Chapter 5

Enzyme Assay Design for High-Throughput Screening

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Abstract

Enzymes continue to be a major drug target class for the pharmaceutical industry with high-throughput screening the approach of choice for identifying initial active chemical compounds. The development of fluorescent- or absorbance-based readouts typically remains the formats of choice for enzyme screens and a wealth of experience from both industry and academia has led to a comprehensive set of standardized assay development and validation guidelines for enzyme assays. In this chapter, we generalize approaches to developing, validating, and troubleshooting assays that should be applicable in both industrial and academic settings. Real-life examples of various enzyme classes including kinases, proteases, transferases, and phosphatases are used to illustrate assay development approaches and solutions. Practical examples are given for how to deal with low-purity enzyme targets, compound interference, and identification of activators. Assay acceptance criteria and a number of assay notes on pitfalls to avoid should provide pointers on how to develop a suitable enzymatic assay applicable for HTS.

Key words: Enzyme, High-Throughput Screening, Fluorescent, Absorbance, Activator, Interference, Kinase, Phosphatase, Methyltransferase.

1. Introduction

Enzyme targets have proven to be one of the most tractable classes for small-molecule drug discovery with many of the new molecular entities approved by the FDA in 2007 targeting enzymes *(1)*. In particular, a number of small-molecule drugs targeting kinases and proteases have progressed through the clinic and into the market *(1)*. High-throughput screening (HTS) has long been the approach of choice in the pharmaceutical industry for the initial identification of novel chemical series *(2)*. The search for small-molecule inhibitors is also becoming of major interest for academic labs *(3)*. Whether in industry or academia, the development of

W.P. Janzen, P. Bernasconi (eds.), *High Throughput Screening, Methods and Protocols, Second Edition, vol. 565*

DOI 10.1007/978-1-60327-258-2_5, Springerprotocols.com

successful screens is typically the result of a collaborative partnership between a screening center/drug discovery core and a biology lab with a novel target or assay technology. It is our goal in this chapter to use our experiences in both industry and academia to provide some recommendations and examples regarding development and implementation of enzymatic screening assays and to illustrate some of the pitfalls that may be encountered.

2. Assay Development Guidance

With the recent advent of the Molecular Libraries Screening Center Network (MLSCN; http://mli.nih.gov/mli/) funded by the NIH Roadmap for Medical Research, academic-based screening centers have emerged with the goal of implementing novel and innovative assays submitted by the academic community into an HTS format with subsequent screening to identify molecular probes. A good deal of the assay development and HTS knowledge gathered from industry has found its way into the academic screening centers *(3)*, and in particular much of enzyme assay development has become standardized. A number of excellent reference texts *(4, 5)* exist describing step-by-step approaches to developing an HTS-ready assay and HTS assay design and implementation has been covered in great detail elsewhere *(5, 6)*. Furthermore, Eli Lilly and the National Institutes of Health Chemical Genomics Center (NIHCGC) have developed a comprehensive assay guidance manual that includes an excellent section on enzymatic assays; a recent update (2008) is available online (http://www.ncgc.nih.gov/guidance/manual_toc.html). In general, the development of an assay for HTS can be summarized in these steps:

Assay Development
↓
Assay Adaptation
↓
Assay Validation and Acceptance
↓
High-Throughput Screening

Recently, the search for small-molecule inhibitors has expanded beyond kinases and proteases with many new enzyme targets originating from academic groups, including phosphatases *(7)*, deacetylases *(8)*, lipases, esterases *(9)*, transferases, isomerases, reductases, recombinases *(10)*, ligases *(11)*, glycosidases, and convertases. Many of these enzyme classes have proven somewhat less

tractable [e.g., phosphatases *(12)*] and have brought new challenges in developing relevant and productive HTS-amenable assays.

3. Assay Development

There are a number of basic decisions and considerations that need to be made in the process of enzyme assay development. Many assays have been developed as a conveyer-belt approach: purify enzyme, identify substrates, select assay format, measure kinetic parameters, and optimize the assay for HTS. The following flow chart describes a typical path to developing a robust enzymatic assay for HTS and each step of the process will be addressed in this section.

Form of Enzyme
↓
Choice of Substrate
↓
Choice of Assay Format
↓
Optimization of pH and Buffer Components
↓
Determine Assay Kinetic Parameters
↓
Identify Control Inhibitor
↓
Kinetic or End Point
↓
Assay Stability and DMSO Tolerance

3.1. Form of Enzyme

Many enzymes consist of multiple domains beyond the catalytic domain and expression of a full-length enzyme may prove difficult. Frequently, a truncated form of the target can be expressed more readily to give active enzyme. This approach is often used for kinases where the target site is the ATP-binding pocket and hence only the catalytic domain is used for the enzyme assay. The downside to this approach is that regulatory control of the full-length enzyme may be lost (e.g., kinases such as Akt *(13)*) and inhibitors that are potent for the truncated enzyme may be ineffective when tested with the full-length enzyme *(13)*. A further

complication is that for an enzyme to be fully active it may have to be expressed as a multiprotein complex rather than as a purified enzyme (e.g., protein kinase C isoforms, histone deacetylases). Compounds effective on isolated component activities may be ineffective when tested against the functional complex. Indeed, isolation of active complexes may be necessary if activators rather than inhibitors are the goal of the screen (e.g., AMP kinase *(14)*). In addition, there is potential to discover novel compounds with a novel binding site that is present in the native full length, but not the truncated enzyme *(15)*.

3.1.1. Enzyme Purity

To unambiguously measure only target enzyme activity, it is preferable to use purified enzyme. However, as shown in the following example, it is possible to develop robust assays using an unpurified source of enzyme. This usually requires a selective substrate (or buffer conditions) that allows the measurement of only the enzyme target of interest. This approach should be carefully validated by using controls such as known inhibitors and/or suitable control crude preparations that do not contain target enzyme. The most important requirement for the use of an enzyme preparation for assay development is enzymatic purity (i.e., all the signal in an assay is due to a single enzyme) as opposed to only mass purity (e.g., "95% pure"). The use of a control inhibitor in the assay to reproduce known IC_{50} values and generation of Hill slope close to 1.0 will go a long way to demonstrating enzymatic purity. A Hill slope much different than 1.0 may indicate the presence of contaminating enzyme activity in the enzyme preparation.

3.1.2. Enzyme Assay Example 1: Using Crude Lysate as Protease Enzyme Source

Attempts to develop an HTS-compatible assay for a protease expressed in *Escherichia coli* were initially unsuccessful using the purified form of the enzyme due to problems with the stability of the purified enzyme preparation. However, a robust HTS assay could be developed using a crude lysate preparation as the source of the enzyme activity. The availability of a specific inhibitor for the enzyme allowed for successful development of this assay. The IC_{50} value of the known inhibitor was determined as 17.8 ± 1.5 nM (mean $\pm$ SD, $n = 16$) (**Fig. 5.1**).

3.2. Assay Formats

Typically enzyme targets have been developed as biochemical assays. There are numerous formats for currently popular classes of enzyme drug targets *(16)* such as kinases, phosphatases, proteases, and ATPases, while other enzymes such as reductases and carboxylases have fewer format options. The ability to identify active compounds from a particular HTS assay depends in part on the suitability or the quality of the assay used in the screening. The estimated cost per well and resultant cost for the planned screen (including at least 20% extra for dead volumes, IC_{50} determinations, etc.) should be determined for the desired assay format

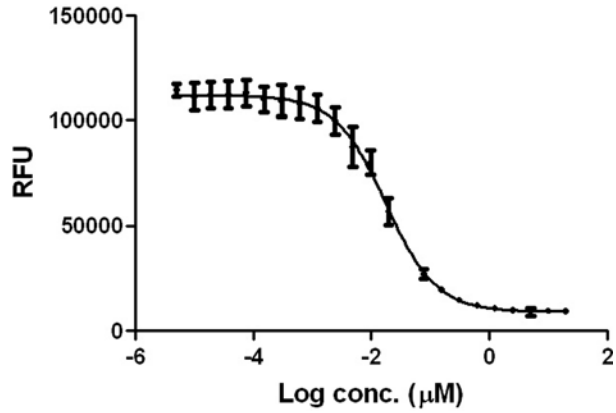

Fig. 5.1. **Replicate inhibition curves for protease enzyme activity**. The assays were performed in 384-well plates with a volume of 50 μl in the presence of different concentrations of inhibitor as indicated. The 16 experiments gave an average of IC_{50} of 17.8 ± 1.5 nM when fit to a sigmoidal dose–response function with a variable slope.

before any wet experiments are done and/or as soon as a working assay is obtained. If the projected cost for the screen is not in the budget, then an alternative format must be chosen.

3.2.1. Homogeneous Versus Nonhomogeneous Assay Format

Assay formats can be classified as homogeneous or nonhomogeneous. Homogeneous formats are often referred to as "mix-and-measure" assays, a name that reflects the simplicity of the assay protocol. Nonhomogeneous formats usually require a separation of substrate from product for enzyme reactions, which can involve plate washing, plate to plate liquid transfers, and/or centrifugation. Although homogeneous assays have some disadvantages, the advantages of homogeneous assay formats (**Table 5.1**) for HTS

Table 5.1
Comparison of attributes for homogeneous versus nonhomogeneous assay formats

Homogeneous	Nonhomogeneous
Fewer steps	Multiple steps
Easy to automate and miniaturize	Difficult to automate
Fast, robust screens	Time consuming, labor intensive
Compound interference can be an issue	Less susceptible to compound interference
Less sensitive, may require higher amounts of enzyme and substrate	Very sensitive to enzyme activity, can tolerate high concentrations of substrate

far outweigh the disadvantages *(17)*. A limited number of assay formats allow a direct readout of inhibition, including microfluidics *(18)*.

One significant pitfall of homogeneous formats is the potential for *compound interference* with the assay detection method since a diversity of compounds are screened, typically at relatively high concentrations in the 1–100 μM range. In contrast, nonhomogeneous formats typically wash away compound after the assay and are thus less susceptible to compound interference. Compound interference in homogeneous formats can be detected by performing secondary assays less susceptible to interference such as nonhomogeneous assays.

Fluorescent and colorimetric readout-based enzyme assays remain a major contributor to HTS labs throughout the field, although there are some advantages to using radioactivity. The use of radioactivity can allow *very sensitive* detection of enzyme activity and in some cases may be the only means to detect enzyme activity in high-throughput mode. There are two types of formats readily available for radioactive homogeneous enzyme assays, scintillation proximity assay *(17)* and Flashplate™ technology (Perkin Elmer, USA).

In general, the goal is to develop *homogeneous nonradioactive* assay formats to measure enzyme activity and so this chapter will focus on absorbance- and fluorescence-based assay formats.

3.2.2. Practical Considerations for Absorbance Assays

Absorbance is by far the most traditional detection method for following enzymatic reactions. Homogeneous absorbance assays have the advantage of being relatively inexpensive, usually simple to design, and they use the ever-prevalent spectrophotometers. For example, there are many enzymes that utilize coenzyme nicotinamide adenine dinucleotide (NAD+ or the reduced form NADH) or nicotinamide adenine dinucleotide phosphate (NADP+ or the reduced form NADPH) in redox reactions or as substrates for posttranslational modifications. Enzyme reactions that generate or consume NADH or NADPH can be detected by change in absorbance. NADH and NADPH absorb strongly at 340 nm, while the oxidized forms (NAD+ and NADP+) do not absorb at this wavelength. Hence, enzyme activity that generates NADH (or NADPH) as one of its products can be detected by an increase in absorbance at 340 nm or if an enzyme uses NADH (or NADPH) as a substrate, a decrease in absorbance indicates activity.

For enzyme reactions that do not use NAD, it has been possible in some cases to utilize a *coupled* assay whereby the initial reaction generates a product that can serve as a substrate by a second NAD-utilizing enzyme. Therefore, the activity of the primary enzyme can be determined by following NADH consumption or generation by the second detection enzyme, which is present at a high nonlimiting concentration. Although the

coupled enzyme assays function well, the assay is more complex requiring more assay development, is more costly due to the requirement of another enzyme, and potentially more difficult to troubleshoot and deconvolute. In addition, for screening chemical libraries, there is potential for inhibition of the detection enzyme causing false positives. Therefore, hits need to be tested in a direct enzyme assay format to rule out this possibility. These disadvantages apply to all enzyme-coupled assays and therefore coupled formats should be a last resort to obtain a homogeneous assay.

Absorbance assays, of which colorimetric assays are a subset, are usually *not very sensitive* in the detection of enzyme activity compared to the other homogeneous detection methods (primarily fluorescence). This lack of sensitivity requires higher concentrations of enzyme and/or substrate, which can be prohibitive depending on the availability and cost of these reagents.

3.2.3. Practical Considerations for Fluorescence Assays

Fluorescence is another commonly used detection method for homogeneous enzyme assays. Fluorescent formats tend to be very sensitive to the detection of enzyme activity, allowing low concentrations of frequently precious and/or expensive target enzyme. There are a number of fluorescence-based assay formats that have been used for HT enzyme assays *(19)*.

3.2.3.1. Fluorogenic Assays

The simplest type of fluorescence assay is the use of an artificial nonfluorescent substrate, which becomes a fluorescent product with enzymatic activity. An example of this is the use of 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP from Invitrogen) for detection of phosphatase activity. DiFMUP is nonfluorescent due to the phosphate group on this small molecule. Upon removal of the phosphate group by a phosphatase, the highly fluorescent 6,8-difluoro-4-methylumbelliferyl molecule (E_x = 360 nm, E = 450 nm) is generated and thus fluorescence is proportional to enzyme activity. This is a very sensitive homogeneous method for detecting either acid or alkaline phosphatase activity and many phosphatases, including protein tyrosine phosphatases, can act on nonphysiological small-molecule substrates like DiFMUP. Similarly, there are commercially available substrates for many hydrolases that increase fluorescence upon hydrolysis by enzymes such as proteases, glycosidases, lipases, and esterases. These types of fluorescence assays can be performed in kinetic or end-point mode and are relatively simple assays to develop.

3.2.3.2. Fluorescence Resonance Energy Transfer Assays

Another fluorescent assay format that is extensively used for enzyme assays is fluorescence resonance energy transfer (FRET). In FRET assays, signal is dependent on the proximity of a fluorophore to a quencher or an acceptor fluorophore and is

intrinsically a homogeneous technique. It is now commonplace to use FRET-based substrates for detection of protease activity. Peptides derived from the natural substrate are synthetically modified to have a donor fluorophore at one end and a quencher or an acceptor dye at the other end. Hence, the fluorescence of the fluorophore is quenched in the intact peptide. When a protease cleaves an internal site within the peptide, the fluorophore is no longer in close proximity to the quencher which results in an increase in donor fluorescence. FRET-based activity assays are also commonly used for nucleic acid-modifying enzymes such as helicases, nucleases, reverse transcriptases, polymerases, and ligases. DNA can be readily and inexpensively modified to have a donor/acceptor pair positioned in close proximity at either end of the same molecule or at the 5'- and 3'-ends of complimentary oligos such that hybridization brings the pair in close proximity. Helicase activity, for example, can be detected by the separation of the labeled strands, resulting in an increase in fluorescence *(20)*.

Time-resolved FRET is a subtype of FRET that employs long half-life lanthanides as donors – most commonly europium and terbium chelates. The long excitation half-life allows a separation in time between excitation and detection of emission photons. This allows short half-life background and compound fluorescence to decay allowing detection of only the acceptor molecule. Thus, TR-FRET has been extensively used for decreasing compound interference and it is also a very sensitive detection technique due to the high fluorescence of lanthanides. TR-FRET has been used for a variety of common enzyme targets including kinases and proteases. Europium- and terbium-labeled reagents, including antibodies, along with acceptor-labeled reagents are commercially available, including reagents for custom-labeling reagents with chelates.

3.2.3.3. Fluorescence Polarization

Fluorescence polarization (FP) is a well accepted and frequently used technology for high-throughput assays *(21)*. FP takes advantage of the inverse relationship between the rotational speed of fluorescent molecules in solution and the size of the labeled molecule or complex. Since FP is a ratiometric fluorescence technique, it is subject to less variability than are other nonratiometric fluorescent assays. A variety of configurations of FP assays are possible for the detection of enzyme activity. Possibly the most common type is the competitive FP immunoassay (FPIA). This method uses antibodies that bind the product of the reaction and a fluorophore-labeled product analog (tracer). Conditions are set up such that product from the reaction competes with tracer for binding product, resulting in the depolarization of the tracer. FP assays are commonly used for the detection of kinase activity using a peptide substrate, an antiphosphopeptide antibody, and a fluorophore-labeled phosphorylated peptide tracer. For maximal sensitivity to enzyme-generated product and minimal use of

antibody, the tracer is usually used at low nanomolar concentration. For assay development, the concentration of tracer is usually set to generate fluorescence that is 10–20-fold above buffer background and then the optimal concentration of antibody determined by titration into this concentration of tracer. The amount of antibody that is slightly less than the amount that binds all the tracer is chosen for maximal sensitivity to product detection. This low concentration of tracer produces an assay that is very sensitive to enzyme activity, but it also makes the assay more sensitive to compound interference. Red-shifted dyes can be used in place of the commonly used fluorescein to dramatically reduce compound interference *(22)*.

In another version of the FPIA for detection of kinase activity, an antibody is employed to detect the ADP reaction product instead of the phosphorylated product. This format has the advantage of being a universal format for measuring the activity of any kinase (or ATPase). The disadvantage though is that any contaminating kinase (or other ATPase) in the enzyme preparation may also be detected and result in a signal that results from a mixture of (or predominately from) nontarget activity.

3.3. Assay Development Approaches for Compound Interference

For absorbance- or fluorescence-based assays, there is a reasonable chance that a certain percentage of compounds will interfere with the signal.

3.3.1. Compound Interference in Absorbance Assays

Absorbance assays are *highly susceptible* to compound interference from certain compounds that absorb at the detection wavelength (for instance, colored compounds in a colorimetric assay). The absorbance of a test compound could potentially result in false negatives or positives, depending on whether an increase or a decrease in absorbance is the measure of activity.

For absorbance assays, compound interference can be reduced by configuring the assay such that actives will be identified as those compounds that *reduce* absorbance instead of increase absorbance. Thus, compounds that absorb at the detection wavelength will not be detected as false positives. However, there is potential for false negatives for absorbing compounds that are also inhibitors. Simple secondary assays such as testing hits with the same enzyme using a totally different assay format or simply adding the compound post-assay termination will eliminate this type of false positives. Despite these shortcomings, absorbance assays remain a viable option for HT enzyme assays due to their simplicity and low cost of detection.

3.3.2. Compound Interference in Fluorescence Assays

All homogeneous fluorescence assay formats are susceptible to compound interference to at least some extent. As a general rule, the higher the fluorescence signal, the less susceptible an assay will

be to compound-related quenching and fluorescence. Thus, an increase in fluorophore used in the assay will make the assay more resistant to compound interference and also generally more robust. However, for assays that employ expensive substrates, antibodies, or other expensive detection reagents, an increase in fluorophore concentration may be cost prohibitive. In this case, one just needs to perform secondary tests to eliminate false positives. Another proven method to reduce the frequency of compound interference is to use red-shifted fluorophores, since most fluorescent small organic molecules emit light in the green light range (as does fluorescein) *(22)*. Despite the widely held belief that TR-FRET is impervious to compound interference, compound interference can still be observed with TR-FRET *(23)* and this should be addressed before a hit is declared.

3.4. Special Considerations for Enzyme Activator Assays

Traditionally, only inhibitors have been sought for enzyme targets to shut down their activity in vivo. As more potential targets are revealed by research, it is apparent that it would be clinically advantageous to enhance the activity of some enzymes. In principle, activators could be identified from any screen as long as the dynamic range for the assay will allow it. However, some assays may have limitations for identifying activators due to the amount of antibody used or the competitive nature of the assay as in the case of the FPIA, unless the assay is specifically designed to detect activators (or both activators and inhibitors). A general problem with finding activators is that certain formats are more susceptible to false positives. For instance, for fluorescence assays, fluorescent compounds can appear to be an activator. One assay approach we have taken is to "preread" before initiating the reaction with substrate, run the reaction, and subtract the prescreen fluorescence, from the final fluorescence thereby subtracting compound fluorescence.

3.4.1. Enzyme Assay Example 2: Prereading for a Phosphatase Assay

For one target, we were interested in inhibitors and *activators* of phosphatase activity. In this screen, the following steps were carried out:

1. 0.5 μl of compound was prespotted on the plate followed by the addition of 25 μl of phosphatase containing assay buffer.
2. The plate was then read in the fluorescence plate reader to obtain a fluorescence value for the compound alone.
3. Subsequently, the reaction was started by the addition of 25 μl of DiFMUP and stopped at the appropriate time followed by the final fluorescence read. The specific fluorescence was obtained by subtracting compound fluorescence from the final fluorescence.

This type of background subtraction, though not a perfect method in terms of eliminating false-positive activators, eliminated

most of the fluorescent compounds in a phosphatase screen of the small Prestwick chemical collection (Prestwick Chemical Company, France) (**Fig. 5.2**). In theory, a weakly fluorescent true activator could still be detected with this method versus just eliminating all fluorescent compounds from follow-up.

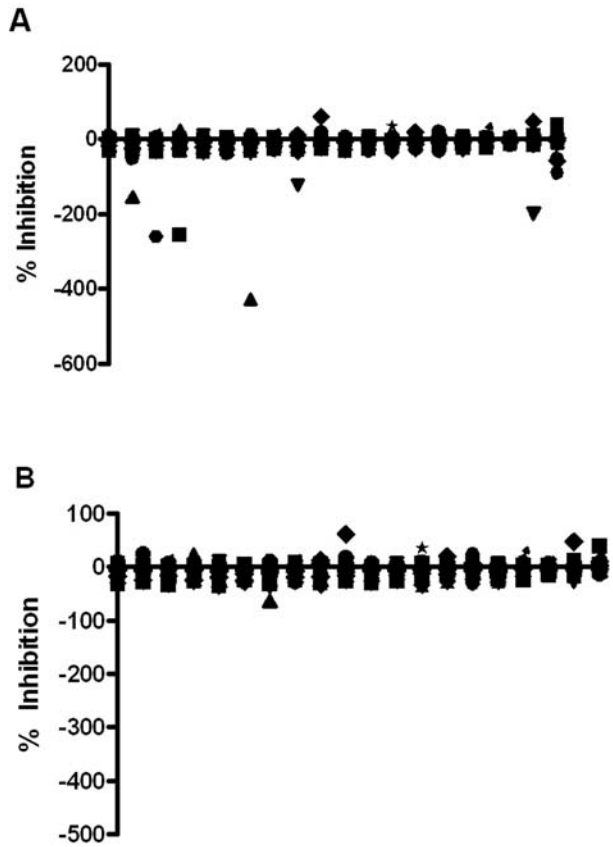

Fig. 5.2. **Enzyme activation activities before and after compound: fluorescence subtraction in a fluorescence-based phosphatase assay**. The phosphatase assay was performed in 384-well plates with a final volume of 50 μl. Twenty-five microliters of phosphatase in assay buffer was added to 0.5 μl of the Prestwick Chemical Collection compound prespotted on the assay plate. The plates were read in the fluorescence plate reader to obtain compound fluorescence values. Subsequently, the reaction was started by the addition of substrate (DiFMUP) in 25 μl assay buffer, incubated, and the reaction terminated followed by fluorescence detection. The percent inhibition values versus plate position (column number) for this library screen are shown before (**A**) and after (**B**) compound fluorescence was subtracted from the postreaction read. A negative percent inhibition is equivalent to activation.

Another approach to reducing the detection of false activators of enzyme activity is to use a kinetic read format such that the baseline for each well will be established with the first read and only an increase in velocity of the reaction (i.e., change in fluorescence or absorbance with time) will indicate a true activator.

3.5. Assay Buffer Considerations

3.5.1. Protein and Detergent Additives

Proteins are inherently sticky and finding nonselective compounds may be more prevalent in assays lacking detergent or with very low protein concentrations *(24, 25)*. In such assays, compounds may form aggregates in solution and inhibit many enzymes nonspecifically. Bovine Serum Albumin (BSA), casein, Tween-80, and Triton X-100 *(26)* are all examples of additives that help reduce either enzyme or compound "stickiness" or aggregation.

For example, the addition of 0.1% BSA has been used in kinase assays to reduce the binding of nonselective compounds *(27)*. However, some legitimate inhibitors bind to BSA and therefore the resulting free compound for enzyme inhibition will be low, resulting in the missing of some hits. Therefore, some groups favor leaving out such protein-based additives to increase the number of hits and use secondary follow-up assays to screen out nonspecific compounds and later modify structure to enhance compound availability in the presence of albumin (or serum).

3.5.2. Enzyme Assay Example 3: Effect of Detergent on Glycosidase Enzyme Linearity

In the course of developing a glycosidase assay, the time course was displaying nonlinear kinetics with the enzyme velocity decreasing with time. This assay was performed with picomolar concentration of purified enzyme in the absence of detergents or BSA. Suspecting that the enzyme might be slowly binding to plastic and being inactivated, we examined enzyme activity over time by preincubating diluted enzyme with and without 0.01% TX-100 from 0 to 2 hrs in a microtiter plate and then started the reaction (**Fig. 5.3**). A subsequent time course using assay buffer containing detergent resulted in a reaction that was linear with respect to time. This type of plastic-binding problem is more prevalent when using very low concentrations of highly purified enzyme in the absence of protein additives like BSA.

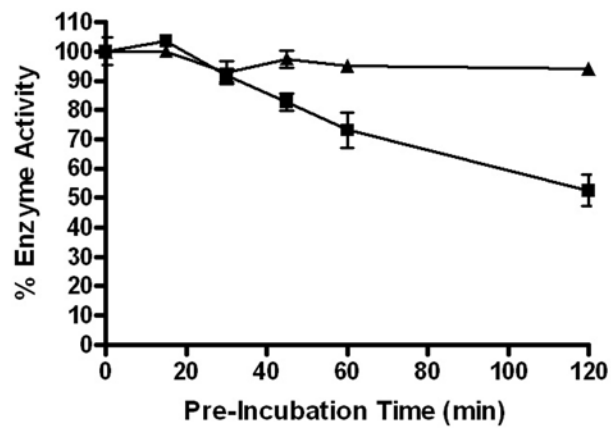

Fig. 5.3. **Effect of detergent on the stability of a glycosidase**. The stability of enzyme activity was followed over time by preincubating diluted enzyme in the presence (▲) or the absence (■) of 0.01% TX-100 from 0 to 2 hrs in a microtiter plate. The reaction was initiated by the addition of substrate and enzyme activity relative to the zero time point (considered 100% activity) was determined.

3.5.3. DMSO Tolerance

Another important issue in enzyme assays is the final concentration of DMSO in the screen reaction mixture; many enzymes are at least somewhat inhibitable by DMSO. Occasionally an increase in detection signal is observed with increasing DMSO concentration – this effect may be due to an affect on the enzyme or on the detection method. Since most compound libraries are initially solubilized in 100% DMSO then subsequently diluted in an aqueous buffer, the final reaction mixture will contain a certain concentration of DMSO. Depending on the initial dilution made, final DMSO percentages can range from below 1% up to 5%. Performing assay development experiments using the same concentration of DMSO as anticipated in screening is a good way to avoid these problems at the outset; however, a full range of DMSO concentrations should be tested (i.e., 0.5–10% DMSO). Should the compound library or dilution scheme change, knowing the acceptable range for the DMSO concentration in the assay is crucial.

3.6. Some Important Kinetic Considerations

Defining parameters (e.g., enzyme assay linearity, substrate concentration, and assay incubation time) that may impact the assay kinetics are important. Irrespective of the enzyme assay format used for HTS, kinetic considerations in assay design are critical for success *(16, 18, 28)*.

3.6.1. Enzyme Linearity

The accuracy of inhibition measurements will be dependent upon the linearity of the enzyme reaction. An enzyme concentration titration and time course should be performed to ensure that the conversion of substrate to product is linear with respect to enzyme concentration and time. These experiments should be done as one of the initial experiments in assay development and also confirmed using final assay conditions after optimization.

3.6.2. Substrate Concentration

The substrate concentration in an assay relative to its K_m is an important consideration *(18)*. The substrate concentration must be low enough to yield hits with a range of affinities, yet it must be sufficiently high to design a robust, low variability assay. It is important to know the type of mechanistic hits desired from the assay, for example, competitive or uncompetitive. Competitive hits will be harder to uncover when increasing substrate concentration above K_m but the opposite is true for hits that are uncompetitive. For competitive inhibitors, too high of a substrate concentration will interfere with the ability to identify weak competitors; conversely, a low substrate concentration will increase the incidence of finding weak competitors. A general rule for developing assays sensitive to competitive inhibitors is to set the substrate concentration in the assay to equal the K_m of the substrate, or if practical considerations preclude this (such as cost), to use a concentration below the K_m. According to

Cheng-Prusoff equation relating IC_{50} to K_i *(29)*, setting the substrate concentration at or below the K_m will result in the IC_{50} values generated being within twofold of the K_i.

3.6.3. End Point Versus Kinetic Read

Many assays are initially developed as kinetic assays, which may be difficult to translate into HTS. If possible, a method for terminating the reaction should be developed using either a known inhibitor or other means. We have found EDTA to be an effective method for terminating kinase reactions and a low percentage of SDS has proven effective for quenching a number of protease reactions. Once the enzyme and the substrate are added and the reaction is started, there is a minimal amount of time necessary to prepare for the termination of the reaction. A short incubation time must thus be avoided. Typically, a 30–60-min incubation time is chosen for enzyme assays. In addition, the stability of the signal from a stopped reaction should be determined over time to know when the assay can be read.

3.6.4. Control inhibitors

The identification and use of a control inhibitor greatly facilitates assay development and validation. A control inhibitor is extremely useful for verifying the enzymatic identity and purity of the enzyme preparation. To obtain reproducible and transferable dose–response curves, it is suggested to work from a single stock concentration of the inhibitor in DMSO. The dose–response curve should be set up as a serial dilution (usually a 1:2 or a 1:3 dilution scheme with 8–12 tested concentrations) in 100% DMSO and equal volumes of this dilution series is transferred into the assay. This dilution scheme minimizes compound solubility problems in aqueous solutions.

4. Is the Assay Ready for HTS?

In many instances, assay methods developed in an academic or a biology lab to monitor a novel enzymatic activity are found to be underdeveloped for HTS. There is typically a need to simplify the assay and move to a more HTS-amenable format.

4.1. Strategies to Adapt and Optimize Assay Protocols for Automated Screening

The goal of assay adaptation is to change an assay configuration for HTS without negatively impacting the validity or the sensitivity of the original assay. Assays should be adapted as follows:

1. Miniaturized as much as possible by moving from cuvette-based assays to 96- or 384-well formats.
2. Converted to operate at room temperature.

3. Assay volumes and order of addition should be adapted, simplified, and optimized for rapid automated assay assembly.
4. Where feasible, reduce reagent addition steps and minimize wash steps.

If possible, a modified protocol should be tested in the laboratory using automation in an identical fashion as the assay will be performed in the HTS lab.

4.1.1. Typical Issues for Adapting Assays to HTS

- *Reagent availability and stability*: When reagents cannot be obtained in relatively pure form and in sufficient amounts, are unstable, or exhibit contaminating activities with the potential to invalidate assay data.
- *Too many addition steps*: Too many liquid handling steps that cannot be consolidated present timing issues during HTS that usually result in high assay variability.
- *Lack of appropriate response to control compounds*: When the pharmacology of control compounds deviates significantly from published results.
- *Temperature*: Not all detection instruments have temperature control. Assays developed at temperatures other than ambient that cannot be performed at room temperature.
- *Assay variability*: Assays that exhibit indeterminate errors where it is not feasible to establish a Z-factor $\geq$0.5 with automation (*see* **Section 4.2**).
- *Incubation steps too short*: Incubation steps should be long enough for handling stacks of plates in an automated robust fashion. Ideally, end-point enzyme reactions should be 30–60 min long with a 30 min window of time allowed to stop multiple plates without negative effect on the assay.

4.1.2. Enzyme Assay Example 4: Adapting a Transferase Assay for HTS

In the following example, methyltransferase was originally developed as a radioactive SDS-PAGE assay. The goal was to convert this methyltransferase assay to a format compatible with HTS and as a result, a competitive fluorescence polarization (FP) immunoassay capable of detecting the activity of any S-adenosylmethionine (SAM)-utilizing methyltransferase was developed *(30)*. The competitive FP assay was developed to detect the S-adenosylhomocysteine (SAH) product of the methyltransferase reaction. The substrate of the reaction, SAM, is similar to chemical structure of the SAH product. The response of the assay to SAH was relatively linear up to about 40 nM product and the limit of detection was ~5 nM (0.15 pmol) SAH in the presence of 3 μM SAM.

An IC_{50} value was obtained for an inhibitor using the FP assay (**Fig. 5.4**). The IC_{50} value obtained with this FP enzyme assay was consistent with the value published using a mass spectrometry-based technique *(31)*. Unlike many other published enzyme

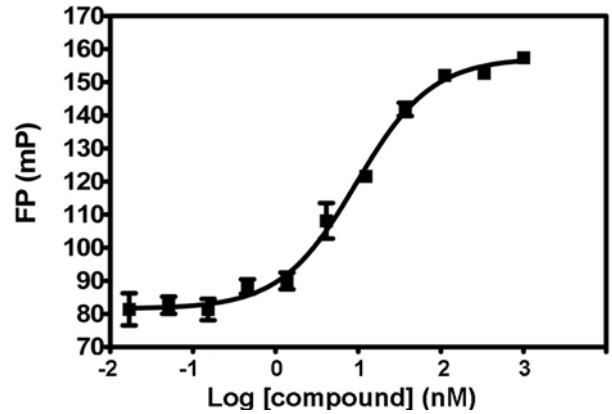

Fig. 5.4. **A typical dose–response curve for the methyltransferase assay**. Methyltransferase and the indicated concentrations of inhibitor were preincubated together for 5 minutes and then the reactions were initiated by the addition of SAM. Reactions were terminated with an EDTA/antibody/tracer solution. Fluorescence polarization was determined 1 hour after terminating the reaction. Average IC_{50} from three independent experiments was 12 nM. Each data point is the average of three replicates and error bars indicate standard deviation.

assays, the FP assay is "universal" because it has the potential to quantify activity of any methyltransferase. It is also 1,000-fold more sensitive in detection of product than previously published homogeneous, enzyme-coupled assays for this enzyme class. This high sensitivity to product was a critical feature of this assay, allowing detection of activity from low amounts of this difficult to express and purify enzyme. This FP assay is miniaturizable, homogeneous, very sensitive, of low cost, and requires only commercially available reagents and equipment *(30)*.

4.2. Determine Assay Variability

Assays adapted to HTS should undergo a series of tests to determine the variability of the assay. Small-scale variability studies provide preliminary data on assay performance. Control compounds or reagents used to validate the assay in assay development should be used to ensure that any changes implemented in the assay have not changed the validity or the sensitivity of the assay. The NIHCGC assay guidance manual is an excellent source for such tests and criteria.

Briefly, small-scale testing (single plates) of an adapted protocol should be performed with whole-plate assays including control wells (typically maximum signal and minimum signal wells) and DMSO in the body of the plate. This experiment provides data concerning the modified assay's overall performance including preliminary Z′ *(32)*, standard deviations (SDs), coefficient of variation (CVs), and intra- and interplate variability. If these preliminary values meet the acceptance criteria, then the assay should be tested with at least one control compound or reagent, e.g., a

known inhibitor with an established IC_{50} in the original assay. If the acceptance criteria are not met and no equipment malfunction is evident, then the protocol should be modified until the assay meets the criteria.

4.2.1. Assay Acceptance Criteria

1. Plate Uniformity Test
   a. Intraplate criteria
      i. CV_{max} and $CV_{mid} < 10\%$
      ii. $SD_{min} \leq$ average min signal
      iii. Normalized $SD_{mid} < 10\%$
      iv. Z'-factor ≥ 0.5
      v. No edge/patterned effects
   b. Interplate and interday criteria
      i. Within and across any 2 days: $\leq$15% difference in percent inhibition of midpoint plates
2. IC_{50} Reproducibility Test
   a. Less than threefold difference between values
   b. Less than threefold difference compared to original assay values
3. Compound Test Set Screen
   a. Z'-factor ≥ 0.5 for each plate
   b. Hit rate $< 3\%$
   c. Determine hit reproducibility

5. Notes

1. Assays that have multiple steps, including incubation times, numerous reagent additions, or washing steps are inherently noisier and are less precise. The more direct the assembly and readout of the assay, the easier to fulfill the requirements of acceptable data quality.
2. Homogeneous formats are generally preferred for HTS assays due to minimal steps, ease of automating the assay, ease of assay volume miniaturization, and typically lower variability.
3. In general, the goal of most HTS assays is to develop homogeneous nonradioactive assay formats to measure enzyme activity.
4. Activity in any homogeneous assay where compound interference with signal can occur should be confirmed in a completely different format (i.e., a nonhomogeneous radioactive assay such as a filtration assay).

5. Regardless of the chosen fluorescent format, the use of a concentration of the fluorophore-labeled substrate that is as high as practically and kinetically acceptable (within the limitations of screen cost and reagent availability) will minimize compound interference. If possible, use of a TR-FRET or red-shifted dyes will further minimize compound interference
6. Many assays are initially developed as kinetic assays and may be difficult to translate into HTS. If possible, a method for terminating the reaction should be developed using either a known inhibitor or other means such as EDTA or pH change.
7. The temperature stability of the enzyme and assay should be checked. Typically, screens are run at room temperature and HTS assays should be developed to operate at room temperature whenever possible. The stability of diluted enzyme solutions over time at room temperature should also be determined.
8. Many assays use truncated or surrogate substrates and there should be a requirement to confirm inhibition using the full-length or natural substrate.
9. The stability of potentially labile reagents, such as enzyme, cofactors, and substrates to freeze–thaw cycles should be tested.
10. "Universal" formats that can detect the activity of any member of a class of enzymes should be used with caution since the enzymatic purity is especially critical as there is no selectivity derived from the protein substrate.
11. The estimated cost for the planned screen using the desired format should be determined *before* any wet experiments are done and confirmed as soon as a working assay is developed.
12. In the authors opinion, it is preferable to use BSA (e.g., $\leq$0.1%) and/or a nonionic detergent in an enzyme assay buffer to reduce nonspecific interactions.

Acknowledgments

The authors would like to thank Dr. Li-An Yeh, the Director of the BRITE at NCCU, for her continued support. We want to thank Mark Hughes and Ginger Smith from the automation group at BRITE for their excellent technical support, and Dr. Tiffany Graves for kindly providing data. We are grateful to them and all our colleagues in the Biomanufacturing Research Institute &

Technology Enterprise (BRITE) at North Carolina Central University; including Dr. Gordon Ibeanu, Dr. Weifan Zheng, Dr. Al Williams, and Dr. Jonathan Sexton for their intellectual support. Finally, we would like to thank the Golden LEAF Foundation and the BIOIMPACT Initiative of the State of North Carolina through the Biomanufacturing Research Institute & Technology Enterprise (BRITE) Center at North Carolina Central University for financial support.

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