts include luciferase – undoubtedly, the most popular reporter gene *(20)* – secreted alkaline phosphate, and beta-lactamase. However, reporter gene assays are of relatively low resolution since they measure effects on an entire pathway at once. Recent advances in cellular imaging have allowed HTS of higher resolution phenomena such as intracellular protein redistribution, GPCR internalization, and other cellular pathway events. Methods using protein complementation assays and bioluminescence resonance energy transfer (BRET) combined with cellular imaging can be very useful *(8)*.

Another cellular phenotype that is commonly measured in HTS is protein secretion. Classical methods to measure protein secretion such as RIA and ELISA are being replaced by improved techniques such as AlphaLISA™ *(21)* and MultiArray® or MultiSpot® electrochemiluminescence-based solutions *(14)*.

3.3. Matching Assay Method to Target Type

Often, one has a choice of assay method for a given target type (**Table 1.1**). To illustrate the various factors that are important when choosing an assay type, let us consider the important GPCR target class. GPCRs can be screened using cell-based assays such as FLIPR, aequorin, and reporter gene or biochemical formats such as SPA and FP. One overriding factor when choosing between functional or binding assays for GPCRs is whether one seeks to find agonists or antagonists. Functional assays are much more amenable to finding agonists than are binding assays, while antagonists can be found with either format. FLIPR assays are relatively easy to develop, but this screening method is more labor-intensive (particularly with respect to cell culture requirements) and more difficult to automate than reporter gene assays. In contrast, the need for longer term incubation time for reporter gene assays (4–6 h vs. min for FLIPR) means that cytotoxic interference by test compounds may be more problematic. On the plus side, reporter gene readouts for GPCRs can sometimes be more sensitive to agonists than FLIPR. Aequorin offers some advantages of FLIPR while being easier to run and less expensive.

Regarding biochemical assays for GPCRs, SPA remains a common format since radiolabeling is often facile and nonperturbing. However, fluorescence assays for GPCRs such as FP and FIDA are becoming more important. Fluorescent labels are more stable, safer, and often more economical than radiolabels. However,

Table 1.1
The most important assay formats for various target types are shown

	Assay formats	
Target type	**Biochemical**	**Cell-based**
GPCRs	SPA, FP, FIDA	FLIPR, reporter gene, aequorin, TR-FRET, AlphaScreen, EFC, cell imaging
Ion channels	SPA, FP	FLIPR, FRET, AAS, automated patch clamp
Nuclear hormone receptor	FP, TR-FRET, SPA, AlphaScreen	Reporter gene, cell imaging
Kinases	FP, TR-FRET, SPA, IMAP	Cellular phosphorylation, cell imaging
Protease	FLINT, FRET, TR-FRET, FP, SPA	Reporter gene, cell imaging
Other enzymes	FLINT, FRET, TR-FRET, FP, SPA, absorbance	
Protein–protein	TR-FRET, FRET. BRET, SPA, ECL, AlphaScreen	BRET, cell imaging, reporter gene

while fluorescent labeling is becoming easier and more predictable, these labels are larger and thus can sometimes perturb the biochemical interaction (in either direction).

These examples illustrate some of the trade-offs one needs to consider when choosing an assay type. In general, one should choose the assay format that is easiest to develop, most predictable, most relevant, and cheapest to run. These factors, however, are not always known in advance. And even worse, they can be at odds with each other and thus must be balanced to arrive at the best option. Additional important quality considerations include compound interference issues and assay variability. It makes little sense to run a cheap and easy assay that is variable or overly sensitive to inhibition. In some cases it makes sense to parallel track two formats during the assay development phase and choose between them based on which is easiest to develop and most facile. Finally, in addition to these scientific considerations, logistical factors such as the number of specific readers or robot types available in the HTS lab and the queue size for these systems must be taken into account.

4. Assay Development and Validation

The final conditions of an HTS assay are chosen following the optimization of quality without compromising throughput, while keeping costs low. The most critical points that must be considered in the design of a high-quality assay are biochemical data and statistical performance. Assay optimization is often required to achieve acceptable HTS performance while keeping assay conditions within the desired range. This usually significantly improves the stability and/or the activity of the biological system studied and has therefore become a key step in the development of screening assays *(22)*.

4.1. Critical Biochemical Parameters in HTS Assays

The success of an HTS campaign in finding hits with the desired profile depends primarily on the presence of such compounds in the collection tested. But it is also largely dependent on the ability of the researcher to engineer the assay in accordance with that profile while reaching an appropriate statistical performance.

A classical example that illustrates the importance of the assay design is how substrate concentration determines the sensitivity for different kinds of enzymatic inhibitors. If we set the concentration of one substrate in a screening assay at 10 times Km, competitive inhibitors of that enzyme–substrate interaction with a Ki greater than one-eleventh of the compound concentration used

in HTS will show less than 50% inhibition and will likely be missed – i.e., competitive inhibitors with a Ki of 0.91 μM or higher would be missed when screening at 10 μM. On the other hand, the same problem will take place for uncompetitive inhibitors if substrate concentration is set at one-tenth of its Km. Therefore, it is important to know what kind of hits are sought in order to make the right choices in substrate concentration; often, one chooses a substrate concentration that facilitates discovery of both competitive and uncompetitive inhibitors.

In this section, we describe the biochemical parameters of an assay that have a greater influence on the sensitivity of finding different classes of hits and some recommendations about where to set them.

4.1.1. Enzymatic Assays

The sensitivity of an enzymatic assay to different types of inhibitors is a function of the ratio of substrate concentration to Km (S/Km).

4.1.1.1. Substrate Concentration

- **Competitive inhibitors:** for reversible inhibitors that bind to a binding site that is the same as one substrate, the more of that substrate present in the assay, the less inhibition observed. The relationship between IC50 (compound concentration required to observe 50% inhibition of enzymatic activity with respect to an uninhibited control) and Ki (inhibition constant) is *(23)*:

$$IC50 = (1 + S/Km)^{*}Ki$$

As shown in **Fig. 1.1**, at S/Km ratios less than 1 the assay is more sensitive to competitive inhibitors, with an asymptotic limit of IC50 = Ki. At high S/Km ratios, the assay becomes less suitable for finding this type of inhibitors.

- **Uncompetitive inhibitors**: if the inhibitor binds to the enzyme–substrate complex or any other intermediate complex but not to the free enzyme, the dependence on S/Km is the opposite to what has been described for competitive binders. The relationship between IC50 and Ki is *(23)*:

$$IC50 = (1 + Km/S)^{*}Ki$$

High substrate concentrations make the assay more sensitive to uncompetitive inhibitors (**Fig. 1.1**).

- **Noncompetitive (allosteric) inhibitors**: if the inhibitor binds with equal affinity to the free enzyme and to the enzyme–substrate complex, the inhibition observed is independent of the substrate concentration. The relationship between IC50 and Ki is *(23)*:

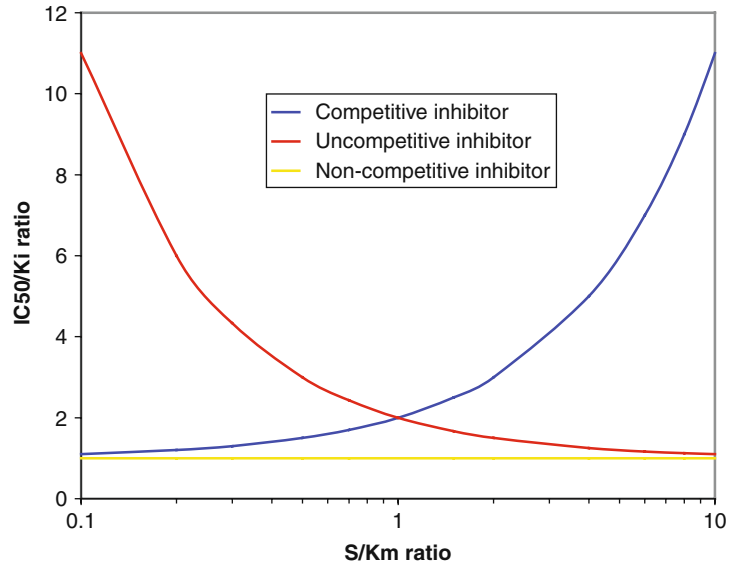


Fig. 1.1. Variation of IC50/Ki ratio with the S/Km ratio for different types of inhibitors. At [S] = Km, IC50 = 2Ki for competitive and uncompetitive inhibitors. For noncompetitive inhibitors IC50 = Ki at all substrate concentrations.

$$\mathrm{IC50} = \mathrm{Ki}$$

- **Mixed inhibitors:** if the inhibitor binds to the free enzyme and to the enzyme–substrate complex with different affinities (Ki1 and Ki2, respectively), the relationship between IC50 and Ki is *(24)*:

$$\mathrm{IC50} = (\mathrm{S} + \mathrm{Km})(\mathrm{Kil} + \mathrm{S}/\mathrm{Ki2})$$

In summary, setting the substrate(s) concentration(s) at the Km value is an optimal way of ensuring that all types of inhibitors exhibiting a Ki close to or below the compound concentration in the assay can be found in an HTS campaign. Nevertheless, if there is a specific interest in favoring or avoiding a certain type of inhibitor, then the S/Km ratio would be chosen considering the information provided above. For instance, many ATP-binding enzymes are tested in the presence of saturating concentrations of ATP to minimize inhibition from compounds that bind to the ATP-binding site.

Quite often the cost of one substrate or the limitations of the technique used to monitor enzymatic activity (**Table 1.2**) may preclude setting the substrate concentration at its ideal point.

As in many other situations found while implementing an HTS assay, the screening scientist must consider all factors involved and look for the optimal solution. For instance, if the sensitivity of a detection technology requires setting $S = 10 \times Km$ to achieve an acceptable signal to background, competitive

Table 1.2
Examples of limitations to substrate concentration imposed by some popular assay technologies. These limitations also apply to ligand in binding assays or other components in assays monitoring any kind of binding event

Assay technology	Limitations
Fluorescence	Inner filter effect at high concentrations of fluorophore (usually >1 μM)
Fluorescence polarization	>30% substrate depletion required
Capture techniques (ELISA, SPA, FlashPlate, BET, others)	Concentrations of the reactant captured must be in alignment with the upper limit of binding capacity
Capture techniques and anyone monitoring binding	Nonspecific binding (NSB) of the product or of any reactant to the capture element (bead, plate, membrane, antibody, etc.) may result in misleading activity determinations
All	Sensitivity limits impose a lower limit to the amount of product detected

inhibitors with a Ki greater than one-eleventh of the compound concentration tested will not likely be identified and will limit the campaign to finding more potent inhibitors. In this case, working at a higher compound concentration would help to find some of the weak inhibitors otherwise missed. If this is not feasible, it is better to lose weak inhibitors while running a statistically robust assay, rather than making the assay more sensitive by lowering substrate concentration to a point of unacceptable signal to background. The latter approach is riskier since a bad statistical performance would jeopardize the discovery of more potent hits (*see* **Section 4.3**).

4.1.1.2. Enzyme Concentration

The accuracy of inhibition values calculated from enzymatic activity in the presence of inhibitors relies on the linear response of activity to the enzyme concentration. Therefore, an enzyme dilution study must be performed in order to determine the linear range of enzymatic activity with respect to enzyme concentration.

As shown in **Fig. 1.2** for valyl-tRNA synthetase, at high enzyme concentrations there is typically a loss of linearity due to substrate depletion, protein aggregation, or limitations in the detection system. If the enzyme is not stable at low concentrations, or if the assay method does not respond linearly to product formation or substrate depletion, there could also be a lack of linearity in the lower end.

In addition, enzyme concentration marks a lower limit to the accurate determination of inhibitor potency. IC50 values lower than one-half of the enzyme concentration cannot be measured;

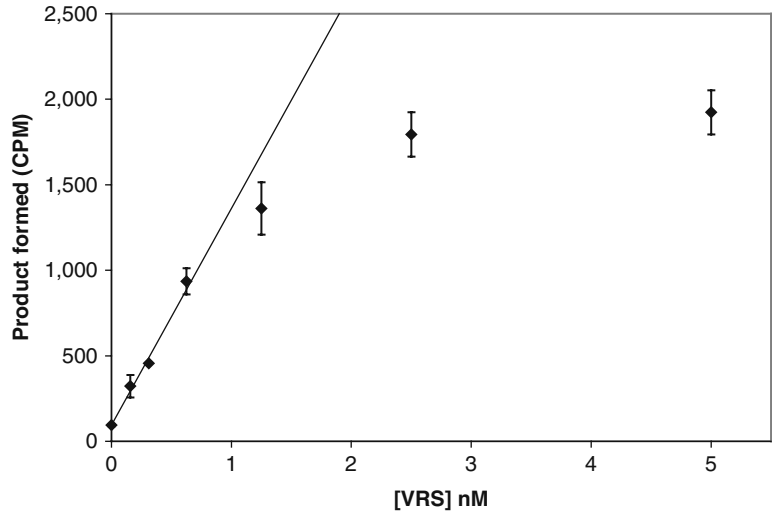


Fig. 1.2. Protein dilution curve for valyl-tRNA synthetase. The activity was measured after 20 min incubation following the SPA procedure described *(25)*.

this effect is often referred to as "bottoming out." As the quality of compound collections improves, this could be a real problem since SAR trends cannot be observed among the more potent hits. Obviously, enzyme concentration must be kept far below the concentration of compounds tested in order to find any inhibitor. In general, compounds are tested at micromolar concentrations (1–100 μM) and as a rule of thumb, it is advisable to work at enzyme concentrations below 100 nM.

On the other hand, the assay can be made insensitive to certain undesired hits (such as inhibitors of enzymes added in coupled systems) by using higher concentrations of these proteins. In any case, the limiting step of a coupled system must be the one of interest, and thus the auxiliary enzymes should always be in excess.

4.1.1.3. Incubation Time and Degree of Substrate Depletion

As described above for enzyme concentration, it is important to assess the linearity vs. time of the reaction analyzed. HTS assays are often end-point and so it is crucial to select an appropriate incubation time. Although linearity vs. enzyme concentration is not achievable if the end-point selected does not lie in the linear range of the progress curves for all enzyme concentrations involved, exceptions to this rule do happen, and so it is important to check it as well.

To determine accurate kinetic constants, it is crucial to measure initial velocities. However, for the determination of acceptable inhibition values, it is sufficient to be close to linearity. Therefore, the classical rule found in Biochemistry textbooks of working at or below 10% substrate depletion [e.g. *(26)*] does not necessarily apply to HTS assays. Provided that all compounds in a

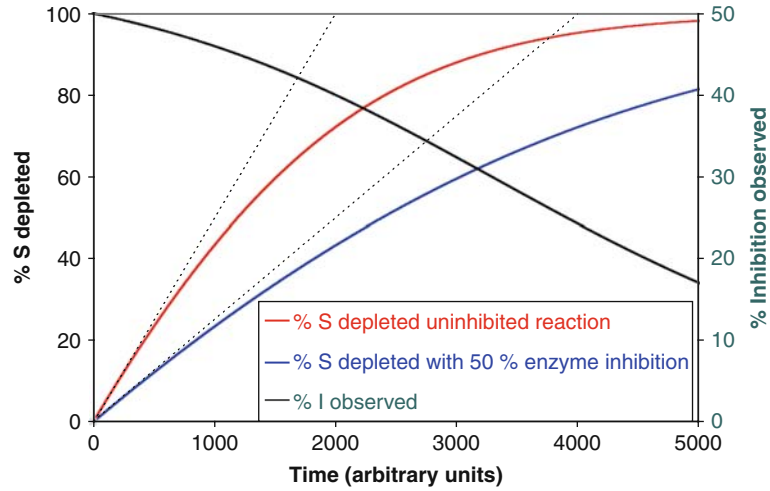


Fig. 1.3. Theoretical progress curves at S = Km of an uninhibited enzymatic reaction and a reaction with an inhibitor at its IC50 concentration. The inhibition values determined at different end-points throughout the progress curve are shown as well. Initial velocities are represented by *dotted lines*.

collection are treated in the same way, if the inhibitions observed are off by a narrow margin, it is not a problem. As shown in **Fig. 1.3**, at 50% substrate depletion with an initial substrate concentration at its Km, the inhibition observed for a 50% real inhibition is 45%, an acceptable error. For higher inhibitions the errors are lower (e.g., instead of 75% inhibition, 71% would be observed). At lower S/Km ratios the errors are slightly higher (e.g., at S = 1/10 Km, a 50% real inhibition would yield an observed 42% inhibition, again at 50% substrate depletion).

This flexibility to work under close-to-linearity but not truly linear reaction rates makes it feasible to use certain assay technologies in HTS – e.g., fluorescence polarization – that require a high proportion of substrate depletion in order to produce a significant change in signal. Secondary assays configured within linear rates should allow a more accurate determination of IC50 s for hits.

In reality, the experimental progress curve for a given enzyme may differ from the theoretical one depicted here for various reasons such as non Michaelis–Menten behavior, reagent deterioration, product inhibition, and detection artifacts. In view of the actual progress curve, practical choices should be made to avoid missing interesting hits.

4.1.1.4. Order of Reagent Addition

The order of addition of reactants and putative inhibitors is important to modulate the sensitivity of an assay for slow binding and irreversible inhibitors.

A preincubation (usually 5–10 min) of enzyme and test compound favors the finding of slow-binding competitive inhibitors. If the substrate is added first, these inhibitors have a lower probability of being found.

In some cases, especially for multisubstrate reactions, the order of addition can be engineered to favor certain uncompetitive inhibitors. For instance, a mimetic of an amino acid that could act as an inhibitor of one aminoacyl-tRNA synthetase will exhibit a much higher inhibition if preincubated with enzyme and ATP before addition of the amino acid substrate.

4.1.2. Binding Assays

Although this section is focused on receptor binding, other binding reactions (protein–protein, protein–nucleic acid, etc.) are governed by similar laws, and so assays to monitor these interactions should follow the guidelines hereby suggested.

4.1.2.1. Ligand Concentration

The equation that describes binding of a ligand to a receptor, developed by Langmuir to describe adsorption of gas films to solid surfaces, is virtually identical to the Michaelis–Menten equation for enzyme kinetics:

$$BL = Bmax^{*}L/(Kd + L)$$

where BL = bound ligand concentration (equivalent to v0), Bmax = maximum binding capacity (equivalent to Vmax), L = total ligand concentration (equivalent to S), and Kd = equilibrium affinity constant also known as dissociation constant (equivalent to Km).

Therefore, all equations disclosed in **Section 4.1.1.1** can be directly translated to ligand-binding assays. For example, for competitive binders

$$IC50 = (1 + L/Kd)^{*}Ki$$

Uncompetitive binders cannot be detected in binding assays; functional assays must be performed to detect this inhibitor class. Allosteric binders could be found if their binding modifies the receptor in a fashion that prevents ligand binding.

Typically, ligand concentration is set at the Kd concentration as an optimal way to attain a good signal (50% of binding sites occupied). This results in a good sensitivity for finding competitive binders.

4.1.2.2. Receptor Concentration

The same principles outlined for enzyme concentration in **Section 4.1.1.2** apply to receptor concentration, or concentration of partners in other binding assays. In most cases, especially with membrane-bound receptors, the nominal concentration of receptor is not known but can be determined by measuring the proportion of bound ligand at the Kd. In any case, linearity of response (binding) with respect to receptor (membrane) concentration should be assessed.

In traditional radiofiltration assays, it was recommended to set the membrane concentration so as to reach at most 10% of ligand bound at the Kd concentration, i.e., the concentration of receptor present should be below one-fifth of Kd *(27)*. Although this is appropriate to get accurate binding constants, it is not absolutely required to find competitive binders in a screening assay. Some formats (FP, SPA in certain cases) require a higher proportion of ligand bound to achieve acceptable statistics, and receptor concentrations close or above the Kd value have to be used.

Another variable to be considered in ligand-binding assays is nonspecific binding (NSB) of the labeled ligand. NSB increases linearly with membrane concentration. High NSB leads to unacceptable assay statistics, but this can often be improved with various buffer additives (*see* **Section 4.2**).

4.1.2.3. Preincubation and Equilibrium

As discussed for enzymatic reactions, a preincubation of test compounds with the receptor would favor slow binders. After the preincubation step, the ligand is added and the binding reaction should be allowed to reach equilibrium in order to ensure a proper calculation of displacement by putative inhibitors. Running binding assays at equilibrium is convenient for HTS assays, since one does not have to carefully control the time between addition of ligand and assay readout as long as the equilibrium is stable.

4.1.3. Cell-Based Assays

The focus of the previous sections has been on cell-free systems. Cell-based assays offer different challenges in their setup with many built-in factors that are out of the scientist's control. Nevertheless, some of the points discussed above apply to them, *mutatis mutandi*.

One of the most important considerations is cell type. The most physiologically relevant cells are primary human cells, but these are very difficult and expensive to procure. Recent advances in stem cell science are beginning to facilitate the provision of cells for HTS that are closer to the primary human cell. However, recombinant cells remain the most commonly used cell type for HTS.

Important considerations when developing cell-based assays include the following *(22, 28)*:

- Cell culture details should be well documented and reproducible. Most problems with cell-based assays can be traced to problems with the cells.
- Consider using cryopreserved cells as an assay source to reduce variability and improve screening scheduling logistics.
- Adherent cells or suspension cells can be used, and the choice is based on the cell type and the assay readout method. In general, try to mimic the physiological conditions as much as possible while considering assay logistics.

- Either stable cell lines or transient transfection can be used. Expression levels of the recombinant protein(s) should be confirmed. Extremely high expression levels should generally be avoided.
- Consider using modified baculovirus (BacMam virus) gene delivery technology for transient expression of target proteins in mammalian cells *(29)*.
- When using stable cell lines, use early passages to avoid cells losing their responsiveness.
- Lower numbers of cells are preferred for cost reasons, but at least 1000 cells per well should generally be used to minimize stochastic single-cell events. The response observed should be linear with respect to the number of cells.
- Pay attention to cell clumps which can cause variability.
- Preincubation of cells with compounds should be considered when applicable (e.g., assays in which a ligand is added).
- Optimal incubation time should be selected in accordance with the rule of avoiding underestimation of inhibition or activation values (*see* **Section 4.1.1.3**). All other factors being equal, shorter incubation times minimize cytotoxic interference problems.
- Cell-based assays tend to be more sensitive to DMSO than biochemical assays. Determine the DMSO sensitivity of the assay and configure the protocol to remain well below this level.
- Use standard inhibitors and/or activators during the screening run to confirm the desired signal is observed.
- Pay attention to edge effects, which occur commonly in cell-based assays due to problems with incubators or uneven cell distribution of cells in the well. Incubating seeded plates at room temperature before placing them in the incubator can help this problem *(30)*.

4.2. Assay Optimization

In vitro assays are performed in artificial environments in which the biological system studied could be unstable or exhibiting an activity below its potential. The requirements for stability are higher in HTS campaigns than in other areas of research. In HTS runs, diluted solutions of reagents are used throughout long periods of time (typically 4–12 h) and there is a need to keep both the variability low and the signal to background high. Additionally, several hundreds of thousands of samples are usually tested, and economics often dictates one to reduce the amount of reagents required. In this respect, miniaturization of assay volumes has been in continuous evolution, from tubes to 96-well plates to 384-well plates to 1536 and beyond. Many times, converting assays from

low-density to high-density formats is not straightforward. Thus, in order to find the best possible conditions for evaluating an HTS target, optimization of the assay should be accomplished as part of the development phase.

HTS libraries contain synthetic or natural compounds that in most cases are dissolved in DMSO. The tolerance of the assay to DMSO must be considered. Typically, compounds are stored at concentrations ranging between 1 and 30 mM. Test compound concentrations in primary screening are in the 1–30 μM range. Therefore, DMSO concentrations from 0 to 10% are tested. It is critical to work at DMSO concentrations in a region of minimal variation, as otherwise compound effects can be obscured by variability in the addition of compound stocks (typically the smallest volume in the assay mix and thus the most sensitive liquid handling step).

If significant decrease in activity/binding is observed at the standard solvent concentration – typically 0.5–1% (v/v) DMSO – lower test compound concentrations may be required. In some cases the detrimental effect of solvent can be circumvented by optimizing assay conditions. In all cases, key biochemical parameters (e.g., Km) should be checked in the final assay conditions (DMSO concentration) before starting the screening campaign.

The stability of reagents should be tested using the same conditions intended for HTS runs, including solvent concentration, stock concentration of reagents, reservoirs, and plates. Quite often signal is lost with time not because of degradation of one biological partner in the reaction but because of its adsorption to the plastics used (reservoir, tips, or plates) (**Fig. 1.4**). Addition of detergents below their critical micellar concentration (CMC) and/or carrier proteins (e.g., BSA) is a common technique to minimize this undesirable phenomenon. These assay components can also aid in reducing nonspecific enzymatic inhibition caused by the aggregation of test compounds *(7)*.

The number of factors that can be tested in an optimization process is immense. Nevertheless, initial knowledge of the system (optimal pH, metal requirements, sensitivity to oxidation, etc.) can help to select the most appropriate ones. Factors to be considered can be grouped as follows:

- Buffer composition
- pH
- Temperature
- Ionic strength
- Osmolarity
- Monovalent ions ($Na^+, K^+, C1^-$)
- Divalent cations ($Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}$)
- Rheological modulators (glycerol, polyethylene glycol)

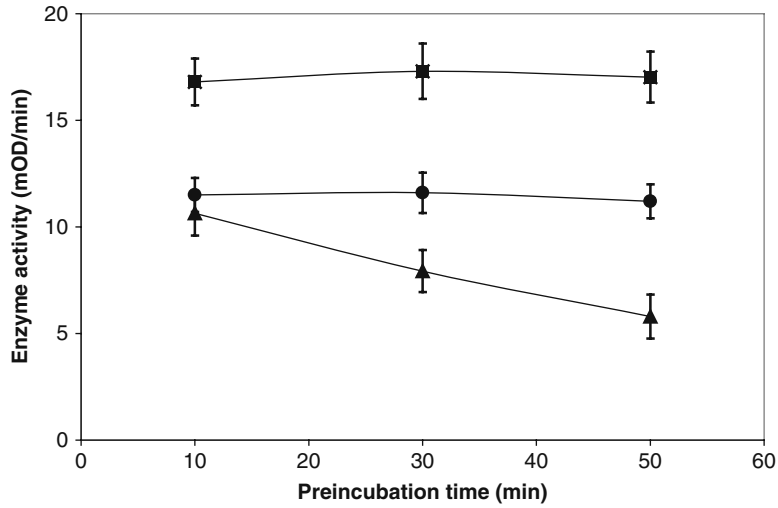


Fig. 1.4. Example of loss of signal in an enzymatic reaction related with adsorption of enzyme (or substrate) to plasticware. The data are from a real assay performed in our lab. Stability of reagents was initially measured using polypropylene tubes and 384-well polystyrene plates, without CHAPS (*circles*). Once HTS was started, using polypropylene reservoirs and polystyrene 384-well plates (*triangles*), a clear loss of signal was observed. Addition of 0.01% (w/v) CHAPS not only solved the problem but also improved the enzyme activity (*squares*). Reactions were initiated at 10, 30, and 50 min after preparation of diluted stocks of reagents that remained at 4°C before addition to the reaction wells.

- Polycations (heparin, dextran)
- Carrier proteins (BSA, casein)
- Chelating agents (EDTA, EGTA)
- Blocking agents (PEI, milk powder)
- Reducing agents (DTT, β-mercaptoethanol)
- Protease inhibitors (PMSF, leupeptin)
- Detergents (Triton, Tween, CHAPS).

Cell-based assays are usually conducted in cell media of complex formulation. Factors to be considered in this case are mainly medium, supplier, selection and concentration of extra protein (human serum albumin, BSA, gelatin, and collagen). One also needs to take into account cell density, plate type, plate coatings, incubation time, temperature, and atmosphere. Since cell-based assays generally have more variables than biochemical assays, extreme care must be taken when documenting and reproducing the cell culture and assay conditions.

Besides analyzing the effect of factors individually, it is important to consider interactions between factors because synergies and antagonisms can commonly occur *(31)*. Full-factorial or partial-factorial designs can be planned using several available statistical

packages (e.g. JMP, Statistica, Design Expert). Experimental designs result in quite complex combinations as soon as more than four factors are tested. This task becomes rather complicated in high-density formats when taking into consideration that more reliable data are obtained if tests are performed randomly. Therefore, an automated solution is necessary because manually running an experiment of this complexity would be extremely difficult. Several commercial packages exist that integrate design of experiments and necessary liquid handling steps to conduct the experiments. A good example is AAO (automated assay optimization) developed by Beckman Coulter (Fullerton, CA) in collaboration with scientists from GlaxoSmithKline *(32)*. An example of the outcome of one assay improved in our lab using this methodology is shown in **Fig. 1.5**. The paper by Taylor et al. *(32)* describes examples of assay optimization through AAO for several types of targets and assay formats.

A typical optimization process starts with a partial-factorial design including many factors (~20). The most promising factors are then tested in a full-factorial experiment to analyze not only main effects but also two-factor interactions. These experiments are done with two levels per factor (very often one level is the absence of the ingredient and the other is the presence at a fairly typical concentration). Finally, titrations of the more beneficial factors are conducted in order to find optimal concentrations of every component.

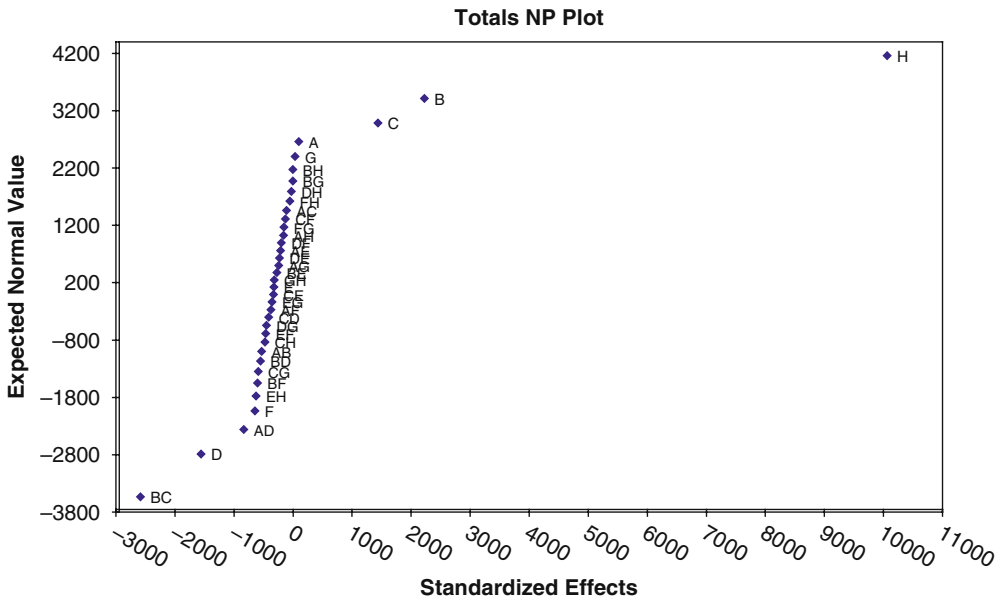
Usually the focus of optimization is on activity (signal or signal to background), but statistical performance should also be taken into account when doing assay optimization. Though this is not feasible when many factors and levels are scrutinized without replicates, whenever possible duplicates or triplicates should be run and the resulting variability measured for every condition. Some buffer ingredients make a reproducible dispensement very difficult, and so should be used only if they are really beneficial (e.g., glycerol).

For some factors it is critical to run the HTS assay close to physiological conditions (e.g., pH) in order to avoid missing interesting leads for which the chemical structure or interaction with the target may change as a function of that factor.

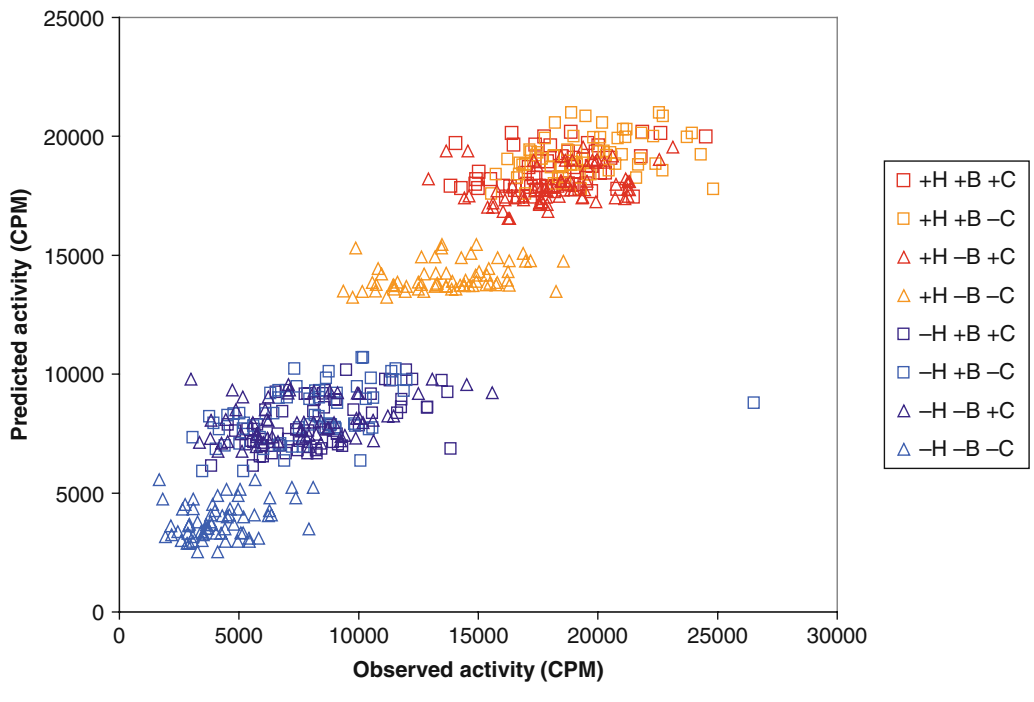
4.3. Statistical Evaluation of HTS Assay Quality

The quality of an HTS assay must be determined according to its primary goal, i.e., to distinguish accurately hits from nonhits in a vast collection of samples.

In the initial evaluation of assay performance, several plates are filled with positive controls (signal; e.g., uninhibited enzyme reaction) and negative controls or blanks (background; e.g., substrate without enzyme). Choosing the right blank is sometimes not so obvious. In ligand–receptor-binding assays, the blanks referred to as NSB controls are prepared traditionally by adding an excess of



A



B

Fig. 1.5. Example of optimization of a radiofiltration assay using Beckman Coulter's AAO program and a Biomek 2000 to perform the liquid handling. The target was to increase activity of this enzyme, bacterial biotin-ligase, aiming to improve assay quality and reduce costs. The initial partial-factorial test included 20 factors, 8 of which were identified as positive. The test shown in this figure used these eight factors and was designed as a two-level full-factorial experiment with duplicates. Five hundred and twelve samples were generated. (**A**) The probability plot resulting from the statistical analysis of experimental data showed three factors being positive (H, B and C) although the interaction of B and C was

unlabelled (cold) ligand; the resulting displacement could be unreachable for some specific competitors that would not prevent nonspecific binding of the labeled ligand to membranes or labware. A better blank could be prepared with membranes from the same cell line not expressing the receptor targeted. Though this is not always practical in the HTS context, it should be at least tested in the development of the assay and compared with the NSB controls to which they should be ideally pretty close.

A careful analysis of these control plates allows identifying errors in liquid handling or sample processing. For instance, an assay with a long incubation typically produces plates with edge effects due to faster evaporation of the external wells even if lids are used, unless the plates are placed in a chamber with humidity control. Analysis of patterns (per row, per column, per quadrant) helps to identify systematic liquid-handling errors.

Obvious problems must be solved before evaluating the quality of the assay. After troubleshooting, random errors are still expected to happen due to instrument failure or defects in the labware used. They should be included in the subsequent analysis of performance (removing outliers is a misleading temptation equivalent to hiding the dirt under the rug).

The analysis of performance can be accomplished by several means. Graphical analysis helps to identify systematic errors (e.g., **Fig. 1.6**). The statistical analysis of raw data involves the calculations of a number of parameters, starting with mean (M) and standard deviations (SD) for signal and background, and combinations of these are as follows:

- **Signal to background**

$$S/B = M_{signal}/M_{background}$$

S/B provides an indication of the separation of positive and negative controls. It can be useful in early assay development to understand the potential of an assay format or to validate reagents under development. But it is a poor indicator of assay quality as it is independent of variability *(33)*.

- **Coefficient of variation of signal and background**

$$CV = 100^{*}SD/M\,(\%)$$

A relative measure of variability provides a good indication of variability. Variability is a function of the assay stability and the precision of liquid handling and detection instruments.

Fig. 1.5. (continued) negative. D showed significant negative effect, while the other four factors had statistically marginal or no effect. (**B**) Applying the statistical model, the correlation between observed and predicted values was very good. The presence of H = CHAPS 0.03% (w/v) (+H, –H) is clearly positive. In the absence of B = 125 mM Bicine (+B *squares*, –B *triangles*) and C = 125 mM TAPS (+C, –C), the enzyme was less active. The original conditions yielded ~5,000 CPM vs. ~25,000 CPM with the optimized buffer (backgrounds were ~100 CPM in all cases).

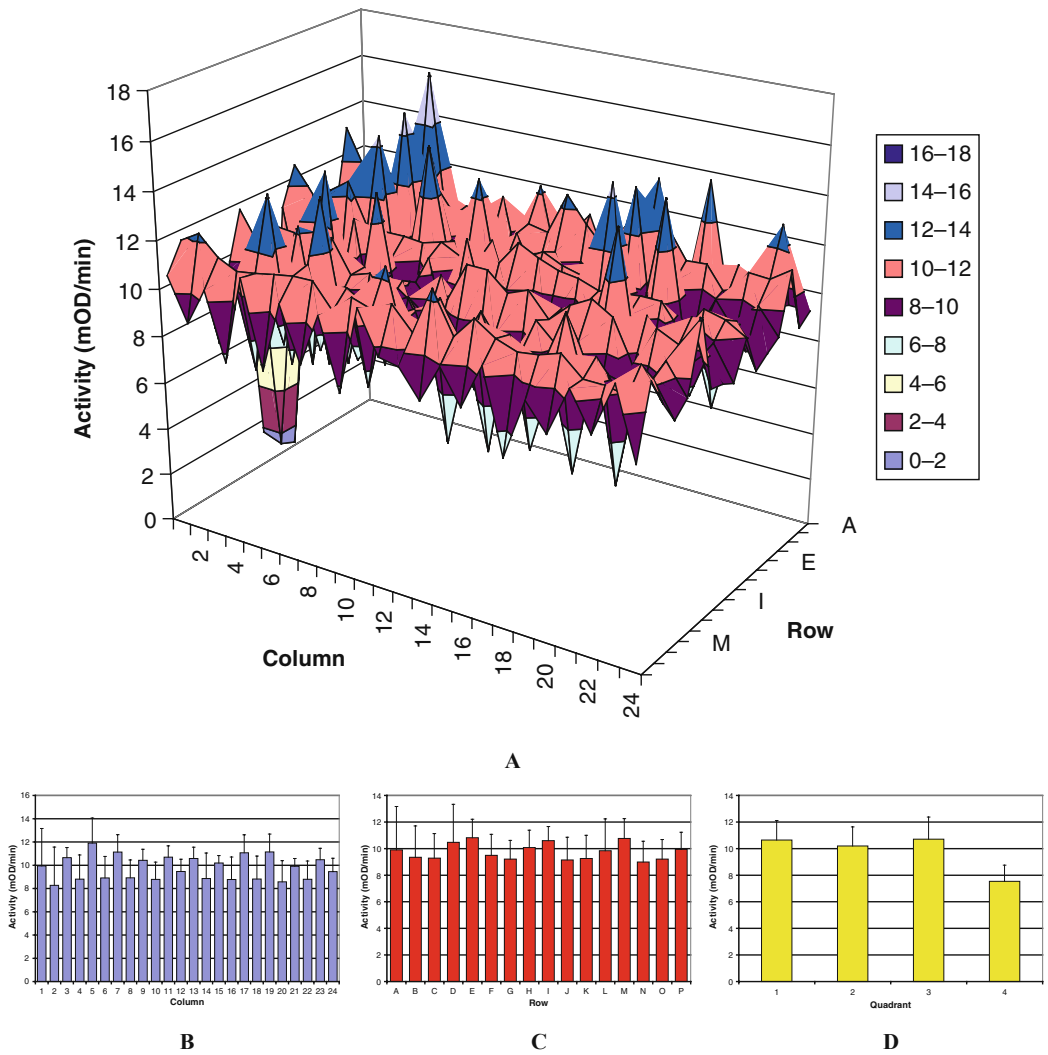


Fig. 1.6. Graphical analysis of a 384-well plate of positive controls of an enzymatic reaction monitored by absorbance (continuous readout). The plate was filled using a pipettor equipped with a 96-well head and indexing capability. (**A**) 3-D plot of the whole plate showing that four wells (I1, I2, J1, and J2) had a dispensement problem. The corresponding tip may have been loose or clogged. Analysis by columns (**B**), rows (**C**), and quadrants (**D**) reveals that the fourth quadrant was receiving less reagent.

- **Signal to noise**

$$S = (M_{signal} - M_{background})/SD_{background}$$

This classic expression of S/N provides an incomplete combination of signal window and variability. Its original purpose was to assess the separation between signal and background in a radio signal *(33)*. It should not be used to evaluate performance of HTS assays.

Another parameter referred to as S/N by some authors is

$$S/N = (M_{signal} - M_{background})\sqrt{(SD_{signal})^2 + (SD_{background})^2}$$

This second expression provides a complete picture of the performance of an HTS assay but as discussed below, the field has converged in using Z′ as the standard measure of HTS assay quality.

- **Z′ factor**

$$Z' = 1 - 3^*(SD_{signal} + SD_{background})/|M_{signal} - M_{background}|$$

Since its publication in 1999 *(33)*, the Z′ factor has been widely accepted by the HTS community as a very useful way of assessing the statistical performance of an assay *(34)*. Z′ is an elegant combination of signal window and variability, the main parameters used in the evaluation of assay quality. The relationship between Z′ factor and S/B is not obvious from its definition but can be easily derived as

$$Z' = 1 - 0.03^*(|S/B|^*CV_{Signal} + CV_{Background})(|S/B| - 1)$$

The value of Z′ factor is a relative indication of the separation of the signal and background populations. It is assumed that there is a normal distribution for these populations, as it is the case if the variability is due to random errors.

Z′ factor is a dimensionless parameter that ranges from 1 (infinite separation) to <0. Signal and background populations start to overlap when $Z' = 0$. In our lab, the minimal acceptable value for an assay is $Z' > 0.4$, although in practice the majority of our assays demonstrate $Z' > 0.6$. A Z′ of 0.4 is equivalent to having an S/B of 3 and a CV of 10%. Low variability allows for a lower S/B, but a minimum of 2 is usually required, provided that CVs are rarely below 5%. **Figure 1.7** shows Z′ at work in three different scenarios. Full analysis of the corresponding data is collected in **Table 1.3**.

Z′ should be evaluated during assay development and validation, and also throughout HTS campaigns on a per plate basis to assess the quality of dispensement and reject data from plates with errors. **Chapter 5** describes in more detail the different tools used to assess statistical performance in HTS campaigns.

4.4. Assay Validation

Once an assay optimized to find compounds of interest passes its quality control with a Z′ greater than 0.6 (or whatever is the applied acceptance criteria), a final step must be done before starting an HTS campaign. The step referred to here as assay validation consists of testing a representative sample of the screening collection in the same way HTS plates will be treated; i.e., on the same robotic system using protocols identical to the HTS run. The purposes of this study are to

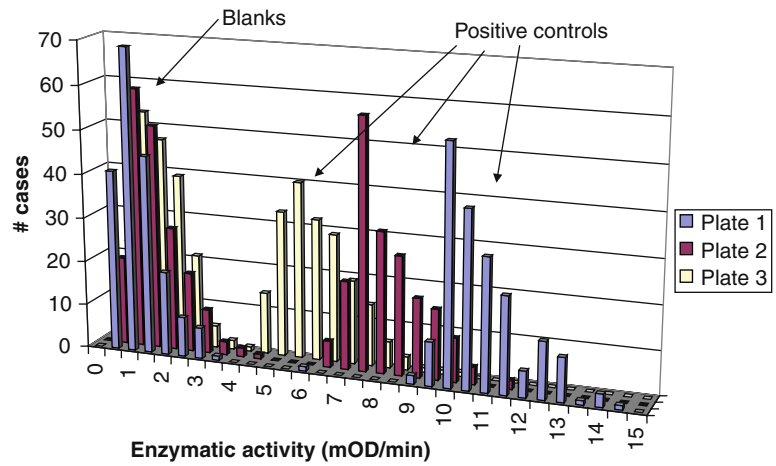


Fig. 1.7. Distribution of activity values (bins of 0.5 mOD/min) for three 384-well plates half-filled with blanks and half-filled with positive controls of an enzymatic reaction monitored by absorbance (continuous readout). Z′ factors were 0.59 for plate 1, 0.42 for plate 2, and 0.10 for plate 3. A complete analysis of performance is shown in **Table 1.3**.

Table 1.3
Statistical analysis of data from the three plates described in Fig. 1.7

Parameter	Plate 1	Plate 2	Plate 3
M_{signal} (mOD/min)	10.09	7.77	5.84
SD_{signal} (mOD/min)	0.84	0.81	0.96
M_{bckg} (mOD/min)	0.30	0.69	0.74
SD_{bckg} (mOD/min)	0.51	0.57	0.57
S/B	34	11	8
SW (mOD/min)	9.80	7.08	5.09
S/N[1]	19	12	9
S/N[2]	10.0	7.1	4.6
CV_{signal} (%)	8%	10%	16%
CV_{bckg} (%)	173%	82%	77%
Z′ factor	0.59	0.42	0.10

[1] $S/N = (M_{signal} - M_{background})/SD_{background}$

[2] $S/N = (M_{signal} - M_{background})/\sqrt{(SD_{signal})^2 + (SD_{background})^2}$

- obtain production data on assay performance;
- assess interferences from screening samples;
- evaluate the reproducibility of results obtained in a production environment;
- estimate the hit rate and determine optimal sample concentration.

A dramatic example of how the test of a pilot collection helps to detect interferences is shown in **Fig. 1.8**. This target, HCV RNA-dependent RNA polymerase, has been found to be

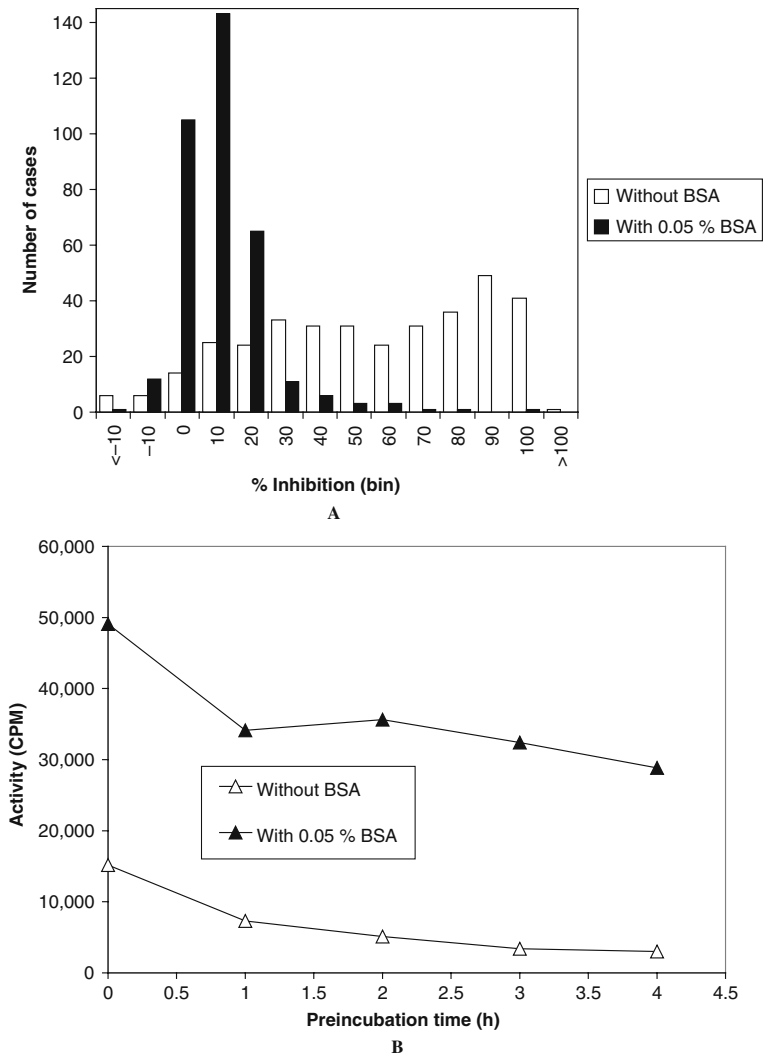


Fig. 1.8. (**A**) Distribution of inhibition values (10% bins) in the validation of an HTS assay of HCV RNA-dependent RNA polymerase tested with and without 0.05% (w/v) BSA. The samples were 352 representative mixtures of compounds (11 components at 9.1 μM each). (**B**) It was shown that the stability and activity of the enzyme was greatly improved in the presence of BSA.

slightly unstable at room temperature (**Fig. 1.8B**, without BSA). Nevertheless, the effect of 352 mixtures of 11 compounds each was tested and an extremely high hit rate was observed (45% of the mixtures inhibited the enzyme activity

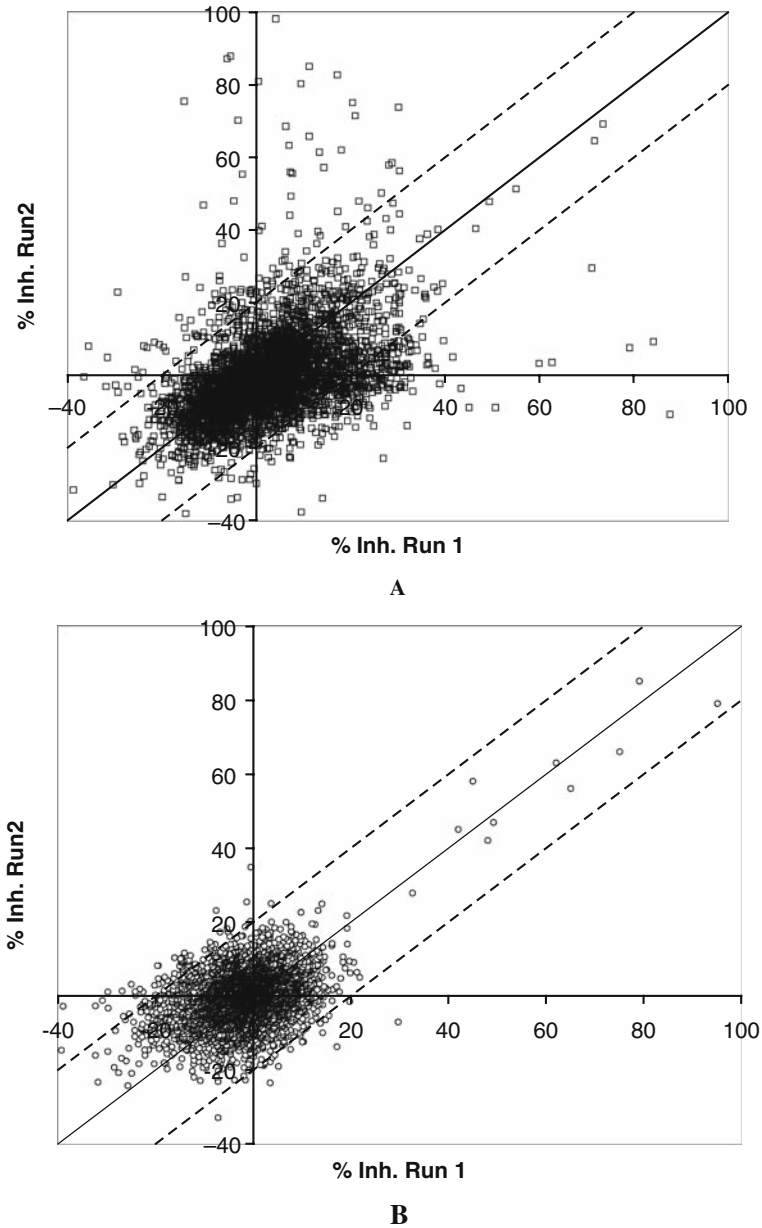


Fig. 1.9. Comparison of duplicates from validation for two HTS assays. (**A**) This enzymatic assay showed a significant number of mismatched results between duplicate runs of the same 4,000 samples. Two actions should be taken in a case like this: liquid handling errors have to be avoided and the assay quality must be improved. (**B**) The data correspond to a ligand-binding assay that showed good reproducibility.

greater than 70%). The problem was solved by stabilization of the system using BSA 0.05%. Similar effects have been observed in several other targets.

It is, therefore, advisable to run a few plates with 500–2,000 random samples just to spot any major interference as soon as possible. The size of the pilot collection can be as small as 1% of the total collection. Its usefulness to predict hit rates and interferences increases with its size. On the other hand, too many plates worth of work and reagents can be lost if any major problem is found in this step, as often happens. Therefore, it is not advisable to go beyond a 5% representation of the collection.

With a randomized sample of 1% of a collection of 50,000 compounds, a hit rate of 1% can be estimated with an SD of 0.5%. For a 5% rate, the estimation's SD would be 1% [approximate figures calculated as described in *(35)*].

Irrespective of the size of the pilot collection, at least 10–20 plates should be run to test the HTS system in real action. Duplicates of the same samples run in independent experiments provide a way to evaluate the reproducibility of results (**Fig. 1.9**). In a duplicated experiment without further retest, false negatives and false positives will be indistinguishable and will all appear as discrepant results. A third replica allows an estimation of the rates of false positives and negatives; additionally hit rate and confirmation rate after retest can be estimated providing the level of information required to assess the quality of the assay and achieve the level of performance required prior to initiation of the HTS efforts.

Acknowledgments

The authors are grateful to the many colleagues at GlaxoSmithKline, which helped over the years to shape the screening process and to build the collective knowledge succinctly described in this introduction.

Abbreviations

AAO:	automated assay optimization
AAS:	atomic absorbance spectroscopy
BRET:	bioluminescence resonance energy transfer
Bicine:	N,N-bis(2-Hydroxyethyl)glycine

Bmax:	maximum binding capacity
BSA:	bovine serum albumin
CHAPS:	3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate
CV:	coefficient of variation
DMSO:	dimethyl sulfoxide
DTT:	dithiothreitol
ECL:	electrochemiluminescence
EDTA:	ethylenediamine-N,N,N′,N′-tetraacetic acid
EFC:	enzyme fragment complementation
EGTA:	ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetra-acetic acid
ELISA:	Enzyme-linked immunosorbent assay
FCS:	fluorescence correlation spectroscopy
FIDA:	fluorescence intensity distribution analysis
FLINT:	fluorescence intensity
FRET:	fluorescence resonance energy transfer
FP:	fluorescence polarization
GPCR:	G protein-coupled receptor
HTS:	high-throughput screening
Kd:	dissociation constant
L:	ligand
M:	mean
NSB:	non specific binding
OD:	optical density unit
PEI:	polyethylene imine
PMSF:	phenylmethylsulfonyl fluoride
RIA:	Radioimmunoassay
S/B:	signal to background ratio
S/N:	signal to noise ratio
SD:	standard deviation
SW:	signal window
SPA:	scintillation proximity assay
TAPS:	N-*tris* (Hydroxymethyl)methyl-3-aminopropane sulfonic acid
TR-FRET:	time-resolved fluorescence resonance energy transfer
Vmax:	maximum velocity

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