Chapter 13

Interpretation of Uniform-Well Readouts

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Summary

High-throughput screening (HTS) covers a range of measurements, from primary screens of either large libraries (>250 K) or small, focused collections (100–1,000 s) of test compounds, to secondary screens used to characterize the mechanism of action of a relatively small number of compounds. Data analysis of assay results from HTS relies upon assay performance and the control wells used to define the assay system. This chapter discusses parameters that must be considered when defining controls and plate maps for primary and secondary assays in HTS. Control wells and plate maps are suggested, which can generally be applied toward a variety of biochemical and cellular assays. The controls and plate-map options can be matched to the scale of the screening campaign; examples are primary screens with % inhibition or % activation as endpoints or secondary screens with IC_{50} or EC_{50} values as endpoints.

Key words: Background signal, Focused screens, High-throughput screening, Plate map, Secondary screening, Total signal, Z' factor.

1. Introduction

Control wells mapped to defined locations on assay plates (plate map) provide key information for HTS data analysis. Data obtained from control wells determine the utility of the assay data set and define the dynamic range of the assay. Data from control wells are also used to identify the variability within a screening assay, thus enabling the user to retain or discard well data for analysis. Without appropriate controls, the user runs the risk of overinterpreting screening results and pursuing molecules for further characterization that are really within the background noise of the screen. Alternatively, true positives will more likely be missed, particularly in cell-based assays, where the dynamic

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range of the assay may be limited or difficult to establish. Control wells are defined by components of the HTS assay and detection technology. Plate maps provide the user with a reproducible configuration on a given plate of two well types: control wells and test wells. Plate maps for HTS are designed to maximize test wells, while providing statistically significant replicates of control wells for data-quality control. Plate maps are an integral part of the standardization of HTS automation and data management. This chapter discusses options for choosing and applying control wells and plate maps to HTS for primary and secondary screens.

High-throughput screening is a multidisciplinary process that brings several scientific fields together *(1, 2)*. There are several activities that must be integrated to ensure a successful HTS campaign. These activities can be generally subdivided into four categories: (1) development of an HTS assay, typically biochemical or cellular *(3, 4)*; (2) handling of unknown samples of chemical, peptide, protein, RNA, or DNA *(5, 6)*; (3) implementation of assay detection technology platform utilizing full robotics, workstation(s), or manual processes *(7)*; and (4) data management and analysis *(8, 9)*.

To integrate the aforementioned activities, a viable HTS campaign will be able to intercalate into the screen the appropriate controls and establish suitable throughput to enable the generation of a large dataset on a collection of test samples. Two key factors toward integrating the various activities of HTS and thus enabling data analysis are establishing screening controls, and plate maps. In this chapter, examples are tailored to small-molecule (SM) HTS. The plate format used in examples is the 96-well format since this format can be easily scaled up (384- and 1536-well, and plate-free conditions) for HTS applications or scaled down (for 6-well, 24-well) to accommodate challenging secondary screening applications.

1.1. Overview of the Four Categories of a HTS Campaign

A well-developed assay is a key component in obtaining useful data from an HTS campaign *(10)*. Assays tend to be either biochemical or cellular endpoints. Whole animal technologies are available (*C. elegans*, zebrafish, etc.) and are usually implemented by specialized organizations *(11, 12)*. Various assay technologies are available to the user, providing choices for assay sensitivity (wide dynamic range of assay window) and throughput (homogenous vs. heterogenous assays).

There are many points to consider for small-molecule screening during assay development prior to HTS and data analysis. Briefly, key points for biochemical assays impacting HTS are the stabilities of assay proteins at time points used for the reaction and detection events of the assay. For example, early knowledge of protein stability will set parameters for the number of plates and samples that can be tested at a given time. The time course

of a biochemical reaction may be relatively short (i.e., period for initial rate of kinase activity may be in minutes), which will also influence the number of plates and samples that can be tested at a given time. Some key points for cellular assays are establishing the cell-line passage number that is acceptable for biological activity and the cell density necessary to measure biological activity. Providing stringent criteria for assays such as those mentioned earlier improve assay reproducibility and decrease the identification of false positives.

Options for assay controls include (a) positive controls, (b) total-signal controls, and (c) background-signal controls. The minimal controls needed to perform data analysis of HTS are the total-signal and background-signal controls. These two parameters provide information regarding the dynamic range of the assay and allow the user to quantify changes within the dynamic range (positive or negative from a reference point). A positive-control well with a known active agent in the assay (small molecule, protein, chelaters, etc.) at an IC_{50} or EC_{50} dose can also be useful for monitoring the dynamic range of the assay. Background-signal and total-signal controls provide the necessary parameters for the calculation of Z', a useful statistical parameter to assess the robustness of an assay *(13)*. A Z' value ≥ 0.5 is typically considered the acceptance limit for assay robustness for HTS *(10)*. However, there are assays, particularly cellular and complex secondary assays, where $Z' \geq 0.5$ may be an insufficient criteria for assessing assay performance. Gribbon et al. have described multiple aspects of quality control of screening data: intrawell, intraplate, and systematic trends in data analysis *(14)*. Systematic approaches to defining assay performance must be defined and applied postscreen to fully capture high-quality HTS data and form relevant conclusions *(15, 16)*.

The composition of a test sample is a key factor for HTS design and data analysis. Although HTS is commonly referred to in the context of screening large collections of small molecules to identify drug candidates, HTS concepts and technologies are also applied to screening of peptide, protein, RNA, or DNA samples. For example, hybridoma and monoclonal antibody screening is a powerful combination used to identify antibodies either as tools or as potential therapeutics *(17)*. Advances in molecular-biology techniques, coupled with chip technologies, have led to HTS applications of gene profiling to characterize up- or downregulation of genes in various cellular and disease states *(18)*.

Points to consider for small-molecule screening prior to initiating HTS and data analysis are the properties of the small-molecule library. Key factors for small molecules are (a) storage, (b) stability, and (c) structure. These points are beyond the scope of this chapter and are reviewed in the literature *(19–21)*. The challenge and costs of maintaining a large high-quality library

have encouraged organizations to use smaller focused libraries, designed by molecular modeling, for testing against specific targets *(22, 23)*. Large compound collections remain in frequent use, as there is a wide range of molecular targets available for drug screening. The large library collections reflect greater diversity of chemical space. However, libraries fraught with degraded compounds increase the difficulty of data analysis.

Implementation of an assay technology platform is a key part of generating robust HTS data for analysis. HTS laboratories have many choices for establishing their infrastructure. Fully automated sample retrieval and assay screening approaches are available where liquid handling is integrated with the assay readout *(24, 25)*. Alternatively, stand-alone technology platforms can be utilized for liquid handling and assay readout. Based on the sample number and the difficulty of the assay, HTS may remain a largely manual process. This is often the case for secondary screens utilizing low-throughput assays to characterize the biological activity of compounds identified by HTS.

Points to consider for small-molecule screening prior to initiating HTS and data analysis are potential erroneous results due to the technology platform being utilized. For example, liquid-handling and storage needs differ significantly between 96- and 1,536-well plates *(26)*. Chip-based technologies and microfluidics have enabled new formats for biochemical and cellular assays *(27)*. HTS technologies must be matched carefully with the appropriate plate and assay format to ensure that datasets are free of technology-based artifacts.

The choice of which detection technology to use is often driven by the biological assay endpoint, coupled with the availability of assay reagents. Radioactive-based detection technologies continue to retain a place in HTS, due to the ability to directly label protein, enabling detection with minimal modification to a protein and thus enabling assays when alternate detection reagents are unavailable. However, in recent years advances in fluorescence detection have led to an increase in the availability of reagents for fluorescence-based biochemical, cellular, and whole animal endpoints *(28, 29)*. The introduction of new fluorescent tags that are coupled to key reagents enabled several new assay formats including time-resolved fluorescence resonance energy transfer and fluorescence polarization *(30, 31)*. These assay types primarily generated biochemical assays with high dynamic range, homogenous, and automation-friendly formats. Cell-based assays have also benefited from the increase in commercially available fluorescently tagged reagents. For example, high-content screening (HCS) assays utilize microscopic fluorescence cell imaging of multiple distinct fluorescent molecules *(32, 33)*. Though HCS may be low-throughput when compared with other assay technology platforms, the value lies with the use of multiple

fluorophores, generating multiple endpoints. Cellular fluorescence imaging endpoints offer an attractive complementary assay format to commonly used cellular reporter-gene assays. Fluorescent technologies and their application in HTS and drug discovery have been reviewed extensively *(34, 35)*.

Data management and analysis are key components of extracting maximal value from an HTS dataset. Once the various components of HTS have been identified (compound collection, assay technology, assay parameters, etc.), one can determine the controls that will be used for the screening campaign. For example, if data is to be analyzed as % inhibition or % activation, control wells must be included that define total signal (100%) and background signal (0%). Quality control of HTS data can be based on the plate map and the assay controls used. It is vital that data be captured in a centralized database with criteria established for data entry. This facilitates analysis of datasets that are free of erroneous results and artifacts.

There are multiple approaches to identifying active compounds, or *hits*, for follow-up study from HTS *(36–40)*. Data analysis can be a largely automated or a manual process, depending on the software systems utilized. There are two frequently used approaches for identifying active compounds from HTS. First, active compounds can be defined as those that meet a given statistical criterion of activity or inhibition relative to a vehicle-control distribution. For example, if the assay endpoint is activation of a signaling event in a cell-based assay, three standard deviations from the mean of vehicle-control wells (designed to measure total signal in the absence of activating compound) can be used as the criterion for identifying active compounds. Since there will be well-to-well variations within a plate, the criterion might be expanded to include three standard deviations from the mean of a total-signal distribution including sample wells. This approach can be applied for generic library screens with success; however, when applied to focused libraries, in which sample wells generate a high hit rate, the approach can have limited value. Second, active compounds can be defined as those that meet an arbitrary readout criteria outside of the variability found within a given assay. For example, if the assay endpoint is the inhibition of cell proliferation at a given time point, an arbitrary definition of actives may be defined at those that have a % inhibition value of ≥50% inhibition. This approach can be beneficial when screening focused library collections or intentionally targeting a hit rate to accommodate follow-up studies.

Sophisticated statistical approaches based on computer-generated models of compound collections and activity can be utilized *(41)*. Relying solely on Z' and control wells for data analysis is a limited approach and does not provide the

user with information about trends across multiple screens or compound collections. However, as control wells are the starting points for analysis of HTS datasets, this chapter focuses on the practical points of defining control wells and the plate maps for data analysis. These parameters can be readily expanded upon by more sophisticated statistical and model-driven approaches.

There are numerous options for plate maps, which can be utilized. Key determinants are the plate type, assay format, and availability of controls. Shown in **Fig. 1** are commonly used plate maps for HTS in a 96-well format.The map can be readily scaled up or down to accommodate alternate density of wells on a plate. The plate map for primary screening is designed to accommodate control wells and a single dose treatment of compound wells (**Fig. 1a**). Based on the availably of reagents for the primary screen and the assay robustness, one can determine the number of replicates needed to test sample wells. Replicates wells of n= 2 are recommended for primary HTS when assay reagents are available. The plate map is simply replicated in sample preparation to generate two daughter plates for HTS to provide the n= 2. The plate map for secondary screening is designed to accommodate control wells and multiple doses of compound for the generation of dose response curves (IC_{50} or EC_{50}) (**Fig. 1b**). The map shown allows for

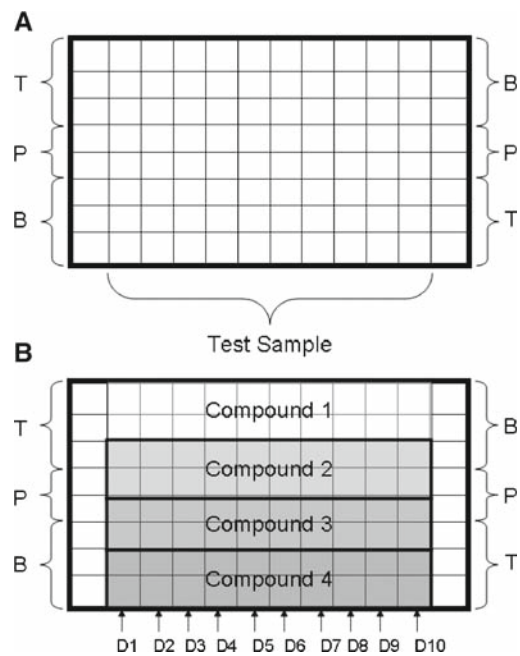

Fig. 1. Test compound sample and dose-response plate maps.

testing of four compounds in a ten-point dose response curve, where each dose is tested in duplicate. A ten-point dose curve can readily accommodate a 3–4 log concentration range of test compound, facilitating a full curve in a single measurement *(42)*. This plate map avoids multiple repeats to identify the appropriate dosing range of a given compound and provides sufficient data points to generate an acceptable curve fit. For example, a ten-point curve using a threefold dilution factor covers from 10 μM to ~0.5 nM (four logs). If throughput is a determining factor to the user for secondary screens, the plate map can be modified to accommodate fewer doses. For example, throughput will be doubled by modifying to a five-point dose curve. However, users must reconcile the quality of curve fit with the throughput obtained.

Control wells in the representative plate maps (**Fig. 1**) contain total-signal, positive-control, and background-signal wells. Total-signal wells and background-signal wells are key control wells, as they define the assay window and set the parameters for data quality control. Data from these control wells are critical in measuring assay robustness (i.e., Z') for a given plate. Thus, the majority of control wells on the plate map are dedicated to total signal (n= 6) and background signal (n= 6). Controls are divided into outer columns and inverted. This allows the user to review edge effects and signal drifts, and compensate accordingly. Positive-control wells are a desirable optional control, if available. These wells provide additional information regarding the assay window and can be particularly informative with assays that have a limited dynamic range. For example, for a given cellular assay measuring activation of a signaling event at a given time point, the positive control well can be defined as the 50% activation point measured by the assay. This layout provides the user with clear guidelines for the midpoint of the assay window on a given plate. If positive controls are unavailable (i.e., limited access to agonist/antagonist, or EC_{50} value is unknown), the plate map can be modified to eliminate positive-control wells in favor of additional total-signal and background-signal control wells.

1.2. Summary

A sample procedure for generating control wells and data analysis from a specified plate map for a 96-well SM HTS assay is described. This protocol utilizes standard liquid-handling approaches and can be readily modified to accommodate alternate density plate formats and screening detection technologies. Data analysis described is based on control wells typically utilized in HTS and provides a framework for further analysis with more sophisticated statistical and modeling approaches.

2. Materials

2.1. Library

1. Master storage plates with lyophilized test SM sample (*see* **Note 1**).
2. DMSO (Sigma-Aldrich, St. Louis, MO).

2.2. Assay and Automation

1. Biochemical or cellular assay for HTS (*see* **Note 2**).

2.3. Data Analysis

1. Database for capture of HTS data (*see* **Note 3**).
2. Software for data analysis (*see* **Note 4**).

3. Methods

3.1. Compound Library Preparation

3.1.1. Preparation of a Master Compound Plate

1. Resuspend powder of test compounds in 100% DMSO at 10 mg/mL or 10 mM in 96-well deep well plates or desired plate format. Fill wells based on test compound location of plate map (**Fig. 1a**). Label plates and track compound identification, well location, and plate label in database (*see* **Note 5**).
2. Mix to resuspend compounds by pipetting up and down. Use a single pipette tip for a single compound to avoid contamination of compounds from well to well.
3. Make a note in database of compounds that do not go into solution. This will serve as a flag for potential problems in assay data interpretation due to compound precipitation.
4. Store plates frozen at 4°C until ready for use. Stock plates can be stored for the duration of a screening campaign and utilized for preparation of plates for secondary screens. DMSO stocks will freeze at 4°C (*see* **Note 6**).

3.1.2. Preparation of Working Compound Plate

1. Prepare control-well solutions and add to specified wells of the plate map (**Fig. 1**). Identify liquid volumes based on HTS assay protocol. For total-signal control wells, options include (a) DMSO, (b) assay buffer, or (c) cell culture media. For background-signal control wells, options include (a) total signal including known inhibitor at specific concentration, or (b) assay reagents excluding a key reagent (cofactor, growth factor, etc.) needed to facilitate the reaction.
2. Prepare test compounds for single-dose (**Fig. 1a**) or dose-response (**Fig. 1b**) testing based on the plate map. Remove the master compound plate from storage and thaw to room temperature. When possible, use freshly made master plates. Make note in database of any new compound precipitation observed.

For HTS, screening concentrations typically vary from 1 to 50 μM. The dose is set higher for testing random large library collections, and lower for screening focused library collections. For IC_{50} or EC_{50} testing, a range of ten doses over 3–4 log units is typically used to generate a reliable curve. The upper limit is generally 10–50 μM. Higher doses of test compounds are subject to solubility problems and interference with assay-detection technologies. Introduce a mixing step for each serial dilution for the dose curve. For HTS or dose-response curve plate preparation, identify stock liquid and dilution buffer volumes, based on the assay protocol, and pipette into the working plate based on the plate map.

3. Store plates frozen at –20°C as needed. If possible, use working compound plates on the day of preparation.

3.1.3. Preparation of Compound Assay Plate

1. For assay protocols in which reagents are added directly to test compounds, prepare two mother–daughter compound plates for testing based on the assay protocol (*see* **Note 7**). Label plates in database for tracking purposes. Use plates immediately for screening (*see* **Note 8**).
2. For assay protocols in which compounds are added directly to an assay plate, draw replicate compounds for two mother–daughter assay plates directly from the working plate (*see* **Note 9**). Label assay plates in database for tracking purposes. In this scenario, addition of compound initiates the assay measurement, or an additional reagent step is needed to initiate the assay.

3.2. Assay

1. Run assay plates, keeping within the parameters of the HTS protocol. Examples of key assay parameters are light, time, temperature, and CO_2.
2. Recommendation: Include a control plate at the beginning and/or end of a given run (**Fig. 2**). Generate control plates based on total- and background-signal control wells (**Fig. 1**) being used for screening (*see* **Note 10**). Inclusion of a control plate allows the user to look for trends in a given testing run, such as a drift in the control-well signal.
3. Upon conclusion of assay, acquire assay readout based on endpoint being measured.

3.2.1. Database

Capture the following information (minimal requirements) in centralized database:

1. Assay name.
2. Assay description (*see* **Note 11**).
3. Screening dose.
4. Date.

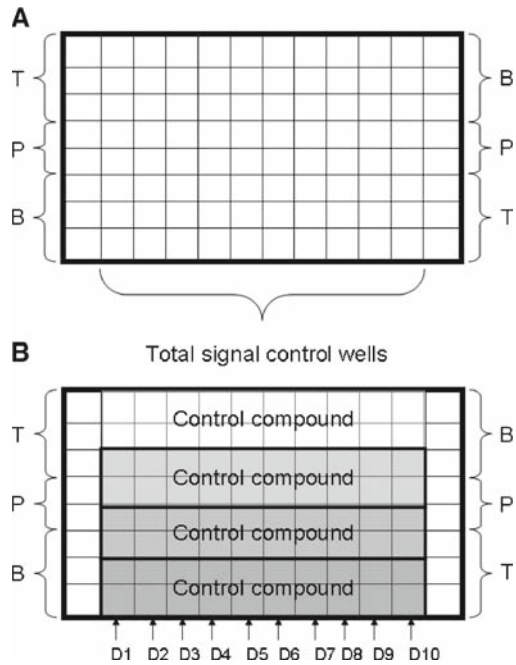

Fig. 2. Control compound sample and dose-response plate maps for assay development, or to accompany test compound sample plates in an HTS run.

5. User.
6. Plate format.
7. Plate map.
8. Compound source identification (working stock and master stock).
9. Data.
   a. Compound identification code.
   b. Screening raw data.
10. Key observations during screen (*see* **Note 12**).

3.2.2. Data Analysis

1. Calculate statistical parameters per plate using control wells on each plate.
2. Calculate signal/background (S/B) ratio and signal/noise (S/N) ratio:

$$S/B = \text{mean}X_2/X_1, \quad (1)$$

$$S/N = \text{mean of } X_2 - \text{mean}X_1/\text{standard deviation of } X_1. \quad (2)$$

where X_1 = minimal assay signal obtained from background control wells of plate map and X_2 = maximal assay signal obtained from total signal control wells of plate map.

3. Calculate Z' value:

$$Z' = 1 - (3S_2 + 3S_1)/X_2 - X_1. \quad (3)$$

where S_1 = standard deviation of X_1 and S_2 = standard deviation of X_2.

4. Using S/B, S/N, and Z' values, apply *pass* or *fail* criteria based on assay performance per plate. Identify plates ready for data analysis (*hit* identification or IC_{50} calculations). If data corresponding to control wells do not meet the *pass* criteria for a given plate, retest the compounds.
5. Calculate percent inhibition (% inhibition) or percent activation (% activity) for primary screen:

$$\%\ \text{inhibition} = 100 \times (1 - (T - \text{mean}X_1)/(\text{mean}X_2 - \text{mean}X_1)), \quad (4)$$

$$\%\ \text{activity} = 100 \times (T - \text{mean}X_1)/(\text{mean}X_2 - \text{mean}X_1)), \quad (5)$$

where X_1 = minimal assay signal obtained from background control wells of plate map, X_2 = maximal assay signal obtained from total signal control wells of plate map, and T = signal obtained from given compound test well of plate map. T can be based on n = 1 or as mean of n (i.e., n = 2 if duplicate measurements of test compound is obtained). The plate map and assay technology used will define the replicates necessary to establish T.

6. Identify active or *hit* compounds from the screen. Based on assay performance, identify threshold for active test compounds. Threshold for specific compound activity is defined per plate or per run based on the total-signal and background-signal control wells. Identify active compounds for follow-up characterization either by their ability to show an arbitrarily defined activity (i.e., 50% inhibition) or by their ability to show activity that is statistically significantly distinct from control wells. For example, define hit compounds as those where the test compound signal is three standard deviations above signal generated by background control wells (*see* **Note 13**).
7. Calculate IC_{50} or EC_{50} for dose-response activity in the secondary screen.
8. Plot fractional activity (y axis) of test compound as a function of the test compound concentration (X axis), based on the ten points of the plate map using curve-fitting software of choice.
9. Fit the data points to a curve using standard four-parameter logistic nonlinear regression analysis (i.e., GraphPad) (*see* **Note 14**).

10. Obtain IC_{50} or EC_{50} from curve fit; by definition, the test compound concentration that generates 50% inhibition of the total signal is the IC_{50} or EC_{50} value.
11. Ensure that there are sufficient data points defining the upper and lower plateaus of the curve to generate an accurate IC_{50} value. The advantage of the ten-point curve plate map is that there are multiple data points for the upper and lower plateau, and that it can define the slope of the curve. If there are not sufficient data points to define the curve, repeat the dose-response curve testing by shifting the dosing concentration as needed and/or modifying the fold dilution of the testing range to ensure full coverage by the dataset.

3.3. General Database and Analysis Management

1. Review data corresponding to compounds with solubility problems carefully. Insoluble compounds can form aggregates and generate spurious results in assays *(9)*.
2. A screening run with multiple plates may undergo a signal loss for total-signal control wells and a signal gain for background-signal control wells over the time course of the run. This effect reduces the assay window, and makes data analysis difficult. To prevent this scenario, perform runs within the range of assay performance (Z'). It may be necessary to run multiple small batches of assay plates for a given assay rather than long runs.

4. Notes

1. A variety of options are available from commercial suppliers of compound libraries for storage and handling. For handling of liquid DMSO solutions of test compound, use gloves and avoid exposure to test compounds. Compound collections may contain agents that are absorbed through skin via DMSO solution.
2. Use assays and automation with well-defined protocols. For example, for a biochemical assay establish parameters such as time point, temperature, and protein stability. For example, for a cellular assay, establish parameters particular to cell line, such as passage number, serum, and time points (with or without stimulation). Common assay-detection parameters to be established are detection reagent stability and stability of readout signal. This allows for flexibility in identifying the number of plates that can be accommodated in a given HTS run. Automation allows for greater precision and *hands-free operation*. However, for focused library collections and limited follow-up testing, automation may be limited to plate washers, multichannel pipettors, etc.

3. Typically, HTS campaigns capture data output in a single database linking chemical data to biological data, facilitating data analysis. Databases can either be generated in-house or purchased from commercial suppliers. It is beyond the scope of this chapter to define all database options. Commercial options include Accelrys® (www.accelrys.com) and IDBS (www.idbs.com). It is worth noting that many laboratories active in HTS generate a fusion between in-house and commercial databases.
4. Data-analysis tools are often integrated into databases (Accelrys Accord, IDBS ActivityBase). In addition, there are several stand-alone software packages, which are routinely applied. Spotfire is commonly utilized for assessing trends in HTS datasets. GraphPad (www.graphpad.com) and Kaleidagraph (www.synergy.com) are commonly utilized for secondary screens.
5. The efficiency with which test compounds are processed for HTS can be improved with a barcoding system. Speed and accuracy minimizes compound degradation and time spent in tracking compound plates during various stages of HTS.
6. If DMSO stocks do not freeze at 4°C, this likely is an indicator that the solution has incorporated water. The stock concentration is now likely inaccurate due to the presence of water. It can be difficult to determine the water content in the compound solution and thus recalculate an accurate compound concentration. Therefore, generate fresh DMSO stock solutions if sufficient test compound is available.
7. This configuration of reagent addition is more common for biochemical assays where proteins are directly added to DMSO or test compound. If multiple assay reagent additions are needed to initiate a reaction, a useful pipetting approach is to aspirate various reagents with an air gap separating each liquid followed by a single dispense. This approach can be used to add reagents simultaneously to test compound and initiate reaction.
8. Use freshly prepared plates to avoid loss of protein activity in the assay and/or compound degradation.
9. Ideally, the order of addition of assay reagents and compound will have been established during assay development for HTS. The addition of high concentrations of DMSO to proteins or cells can be a problem. Order of addition of assay reagents allows the user to control reaction kinetics ($t = 0$) and the introduction of DMSO to the assay.
10. Define the map for control plate (**Fig. 2**) using either total-signal wells (**Fig. 2a**) for a primary screen or control dose-response curve (**Fig. 2b**) for secondary screen. Utilize a plate map where the outer wells of the plate retain the control-well

configuration on each plate of an HTS run. Inclusion of a control plate allows user to identify and address edge effects, which can be pronounced during long testing runs. Corrective measures may be technical *(43)* or based on statistical data analysis *(8)*. For example, edge effects have been shown to be minimized in a cell-based assay by incubating freshly seeded plates of cells at room temperature prior to placing in an incubator.

11. A brief assay description with a link to a detailed protocol can be a valuable reference point to the user when comparing datasets from various assays. For example, if comparing cellular proliferation assay data across various cell lines, important factors for comparison are the cell type, readout (e.g., BrdU), time points, and starting cell densities. Ideally, these key parameters are captured in the database to facilitate queries across all assays.
12. Observations noted during the course of a given screen aid the user in performing data quality control of a given HTS run. Tracking events out of the ordinary (e.g., robotic crashes, clogging of liquid handlers, compound precipitation) allow the user to determine which data can be used and which compounds require retesting to acquire useful data.
13. Positive-control wells where the control compound is introduced to wells at its IC_{50} or EC_{50} value provide a benchmark for establishing the midpoint of the dynamic range of the assay on a given plate. Positive-control wells are a useful practical reference point for identifying a range of potency values for active compounds on a given plate; particularly if an arbitrary potency (i.e., 50% inhibition) is chosen as the criteria for the identification of active compounds. The contents of the positive-control wells can be modified readily to suit the potency range targeted by the user (e.g., 25%, 50%, 75%).
14. Shown below is a commonly used four-parameter logistic equation for curve fitting. Details can be found at http://www.graphpad.com and *(42)*.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - X)\,\text{HillSlope}}}.$$

References

1. Verkman, A. (2004) Drug discovery in academia. *Am. J. Physiol. Cell Physiol.* **286**, 465–474.
2. Root, D., Kelly, B., and Stockwell, B. (2002) Global analysis of large-scale chemical and biological experiments. *Curr. Opin. Drug. Discov. Dev.* **5**, 355–360.
3. Butcher, E. (2005) Can cell systems biology rescue drug discovery? *Nat. Rev. Drug Discov.* **4**, 461–467.
4. Liu, B., Li, S. and Hu, J. (2004) Technological advances in high-throughput screening. *Am. J. Pharmacogenomics* **4**, 263–276.
5. Archer, R. (2004) History, evolution and trends in compound management for high throughput screening. *Assay Drug Dev. Technol.* **2**, 675–681.
6. Echeverri, C. and Perrimon, N. (2006) High-throughput RNAi screening in cultured cells: A user's guide. *Nat. Rev. Genet.* **7**, 373–384.

7. Harding, D., Bradford, D., and Brown, G. (2002) Transferring industrial automation technology to the laboratory. *J. Assn. Lab. Automat.* **7**, 84–88.
8. Malo, N., Hanley, J., Cerquozzi, S., Pelletier, J., and Nadon, R. (2006) Statistical practice in high throughput screening data analysis. *Nat. Biotechnol.* **24**, 167–175.
9. McGovern, S., Caselli, E., Grigorieff, N., and Shoichet, B. (2002) A common mechanism underlying promiscuous inhibitors from virtual and high throughput screening. *J. Med. Chem.* **45**, 1712–1722.
10. Carter, J. (ed.) (2003) A Guide to Assay Development. D&MD, Westborough, MA.
11. Rabitsch, K., Toth, A., Galova, A., Schleiffer, G., Schaffer, E., Aigner, C., Rupp, A., Penkner, A., Moreno-Borchart, A., and Primig, M. (2001) A screen for genes required for meiosis and spore formation based on whole-genome expression. *Curr. Biol.* **11**, 1001–1009.
12. Parng, C., Weng, W., Semino, C., and McGrath, P. (2002) Zebrafish: A preclinical model for drug screening. *Assay Drug Dev. Techmol.* **1**, 41–48.
13. Zhan, J., Chung, T., and Oldenburg, K. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73.
14. Gribbon, P., Lyons, R., Laflin, P., Bradley, J., Chambers, C., Williams, B., Keighley, W., and Sewing, A. (2005) Evaluating real life high throughput screening data. *J. Biomol. Screen.* **10**, 99–107.
15. Sun, D., Whitty, A., Papadatos, J., Newman, M., Donnelly, J., Bowes, S., and Josiah, S. (2005) Adopting a practical statistical approach for evaluating assay agreement in drug discovery. *J. Biomol. Screen.* **8**, 508–516.
16. Eastwood, B., Farmen, M., Iversen, P., Craft, T., Smallwood, J., Garbison, K., Delapp, N., and Smith, G. (2006) The minimum significant ratio: A statistical parameter to characterize the reproducibility of potency estimates from concentration-response assays and estimation of replicate-experiment studies. *J. Biomol. Screen.* **11**, 253–261.
17. Lal, S., Christopherson, R., and Remedios, C. (2002) Antibody arrays: An embryonic but rapidly growing technology. *Drug Discov. Today* **7**, 141–149.
18. Stegmaier, K., Ross, K., Colavito, S., O'Malley, S., Stockwell, B., and Golub, T. (2004) Gene expression-based high-throughput screening (GE-HTS) and application to leukemia differentiation. *Nat. Genet.* **36**, 257–263.
19. Archer, R. (2004) History, evolution and trends in compound management for high throughput screening. *Assay Drug Dev. Technol.* **2**, 675–681.
20. Chan, J. and Hueso-Rodriguez, J. (2002) Compound library management. *Methods Mol. Biol.* **190**, 117–127.
21. Holden, K. (2003) The significance of effective compound management. *Curr. Drug Discov.* **9**, 9–10.
22. Bajorath, J. (2002) Integration of virtual and high-throughput screening. *Nat. Rev. Drug Discov.* **1**, 882–894.
23. Sun, D., Chuaqui, C., Deng, Z., Bowes, S., Chin, D., Singh, J., Cullen, P., Hankins, G., Lee, W., Donnelly, J., Friedman, J., and Josiah, S. (2006) A kinase-focused compound collection: Compilation and screening strategy. *Chem. Biol. Drug Des.* **67**, 385–394.
24. Sundberg, S., Chow, A., Nikiforov, T., and Wada, H. (2000) Microchip-based systems for target validation and HTS. *Drug Discov. Today* **5**, 92–103.
25. Falconer, M., Smith, F., Surah-Narwal, S., Congrave, G., Lui, Z., Hayter, P., Ciaramella, G., Keighley, W., Haddock, P., Garethwaldron, G., and Sewing, A. (2002) High-throughput screening for ion channel modulators. *J. Biomol. Screen.* **7**, 460–465.
26. Dunn, D., Orlowski, M., McCoy, P., Gastegeb, F., Appell, K., Ozgur, L., Webb, M., and Burbau, J. (2000) Ultra-high throughput screen of two-million member combinatorial compound collection in a miniaturized, 1536-well assay format. *J. Biomol. Screen.* **5**, 177–187.
27. Battersby, B. and Trau, M. (2002) Novel miniaturized systems in high-throughput screening. *Trends Biotech.* **20**, 167–173.
28. Bailey, S., Sabatini, D., and Stockwell, B. (2004) Microarrays of small molecules embedded in biodegradable polymers for use in mammalian cell-based screens. *Proc. Natl. Acad. Sci. USA* **101**, 16144–16149.
29. Miller, L. and Cornish, W. (2005) Selective chemical labeling of proteins in living cells. *Curr. Opin. Chem. Biol.* 9, 56–61.
30. Hertzberg, R. and Pope, A. (2000) High-throughput screening: A new technology for the 21st century. *Curr. Opin. Chem. Biol.* **4**, 445–451.
31. Ozkan, M. (2004) Quantum dots and other nanoparticles: What can they offer to drug discovery? *Drug Discov. Today* **9**, 1065–1071.
32. Bowen, W. and Wylie, P. (2006) Application of laser-scanning fluorescence microplate cytometry in high content screening. *Assay Drug Dev. Tech.* **4**, 209–221.
33. Dove, A. (1999) Drug screening-beyond the bottleneck. *Nat. Biotechnol.* **17**, 859–863.

34. Buttner, F. (2006) Cell-based assays for high-throughput screening. *Exp. Opin. Drug Discov.* **1**, 373–378.
35. Jager, S., Brand, L., and Eggeling, C. (**2003**) New fluorescence techniques for high-throughput drug discovery. *Curr. Pharm. Biotechnol.* **4**, 463–476.
36. Iversen, P. W., Eastwood, B. J., Sittampalam, G. S., and Cox, K. L. (2006) A comparison of assay performance measures in screening assays: Signal window, Z′ factor, and assay variability ratio. *J. Biomol. Screen.* **4**, 247–252.
37. Bleicher, K., Bohm, H., Muller, K. and Alanine, A. (2003) Hit and lead generation: Beyond high-throughput screening. *Nat. Rev. Drug Discov.* **2**, 369–379.
38. Walters, W. and Namchuk, M. (2003) Designing screens: How to make your hits a hit. *Nature Rev. Drug Discov.* **2**, 259–266.
39. Gagarin, A., Makarenkov, V., and Zentilli, P. (2006) Using clustering techniques to improve hit selection in high throughput screening *J. Biomol. Screen.* **11**, 903–914.
40. Woodward, P.W., Williams, C., Sewing, A., and Benson, N. (2006) Improving the design and analysis of high throughput screening technology comparison experiments using statistical modeling. *J. Biomol. Screen.* **11**, 5–12.
41. Konstantin, B. and Nikolav, S. (2006) Computational methods for analysis of high-throughput screening data. *Curr. Comp. Drug Des.* **2**, 1–19.
42. Copeland, R. A. (ed.) (2000) *Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis.* Wiley-VCH, New York.
43. Lundholt, B., Scudder, K. and Pagliaro, L. (2003) A simple technique for reducing edge effect on cell-based assays. *J. Biomol. Screen.* **8**, 566–570.