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Design and Implementation of High Throughput Screening Assays

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1. Introduction

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 study 100 targets or more each year, and in order to progress these targets, lead compounds must be found. Increasingly, pharmaceutical companies are relying 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:

Target Choice
⇓
Reagent Procurement ⇔ Assay Development and Validation
⇓
Screening Collections ⇒HTS Implementation
⇓
Data Capture, Storage and Analysis
⇓
Leads

It is critically important to align the target choice and assay method to ensure that a biologically relevant and robust screen is configured. Every screening

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laboratory can relate stories of assays being delivered that are incompatible with modern robotic screening instruments and unacceptable in terms of signal to background or variability. To avoid this problem, 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 production 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. To improve scheduling, modern HTS laboratories are moving toward a supply-chain model similar to that used in industrial factories.

Successful HTS implementation is multidisciplinary and requires close alignment of personnel maintaining and distributing screening collections, technology specialists responsible for setting up and supporting HTS automation, biologists and biochemists with knowledge of assay methodology, information technology (IT) personnel capable of collecting and analyzing large data sets, and 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

While disease relevance should be the main driver when choosing a therapeutic target, one should also consider factors important to the HTS process. These factors are technical, i.e. whether a statistically robust and sufficiently simple assay can be configured, as well as chemical. Chemical considerations relate to the probability that compounds capable of producing the therapeutically relevant effect against a specific target are: 1) present in the screening collection, 2) can be found through screening, and 3) have drug-like physicochemical properties.

Years of experience in HTS within the industry have suggested that certain targets are more ‘chemically tractable’ than others. Recent studies of top-selling prescription drugs have shown that G-protein coupled receptors (GPCRs), ion channels, nuclear hormone receptors and proteases are among the most exploit-

able target classes, i.e., drugs against these targets produce the highest sales. Among these targets, GPCRs are normally thought of as the most chemically tractable, since there are more GPCR drugs on the market than drugs for any other target class. Furthermore, evidence indicates that HTS campaigns against GPCRs produce lead compounds at a higher rate than many other target classes ***(1)***. Kinases are another chemically tractable class that often affords lead compounds from screening (*see* Chapter 4); however, while many kinase inhibitors are in clinical trials, none have yet reached the market.

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 (*see* Chapter 9). 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 begun to tackle this problem by screening for small fragments that inhibit the interaction and joining them together to produce moderate-sized potent inhibitors.

Certain subsets of protein-protein interaction targets have been successful from an HTS point of view. For example, chemokines 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 ***(2)***. Similarly, certain integrin receptors that rely on small epitopes (i.e., RGD sequences) have also been successful at producing lead compounds ***(3)***. 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 makes sense from a cost-vs-benefit point of view, one should be careful not to eliminate entirely 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.

A second and equally important factor to consider when choosing targets is 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* **Subheading 3.**). Nevertheless, some targets are more technically difficult than others. Of the target types mentioned earlier, GPCRs, kinases, proteases, nuclear hormone receptors, and protein-protein interactions are often relatively easy to establish screens for. Ion channels are more difficult, although new technologies are being developed that make these more approachable from an HTS point of view ***(4)***. Enzymes other than kinases and proteases must be considered on a case-by-case basis depending on the nature of the substrates involved.

Reagent procurement is also a factor to consider, obtaining sufficient reagents for the screening campaign can sometimes be time-consuming, expensive, and unpredictable. In the case of protein target, this depends on the ease with which the particular protein(s) can be expressed and purified; the amount of protein needed per screening test; and the commercial cost of any substrates, ligands, or consumables.

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 target must be balanced against the degree of validation and biological relevance. While the perfect target is chemically tractable, technically easy, inexpensive, fully validated, 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 or a cell-based assay (*see* Chapter 6). By biochemical we understand an assay developed to look for compounds that interact with an isolated target in an artificial environment. This has been the most popular approach in the early 1990s, the decade in which HTS became a mature and central area of drug discovery. This bias toward biochemical assays for HTS is partly driven by the fact that cell-based assays are often more difficult to run in high throughput. However, recent advances in technology and instrumentation for cell-based assays have occurred over the past few years. Among these is the emergence of HTS-compatible technology to measure GPCR ***(5)*** and ion channel function ***(4)***, confocal imaging platforms for rapid cellular and subcellular imaging, and the continued development of reporter-gene technology.

3.1. Biochemical Assay Methods

While laborious separation-based assay formats such as radiofiltration and enzyme-linked immunosorbent assays (ELISAs) were common in the early 1990s, most biochemical screens in use today use simple homogeneous "mix-and-read" formats (Chapter 3 provides more details). These technologies—including scintillation proximity assay (SPA), fluorescence intensity (FLINT), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), time-resolved energy transfer (TRET) and others—are now the workhorses of the modern HTS laboratory ***(6)***.

The most common assay readouts used in biochemical assay methods for HTS are optical, including scintillation, fluorescence, absorbance, and luminescence. Among these, fluorescence-based techniques are among the most important detection approaches used for HTS. 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 versatile fluorescence technique is FP, which can be used to measure bimolecular association events ***(7)***. Many examples of HTS applications of FP have now been reported, including ligand-receptor binding and enzyme assays in 1536-well plates. Another important fluorescence readout is TRET ***(7)***. This is a dual-labeling approach that is based on 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 TRET. This technique is highly suited to measurements of protein-protein interactions.

One area of fluorescence spectroscopy that is just starting to be applied to HTS is that of single-molecule fluctuation-based measurements. These methods are performed using confocal optics in which the observation volume is extremely small (~ 1 fL). The classical form of confocal fluctuation spectroscopy, known as fluorescence correlation spectroscopy (FCS), has now been demonstrated to be a viable approach to HTS ***(7,8)***. Fluorescence intensity distribution analysis (FIDA), a related method for analyzing fluctuation data that may be more versatile than FCS, involves the measurement of molecular brightness within a confocal observation volume ***(8)***.

While fluorescence assay technologies are growing in importance, current estimates from various surveys of HTS laboratories indicate that radiometric assays presently constitute between 20 and 50% of all screens performed. Important radiometric techniques include scintillation proximity techniques such as SPA/Leadseeker™ (Amersham Pharmacia Biotech, Cardiff, Wales) and FlashPlates™ (NEN Life Science Products, Boston, MA). These techniques have been used for a wide variety of applications including kinases, nucleic acid-processing enzymes, ligand-receptor interactions, and detection of cAMP levels ***(6)***. Of course, radiometric assays have several disadvantages including safety, limited reagent stability, relatively long read-times, and little intrinsic information on the isotope environment. However, imaging plate readers are now emerging to address the issue of read-time and assay miniaturization.

3.2. Cell-based Assay Methods (see also Chapter 6)

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. The FLIPR™ (Molecular Devices, Sunnyvale, CA) is 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 ***(5)***. This format is now commonly used for GPCR and ion channel targets. Another promising technology for ion channels is based on voltage-sensitive fluorescence resonance energy transfer (VIPR™; Aurora Biosciences, La Jolla, CA) ***(4)***.

The reporter gene assay is another common cell-based format amenable to HTS. This method offers certain advantages over FLIPR™ and VIPR™, in that it requires fewer cells, is easier to automate and can be performed in 1536-well plates. Recent descriptions of miniaturized reporter gene readouts include luciferase, secreted alkaline phosphate, and beta-lactamase. Another cell-based screening format based on cell darkening in frog melanophores has been applied to screening for GPCR and other receptor targets ***(6)***.

Recently, imaging systems have been developed that quantify cellular and subcellular fluorescence in whole cells. These systems have the capability of bringing detailed assays with high information content into the world of HTS. One of the most advanced systems is the ArrayScan™ (Cellomics, Pittsburgh, PA), which has been used to measure GPCR internalization as well as a range of other applications ***(6)***.

3.3. Matching Assay Method to Target Type

Often, one has a choice of assay method for a given target type (**Table 1**). To illustrate the various factors that are important when choosing an assay type, let's consider the important GPCR target class. GPCRs can be screened using cell-based assays such as FLIPR and reporter gene; or biochemical formats such as SPA, FP, or FIDA. One overriding factor when choosing between functional or binding assays for GPCRs is whether one seeks to find agonists or antagonists. Functional assays such as FLIPR and reporter gene 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 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 times 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.

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

In general, one should choose the assay format that is easiest to develop, most predictable, most relevant, and easiest 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. 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. Achieving an accept-

Table 1
The Most Important Assay Formats for Various Target Types

	Assay formats	
Target type	Biochemical	Cell-based
GPCRs	SPA, FP, FIDA	FLIPR, reporter gene, melanophores
Ion channels		FLIPR, VIPR
Nuclear hormone receptor	FP, TRET, SPA	Reporter gene
Kinases	FP, TRET, SPA	
Protease	FLINT, FRET, FP, SPA	Reporter gene
Other enzymes	FLINT, FRET, FP, SPA, TRET, colorimetry	
Protein-protein	TRET, BET, SPA	Reporter gene

able performance while keeping assay conditions within the desired range often requires an assay-optimization step. This usually significantly improves the stability and/or activity of the biological system studied, and has therefore become a key step in the development of screening assays.

4.1. Critical Biochemical Parameters in HTS Assays

The success of an HTS campaign in finding hits of 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 kind of enzymatic inhibitors. If we set the concentration of one substrate in a screening assay at 10 × Km, competitive inhibitors of that enzyme-substrate interaction with a Ki greater than 1/11 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 μ*M* or higher would be missed when screening at 10 μ*M*. On the other hand, the same problem will take place for uncompetitive inhibitors if substrate concentration is set at 1/10 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

4.1.1.1. Substrate Concentration

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

- 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 will be observed. The relationship between IC_{50} (compound concentration required to observe 50% inhibition of enzymatic activity with respect to an uninhibited control) and Ki (inhibition constant) is ***(9)***:

$$IC_{50} = (1 + S/Km) \times Ki$$

  As shown in **Fig. 1**, at S/Km ratios less than 1 the assay is more sensitive to competitive inhibitors, with an asymptotic limit of IC_{50} = Ki. At high S/Km ratios, the assay becomes less suitable for finding this type of inhibitors.

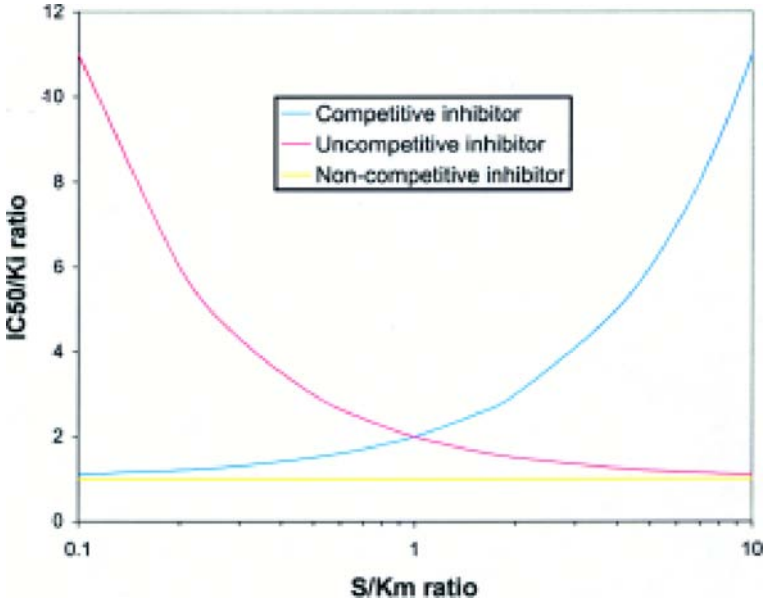


Fig. 1. Variation of IC_{50}/Ki ratio with the S/Km ratio for different type of inhibitors. At [S] = Km, IC_{50} = 2Ki for competitive (blue line) and uncompetitive (red line) inhibitors. For non-competitive inhibitors (yellow line) IC_{50} = Ki at all substrate concentrations.

- 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 IC_{50} and Ki is ***(9)***:

$$IC_{50} = (1 + Km/S) \times Ki$$

  High substrate concentrations make the assay more sensitive to uncompetitive inhibitors (**Fig. 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 IC_{50} and Ki is ***(9)***:

$$IC_{50} = Ki$$

- 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 IC_{50} and Ki is ***(10)***:

$$IC_{50} = (S + Km)/(Km/Ki1 + S/Ki2)$$

Table 2
Examples of Limitations to Substrate Concentration Imposed by Some Popular Assay Technologies

Assay Technology	Limitations[a]
Fluorescence	Inner filter effect at high concentrations of fluorophore (usually > 1 μ*M*)
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

[a]These limitations also apply to ligand in binding assays or other components in assays monitoring any kind of binding event.

In summary, setting the substrate(s) concentration(s) at the Km value is an optimal way of ensuring that all type 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 earlier. 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 2**) may preclude setting the substrate concentration at its ideal point.

As in many other situations found while implementing a 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 × Km to achieve an acceptable signal to background, competitive inhibitors with a Ki greater than 1/11 of the compound concentration tested will not be found 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

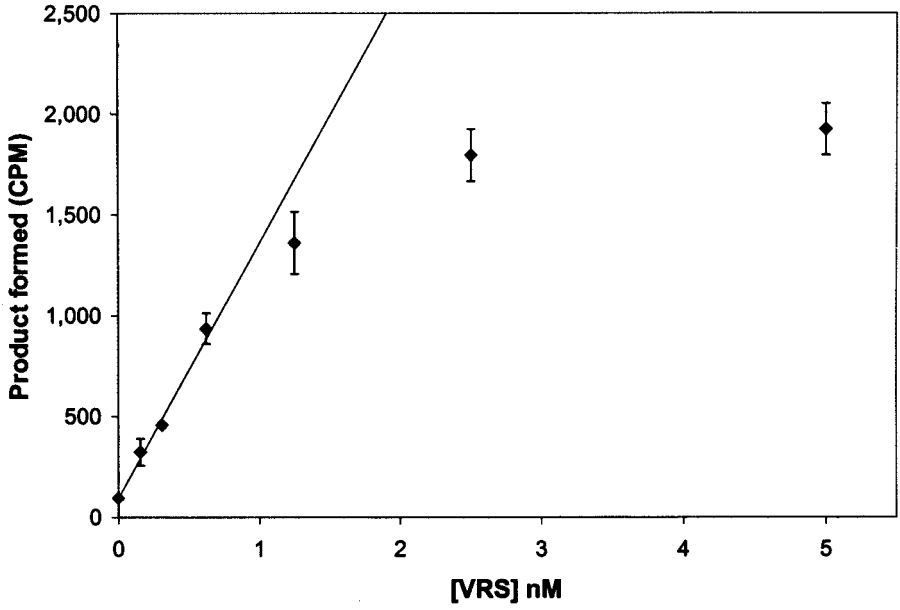


Fig. 2. Protein dilution curve for valyl-tRNA synthetase. The activity was measured after 20 min incubation following the SPA procedure described *(11)*.

statistical performance would jeopardize the discovery of more potent hits (*see* **Subheading 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. 2** for valyl-tRNA synthetase, at high enzyme concentrations there is typically a loss of linearity due to either 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. IC_{50} values lower than one half of the enzyme concentration cannot be measured; this effect is often referred to as "bottoming out." As the quality of compound collections improves, this could be a real

problem since structure activity relationship (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 μ*M*) and, as a rule of thumb, it is advisable to work at enzyme concentrations below 100 n*M*.

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 earlier for enzyme concentration, it is important to assess the linearity vs time of the reaction analyzed. Most HTS assays are 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., **ref. *12***) does not necessarily apply to HTS assays. Provided that all compounds in a 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. 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 4 % 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 IC_{50}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, inhibition by product, detection artifacts, etc. In view of the actual progress curve, practical choices should be made to avoid missing interesting hits.

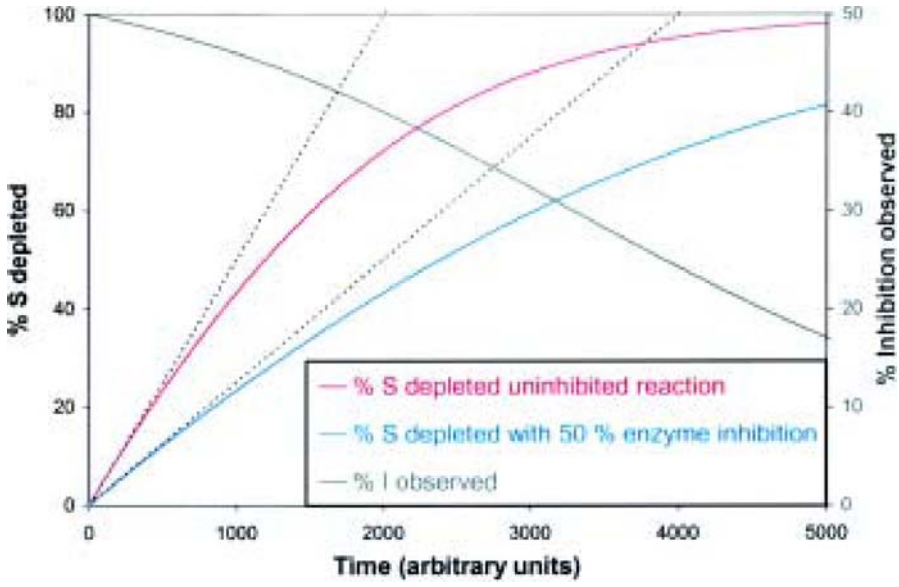


Fig. 3. Theoretical progress curves at S = Km of an uninhibited enzymatic reaction (red) and a reaction with an inhibitor at its IC_{50} concentration (blue). The inhibition values determined at different end-points throughout the progress curve are shown in green. Initial velocities are represented by dotted lines.

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 (for more details, *see* Chapters 2 and 3).

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:

$$BL = B_{max} \times L / (Kd + L)$$

where BL = bound ligand concentration (equivalent to v_0), B_{max} = maximum binding capacity (equivalent to V_{max}), 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 **Subheading 4.1.1.1.**, can be directly translated to ligand-binding assays. For example, for competitive binders,

$$IC_{50} = (1 + L/Kd) \times Ki$$

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 **Subheading 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. It 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 1/5 of Kd ***(13)***. 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* **Subheading 4.2.**).

4.1.2.3. Pre-incubation 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 set-up with many built-in factors that are out of the scientist's control. Nevertheless, some of the points discussed earlier apply to them, *mutatis mutandi*. A few general points to consider are:

- The response observed should be linear with respect to the number of cells;
- Pre-incubation of cells with compounds should be considered when applicable (e.g., assays in which a ligand is added); and
- Optimal incubation time should be selected in accordance to the rule of avoiding underestimation of inhibition or activation values (*see* **Subheading 4.1.1.3.**).

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 compounds or natural extracts that in most cases are dissolved in dimethyl sulfoxide (DMSO). The tolerance of the assay to DMSO should be considered. If significant decrease on activity/binding is observed at the standard solvent concentration—typically 0.5–1% (v/v) DMSO—lower concentrations may be required. In some cases the detrimental effect of solvent can be circumvented by the optimized assay conditions.

The stability of reagents should be tested using the same conditions intended for HTS runs, including solvent concentration, stock concentration of reagents, reservoirs, plates, etc. Sometimes 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. 4**).

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^+, Cl^-)
- Divalent cations (Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+})
- Reological modulators (glycerol, polyethyleneimine glycol [PEG])
- Polycations (heparin, dextran)
- Carrier-proteins (bovine serum albumin [BSA], casein)
- Chelating agents (ethylene diamine tetraacetic acid [EDTA], ethylene glyrol tetraacetic acid [EGTA])
- Blocking agents (polyethyleneimine [PEI], milk powder)
- Reducing agents (dithiothreitol [DTT], β-mercaptoethanol)
- Protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], leupeptin)
- Detergents (Triton, Tween, CHAPS)

In addition, there are other factors that need to be specifically optimized for some techniques. For instance, SPA for receptor-binding assays can be performed with different types of beads, and the concentration of bead itself should be carefully selected according to the behavior of every receptor (Bmax, NSB, Kd, etc.). 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, collagen). One also needs to take into account the possible physiological role of the factors chosen and also the cell's tolerance to them.

Besides analyzing the effect of factors individually, it is important to consider interactions between factors because synergies and antagonisms can commonly occur ***(14)***. 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

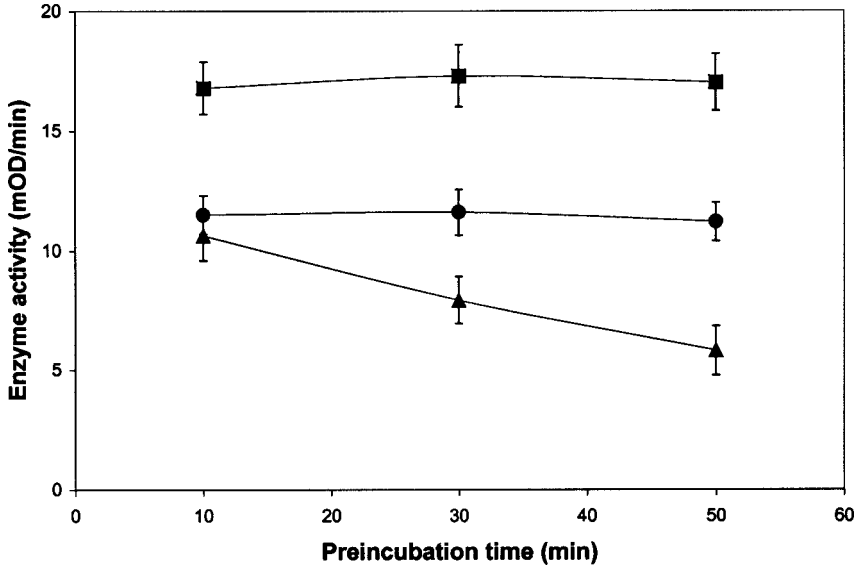


Fig. 4. Example of loss of signal in an enzymatic reaction related with adsorption of enzyme (or substrate) to plasticware. The data is from a real assay performed in our lab. Stability of reagents was initially measured using polypropylene tubes and 384-well polystyrene plates, without CHAPS (circles). 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 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.

tests are performed randomly. Therefore, an automated solution is necessary because manually running an experiment of this complexity would be extremely difficult. A full package called AAO (automated assay optimization) has been recently launched by Beckman Coulter (Fullerton, CA) in collaboration with scientists from GlaxoSmithKline ***(15)***. An example of the outcome of one assay recently improved in our lab using this methodology is shown in **Fig. 5**. The paper by Taylor et al. ***(15)*** describes examples of assay optimization through AAO for several type of targets and assay formats.

A typical optimization process starts with a partial factorial design including many factors (**ref. *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 absence of the ingredient and the other is presence at a fairly typi-

cal 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 only be used 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.

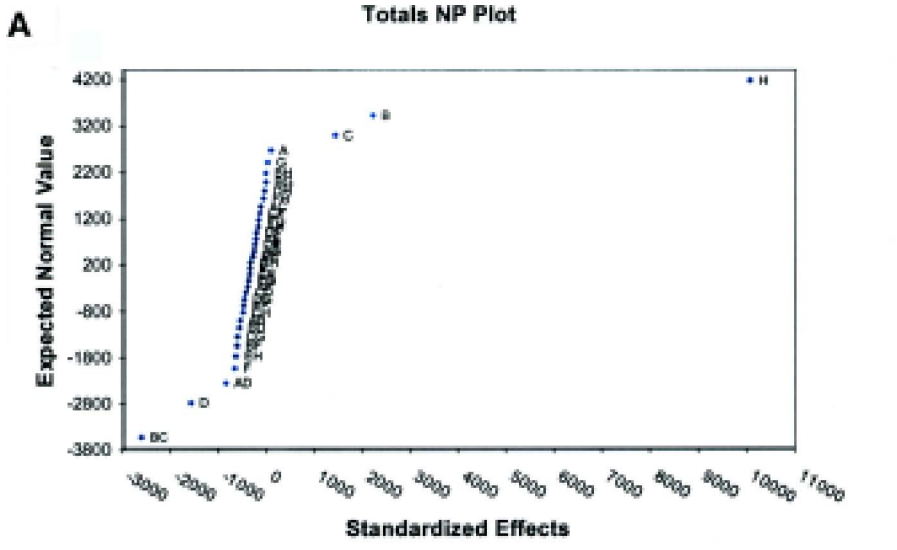
4.3. Statistical Evaluation of HTS Assay Quality

The quality of a 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 unlabeled (cold) ligand; the resulting displacement could be unreachable for some specific competitors that would not prevent NSB 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, fairly 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 carpet).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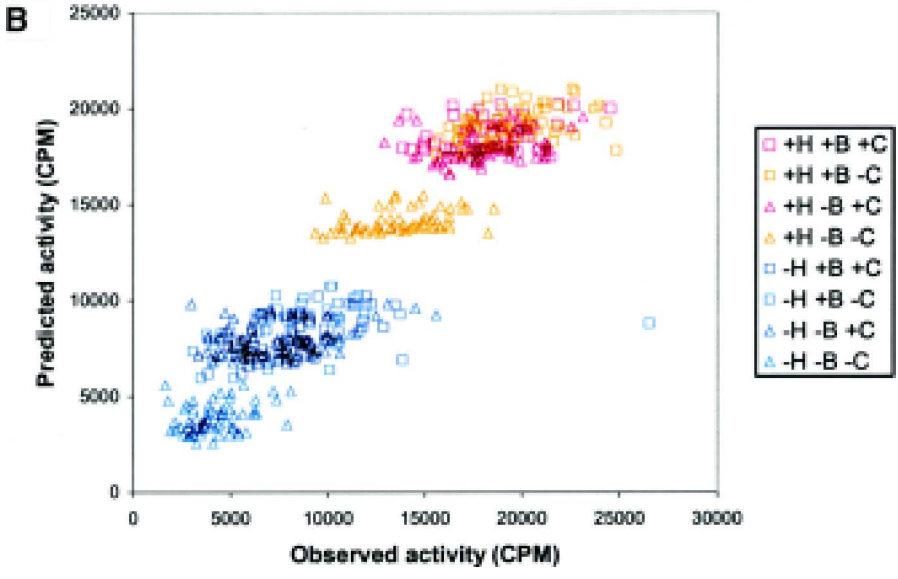
B
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25000
30000
Observed activity (CPM)
+H +B +C
+H +B -C
+H -B +C
+H -B -C
-H +B +C
-H +B -C
-H -B +C
-H -B -C

The analysis of performance can be accomplished by several means. Graphical analysis helps to identify systematic errors (e.g., **Fig. 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 as follows:

1. Signal to background

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

   S/B provides an indication of the separation of positive and negative controls. It has to be reviewed in the context of the assay technique used. In our experience, a S/B of 3 is the minimal requirement for a robust assay, though some techniques less prone to variability allow for lower S/B ratios (e.g., FP). While assay variability is instrument dependent and can change from experiment to experiment, S/B is mainly assay-dependent and can be used early on to estimate the quality of an assay.

2. Specific signal or signal window

$$SW = M_{signal} - M_{background}$$

   SW gives another idea of the magnitude of the signal. It has to be reviewed in the context of the assay technique used. For instance, at least 1,000 CPM in a radiometric assay or 5 mOD/min in a continuous absorbance assay are required to avoid reproducibility problems in the subsequent HTS campaign.

Fig. 5. (Left) 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, 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 8 factors and was designed as a 2-level full factorial experiment with duplicates. 512 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 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 red and orange, –H dark and light blue) is clearly positive. In the absence of B = 125 m*M* Bicine (+B squares, –B triangles) and C = 125 m*M* TAPS (+C red and dark blue, –C orange and light blue), the enzyme was less active. The original conditions yielded ca. 5,000 CPM vs ca. 25,000 CPM with the optimized buffer (backgrounds were ca. 100 CPM in all cases).

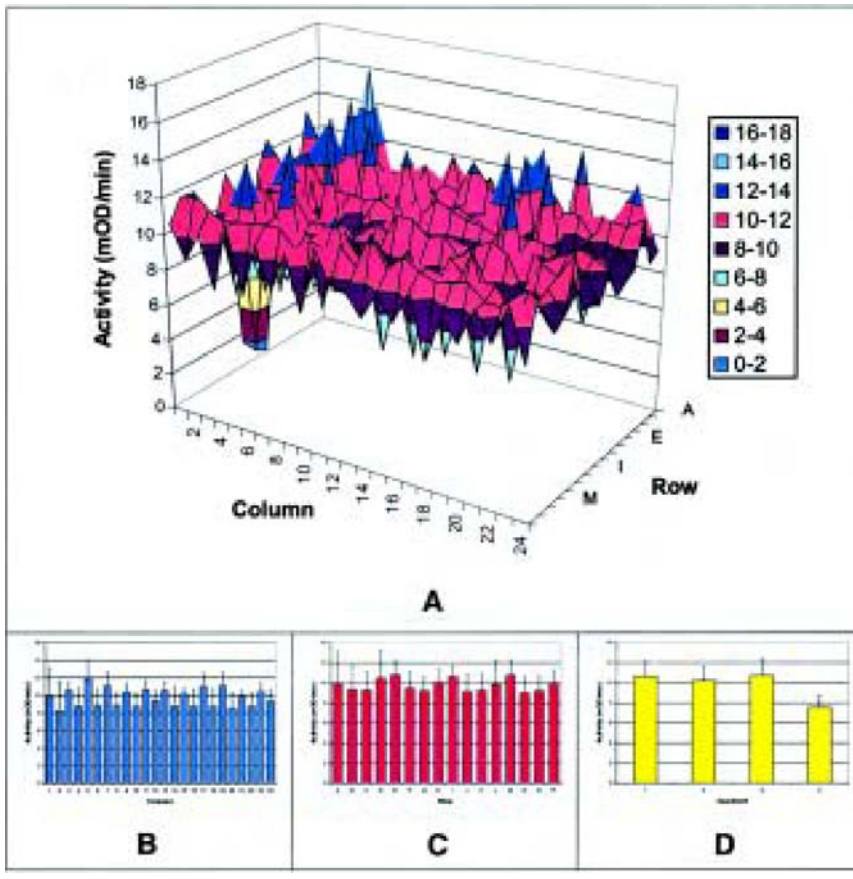

Fig. 6. Graphical analysis of a 384-well plate of positive controls of an enzymatic reaction monitored by absorbance (continuous read-out). The plate was filled using a pipettor equipped with a 96-well head and indexing capability. **(A)** Three-dimensional plot of the whole plate showing that four wells (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 4th quadrant was receiving less reagent.

3. Signal to noise

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

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 *(16)*. 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 a HTS assay. Typically, assays with values of S/N greater than 10 are considered acceptable.

4. Coefficient of variation of signal and background

$$CV = 100 \times SD/M \ (\%)$$

A relative measure of variability, it provides a good indication of variability for the signal. For backgrounds it is less useful, as values close to 0 for the mean distort the CV. Variability is a function of the assay stability and precision of liquid handling and detection instruments.

5. Z' factor

$$Z' = 1 - 3 \times (SD_{signal} + SD_{background}) / \left| M_{signal} - M_{background} \right|$$

Since its publication in 1999 ***(16)*** the Z' factor has been widely accepted by the HTS community as a very useful way of assessing the statistical performance of an assay. 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:

$$Z' = 1 - 0.03 \times (\left| S/B \right| \times CV_{signal} + CV_{background}) / (\left| S/B \right| - 1)$$

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 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 acceptance criteria for an assay is Z' > 0.4, equivalent to having a S/B of 3 and a CV of 10%. Higher S/B ratios allow for higher variability, but as a rule, the CV_{signal} must be below 20% . Low variability allows for a lower S/B, but a minimum of 2 is usually required provided that CV_{signal} is rarely below 5%. **Figure 7** shows Z' at work in 3 different scenarios. Full analysis of the corresponding data is collected in **Table 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.

4.4. Assay Validation

Once an assay optimized to find compounds of interest passes its quality control with a Z' greater than 0.4 (or whatever is the applied acceptance criteria),

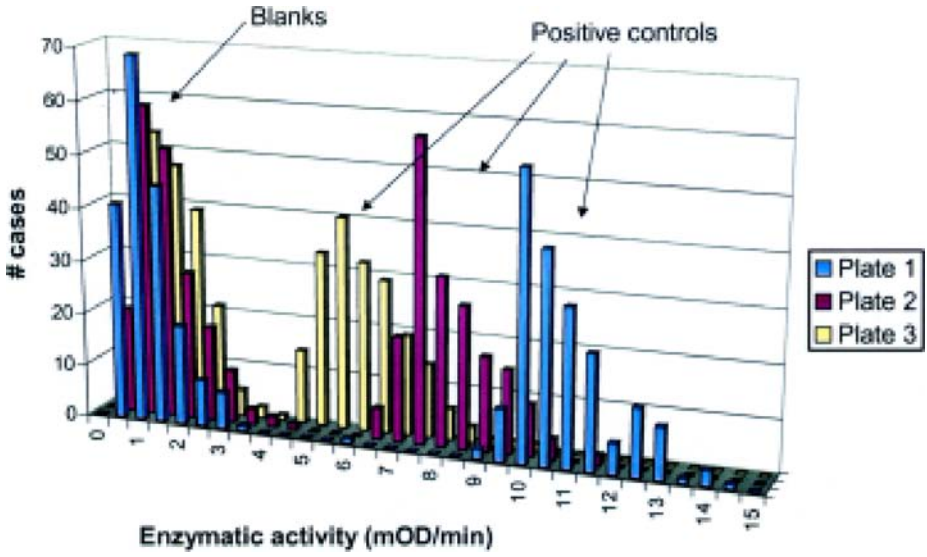

Fig. 7. Distribution of activity values (bins of 0.5 mOD/min) for three 384-well plates half-filled with blanks and half-filled with positive controls of an enzymatic reaction monitored by absorbance (continuous read-out). Z' factors were 0.59 for plate 1, 0.42 for plate 2, and 0.10 for plate 3. A complete analysis of performance is shown in **Table 3**.

Table 3
Statistical Analysis of Data from the Three Plates Described in Fig. 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
$M_{background}$ (mOD/min)	0.30	0.69	0.74
$SD_{background}$ (mOD/min)	0.51	0.57	0.57
S/B	34	11	8
SW (mOD/min)	9.80	7.08	5.09
S/N[a]	19	12	9
S/N[b]	10.0	7.1	4.6
CV_{signal} (%)	8%	10%	16%
$CV_{background}$ (%)	173%	82%	77%
Z' factor	0.59	0.42	0.10

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

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

a final step must be done before starting a 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:

1. Obtain field data on assay performance,
2. Estimate the hit rate and determination of optimal sample concentration,
3. Assess interferences from screening samples, and
4. Evaluate the reproducibility of results obtained in a production environment

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 a SD of 0.5%. For a 5% rate, the estimation's SD would be 1% (approximate figures calculated as described in ***17***).

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. 8**).

A dramatic example of how the test of a pilot collection helps to detect interferences is shown in **Fig. 9**. This target had been tested for and found to be slightly unstable at room temperature (**Fig. 9B**, without BSA). Nevertheless, the effect of 352 mixtures of compounds was tested and an extremely high hit rate was observed (45% of the mixtures inhibited the enzyme activity 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.

4.5. Summary

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 can 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.

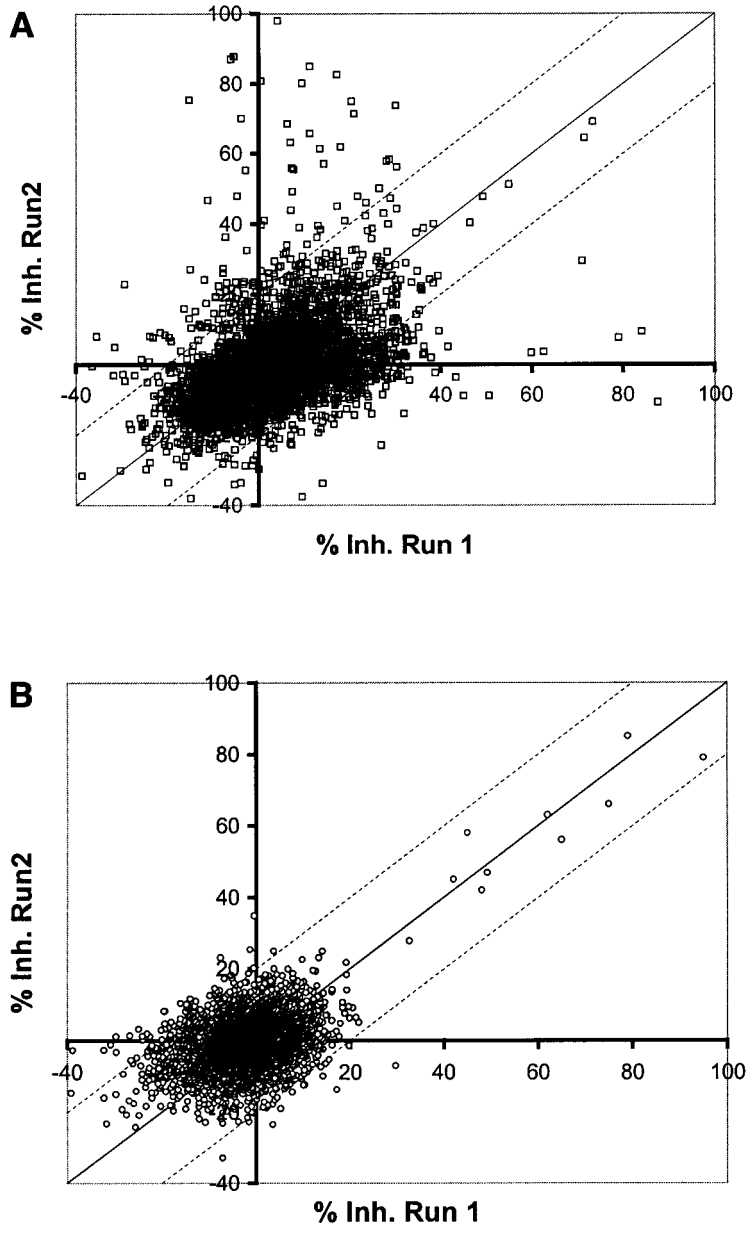

Fig. 8. Comparison of duplicates from validation for two HTS assays. **(A)** This enzymatic assay showed a significant number of mismatched results between duplicate runs of the same 4,000 samples. Two actions should be taken in a case like this: liquid handling errors have to be avoided, and the assay quality must be improved. **(B)** The data corresponds to a ligand-binding assay that showed a good reproducibility.

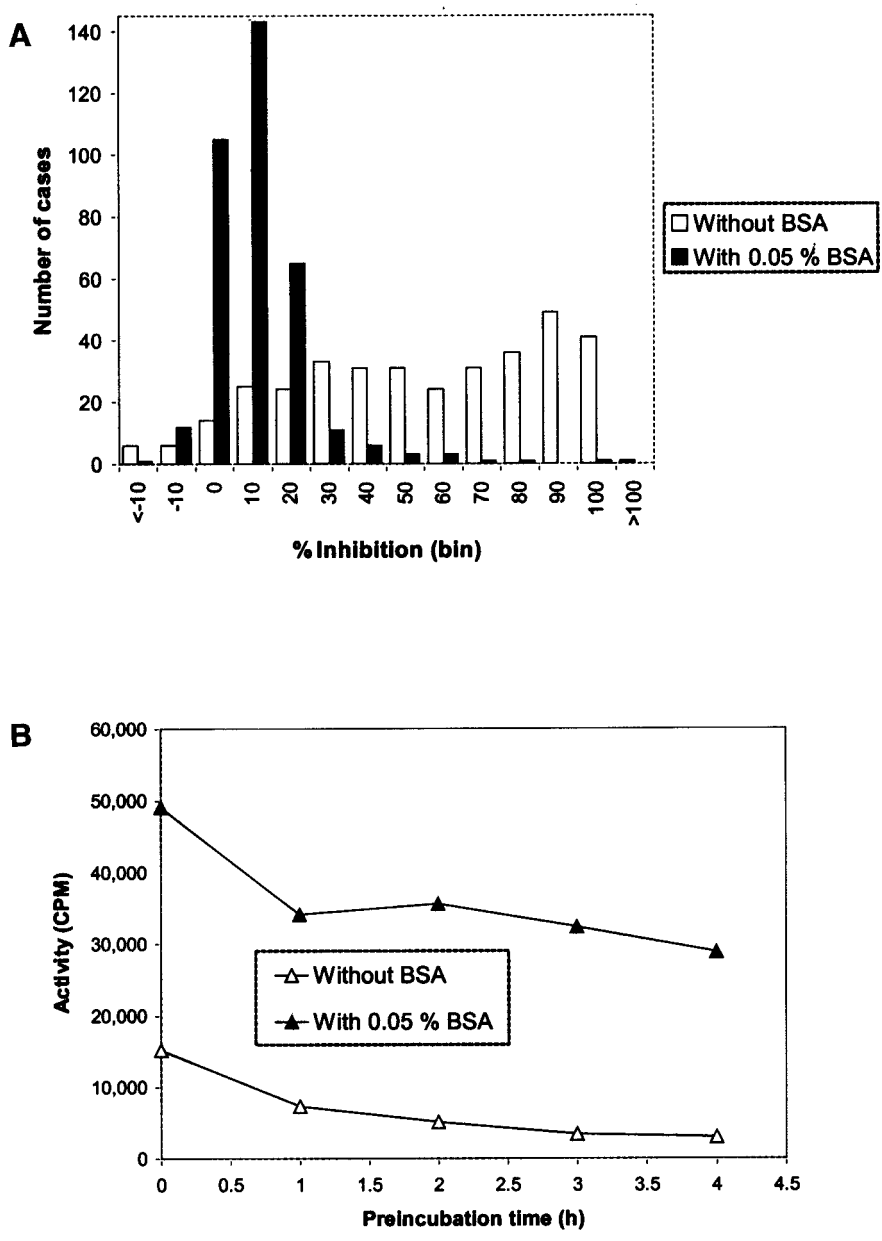

Fig. 9. **(A)** Distribution of inhibition values (10% bins) in the validation of a HTS assay of an enzyme tested with and without 0.05% (w/v) BSA. The samples were 352 representative mixtures of compounds (11 components at 9.1 μ*M* each). **(B)** It was shown that the stability and activity of the enzyme was greatly improved in the presence of BSA.

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