Reporting data from high-throughput screening of small-molecule libraries

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Publications reporting results of small-molecule screens are becoming more common as academic researchers increasingly make use of high-throughput screening (HTS) facilities. However, no standards have been formally established for reporting small-molecule screening data, and often key information important for the evaluation and interpretation of results is omitted in published HTS protocols. Here, we propose concise guidelines for reporting small-molecule HTS data.

High-throughput screening (HTS) is becoming a routine method for identifying probes used in chemical biology. The flexibility of the process has allowed numerous and disparate areas of biology to engage with an equally diverse palate of chemistry. However, with this diversity, the many components associated with HTS—the bioassay, the assay format, the nature of the library, reagent and sample delivery methods, the detection instrumentation, the level of automation, and the data analysis algorithms—all conspire to make HTS one of the least standardized processes used in academia today. Nonetheless, there exists commonality in HTS that allows reporting of data describing fundamental parameters of the assay, screen, library, and outcome that are useful for gaining insight into these HTS processes and for comparing results between screens.

Here we suggest data and descriptive information to include in HTS protocols in manuscripts and databases that should aid in providing a basis for evaluation, comparison and replication of small-molecule screens. We have divided small-molecule screen protocol information into five categories: the assay, the library, the HTS process, the post-HTS analysis of data and compound structures, and the screen results (**Fig. 1**). Within each category, we describe key pieces of information that are important for interpretation and replication of a screen (**Table 1**).

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Assay

Assays fall into three general types: isolated molecular target assays, cell-free multicomponent assays, and cell- or organism-based assays. Assays on purified enzymes such as proteases or kinases, and assays on activities associated with cell extracts, membranes or reconstituted signaling cascades, are representative examples of the first two assay types. Cellular assays can be subdivided into 'reporter gene'–type assays and phenotypic assays that measure outputs resulting from intact cellular processes.

A description of the logic behind the assay, including positive and negative control conditions, is critical to understanding how library compounds will be scored as active in the assay. This description provides context for the assay's sensitivity to specific types of interference. Positive controls are conditions (for example, small-molecule addition, RNA interference knockdown, or mutations) that produce the same result in the assay as a desired active compound. Negative controls are usually 'vehicle'-only conditions (for example, DMSO) or, where appropriate, small molecules demonstrated to have no activity in the assay. Controls are used to determine an assay 'window' and validate the biological response. It is often desirable to provide an indication as to the efficacy of the controls. For example, here is an assay strategy description that might be written for a fluorescence polarization assay:

> This screen was carried out to identify compounds that disrupt formation of the X–Y protein complex. The assay uses fluorescence polarization to monitor binding of a fluorescein-labeled peptide, X36* (derived from the protein X extracellular domain), to full-length protein Y. Screening positives are compounds that block binding of X36* to protein Y. Addition of unlabeled X36 peptide (5 μM) completely blocks binding of X36* in this assay and is used as a positive control. DMSO alone is the best negative control for this assay during HTS.

For some assays (in particular for cell-free assays), it is possible to get quantitative measurements of the dynamic range and sensitivity of the assay. When available, this information is useful to help evaluate the quality of an assay. For example, this information might be presented for a fluorescence polarization assay as "This assay was linear in the range of 60 mP units (the polarization observed for free peptide) to 170 mP units (the polarization observed for fully bound peptide) with an error of ± 5 mP units. Under screening conditions, we estimate that differences of 5 to 10% of peptide bound could be reliably distinguished."

The sources of all reagents used in the screen should be documented. Catalog and batch numbers (if relevant) for all commercially

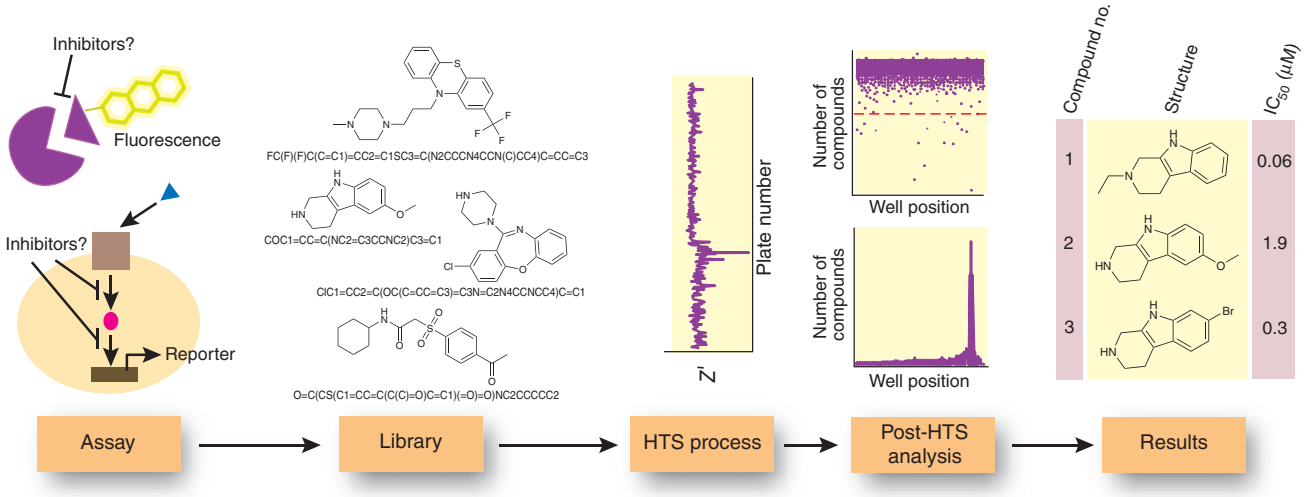

Figure 1 The flow of materials and data from assay to reported results in HTS.

available components should be listed. Descriptions of how all noncommercial reagents were generated or obtained should be provided. Availability of proprietary reagents should be described. It is helpful if the amounts of key assay reagents required to screen a library of a defined size in a specified assay format are mentioned explicitly.

A clear summary of the assay protocol should be provided for each screen. The instruments used to perform each step should be indicated. This can be written in paragraph form; for example, "Assay plates were filled with 1,000 HeLa cells per well in 30 μl of medium (Matrix Wellmate plate filler) and incubated in a Liconic STX200 incubator for 16 hours before compound addition...." Presenting HTS assay protocols in table form is also a good option, especially for more complex protocols. **Table 2** shows an example adapted from Davis *et al.*[1].

Library

In order to allow others to evaluate the nature of the results and to replicate work in the future, it is essential to provide sufficient knowledge regarding the nature of the library that has been screened. First, the constituency of the library should be described (for instance, peptide, natural product, known drugs), with corresponding detail provided about the type of compounds (for instance, core scaffolds or privileged structures) represented. An indication of the size or number of members in the library and how samples are presented to the assay (for instance, arrayed individually or pooled) should be provided. If the library was screened as mixtures then a description of the procedures needed to identify the active compound from the mixture (deconvolution) should be provided. For natural products, the nature of the samples should be described (that is, are the samples purified and structures determined, or are extracts being used?). Finally, information should be provided about the quality control procedures used to acquire and maintain the library, and about the source of the library. If available a web link can be included providing additional library information. When files detailing the composition of a library are provided, where possible they should include structure and unique/vendor ID information. Example:

> The library screened consisted of 50,000 compounds arrayed in 384-well plates as single compounds at 10 mM in DMSO (additional detail describing the library may be obtained from the links included in the supplementary information). The quality of all compounds was assured by the vendor as greater than 90% pure, with provided quality control data; this was verified internally on 5% random sampling. The library was screened at a constant 1:1,000 dilution, with a 10-μM final concentration of compound in each well (0.1% DMSO). Supplementary information: http://www.msdiscovery.com/spectrum.html for library .xls or .sdf files.

HTS process

Though most HTS laboratories use microtiter plate–based platforms, the following descriptive information should serve as a general guide for nonplate-based processes as well. Assay controls are critical to evaluation of assay response and to the performance of any assay, and for microtiter plate assays they are often arranged as follows: interplate controls are used to assess and correct, when possible, systematic variations in the biological response over time (for example, slow clogging of a dispenser tip). Intraplate controls are essential to the establishment of the assay window (as described above), and over the course of a screen they permit the analysis of the uniformity of the biological response. The following two examples from Davis *et al.*[1] illustrate how this information can be described:

> Interplate controls: plates containing vehicle only (in place of test compounds) were uniformly distributed throughout the screen at ten-plate intervals to monitor systematic variation in background.
>
> Intraplate controls: columns 1–4 of the 1,536-well plates were used for arraying of controls. Columns 1 and 2 contained a 16-point dose-response curve of MG132, with each concentration present in duplicate. In column 3, the top 24 wells contained doxycycline only (for use as normalization to the minimum signal), and the bottom 8 wells contained assay medium alone for use as a reference for induction by doxycycline. In column 4, the top 24 wells contained the highest concentration of MG132 (for use as a 100% activation reference), and the bottom 8 wells again contained assay medium alone.

The number of assay plates and screen duration are useful metrics that should be incorporated into a description of the HTS. Reagent and compound dispensing systems, detectors (type, model, settings), and output mode (end point, kinetic, and so on) should be adequately detailed. Availability of custom detectors or data handling methods should be included, as well as critical details to optimize output reads, such as spectral overlap 'spillover corrections' for multifluorophore[2] or multireporter gene–based assays[1]. If these instruments are peripheral devices on a robotic platform, additional details regarding the nature of the

software controlling the system should be included. Two examples:

> Seventy-eight 1,536-well plates representing a library of 100,000 compounds and interplate controls (7 plates) were screened over an 8-h period. Cells were dispensed into compound-containing 1,536-well microtiter plates previously loaded using an Echo555 liquid handler (Labcyte, Inc.), with a single-channel BioRAPTR FRD (Beckman Coulter, Inc.). Microtiter plates were read on an EnVision Multilabel Plate Reader (Perkin Elmer, Inc.) with 405 nm excitation, dual emission at 460 and 530 nm using a bottom read.

> Target enzyme was added to 384-well plates using a Freedom EVO75 (Tecan), followed by addition of compound with a 384-hydrophobic-coated FP3S100 pin tool array (V&P Scientific).

Correction and normalization procedures describe the methods by which screening results were corrected for systematic error, as, for example, discussed by Gunter *et al.*[3] using the B-score method, and normalized to controls. Typically data normalization is expressed as either 'percentage inhibition', 'percentage activity', or potency values (for example, EC_{50}, IC_{50}, AC_{50}) if multiple concentrations are tested in the screen (for example, % inhibition = 100 × (corrected sample result – average of positive control)/(average of negative control – average of positive control)).

Performance of the assay during the screen is commonly calculated using the *Z* factor, a statistical parameter that takes into account the signal to background and assay signal variation[4]. Plate-based *Z* factor determinations provide a measure of the screen performance. Both control (*Z*′) and sample (*Z*) factors can be reported. The minimum significance ratio (MSR) can be reported for in-plate reference titrations[5].

Post-HTS analysis

After HTS, the primary screening data are analyzed and follow-up assays are carried out to confirm the activity of compounds that score as 'positives' in the primary screen. Chemical structures of active compounds should be verified before being reported. Addressing the following questions should provide the audience with an understanding of how the investigator arrived at the compounds of interest: how were compounds selected as active in the primary screen? How were the initial active compounds retested to confirm activity? How was compound chemical structure confirmed? Have active compounds been further purified or resynthesized? Two examples:

> Data were normalized as percentage activity relative to positive control. Active compounds were defined as those in the 99.5th percentile. Individual samples of actives were rearrayed in 384-well plates from separately maintained master samples and resubjected to the original screening assay, at 10 μM fixed concentration, with triplicate sampling for each active. Those giving a reproducible (2 of 3 or 3 of 3) activity were used to

Table 1 Reporting parameters for small-molecule screening data

Category	Parameters	Examples (see text for more detail)
Assay	Nature of the assay	Cell-free multicomponent assay, or mammalian cell-based imaging assay
	Assay strategy	Detection of double-stranded DNA using intercalated fluorescence enhancement of fluorophore, or cellular cytosol-to-nuclear translocation of GFP-tagged nuclear hormone receptor
	Reagents and sources	Standard information
	Assay protocol	Key steps are outlined in **Table 2**
Library screened	Nature of the library	Rule of 5–compliant or stochastic clustering analysis gave 332 fingerprint diversity clusters, normalized to 17.1 clusters per 100 compounds (http://ccc.chem.pitt.edu/upcmld/Library_Diversity_Analysis.html)
	Size of the library	50,000 compounds arrayed in 384-well plates as single compounds at 10 mM in DMSO
	Source	University of Kansas Chemical Methodology and Library Development Center
	Details	An SD-format file of the UPCMLD library containing structure, ID, etc. is available at http://pubchem.ncbi.nlm.nih.gov/ (use PubChem compound UPCMLD)
	Quality control	All compounds assured by vendor as >90% pure with provided QC data; verified internally on 5% random sampling
	Concentration tested	Constant 10 μM concentration, 0.1% DMSO, 1:1,000 dilution
HTS process	Format	96-well imaging plate (BD BioSciences)
	Plate controls	Positive control: EC_{50} agonist (A1-D1); negative control: EC_{50} agonist + 10× IC_{50} antagonist (E1-H1); 12A-H: titration of agonist
	Plate number and duration	150 96-well plates over 3 d
	Reagent and compound dispensing systems	Reagents and compounds delivered using a VPrep (Velocity11)
	Output, detector, analysis software	Fixed endpoint; imaging microscopy using ArrayScanV^{Ti} (Cellomics, Inc.); Molecular Translocation BioApplication (Cellomics, Inc.)
	Correction factors	B-score analysis and correction
	Normalization	% inhibition = 100 × (corrected sample result – average of positive control)/(average of negative – average of positive control)
	Performance	*Z* and *Z*′ plotted per plate for 100-plate screen. Interplate EC_{50} MSR = 2.5
Post-HTS analysis	Selection of actives	Actives were selected from the primary screen using a threshold based on statistical criteria
	Retesting of initial actives	Original samples rearrayed and retested using screening assay; compounds with replicated activity tested in dose-response mode
	Structure confirmation	Compound structure verified by analytical chemistry methods
	Compound purification/resynthesis	Validated actives resynthesized or repurchased and retested
Screen results	List of all screening positives	List of positives ranked by % activity at fixed concentration and defined selection cutoff threshold
	List of validated compounds	Rank order of compounds, based on score in selection criteria
	Comments on active compound selection	Potency, cellular efficacy, pharmacological parameters and toxicity used to rank actives

Table 2 Example HTS assay protocol table

Step	Parameter	Value	Description
1	Plate cells	3 µl	5,000 OCI-Ly3 cells
2	Controls	20 nl	±doxycycline, media, MG132
3	Library compounds	20 nl	57 µM to 0.7 nM dilution series
4	Reporter induction	1 µl	Induce CBR and CBG68 luciferases
5	Incubation time	4 h	37°C, 5% CO_2
6	Reporter reagent	4 µl	Chroma-Glo detection
7	Incubation time	10 min	Ambient temperature
8	Assay readout	540 and 618 nm	CCD imager, luminescent mode

Step	Notes
1	Solid white tissue culture–treated plates, 1-tip dispense cells all wells
2	Columns 1–2, 16-pt MG132 titration, duplicate; column 3, rows 1–24 doxycycline only, rows 25–32 medium; column 4, rows 1–24, 10 µM MG132, rows 25–32 medium only. MG123 added with Pintool (V&P Scientific), media ± doxycycline added with nanoliter reagent dispenser
3	Pintool transfer (tip wash sequence: DMSO, iPA, MeOH, 3-s vacuum dry)
4	20 ng ml^{-1} stock concentration doxycycline
5	Plates covered with stainless steel gasket-lined lids containing pinholes for gas exchange
6	8-tip dispense reagent all wells
7	Plates lidded until read
8	$G' = \frac{Lgf - (R' \times (Rgf/R))}{Ggf/G}$ $R' = \frac{Lrf - (Lgf \times (Grf/Ggf))}{(Rrf/R) - (Rgf/R) \times (Grf/Ggf)}$ Green filter (540/20 nm); red filter (618/8 nm); 15-s exposure; correction factors for spectral overlap between green and red luminescence

Adapted from ref. 1.

produce 10-point dose-response curves ranging from 30 µM to 1 nM, with triplicate sampling. Active compounds of interest for further study were defined as those with a reproducible EC_{50} of less than 1 µM. These compounds were subjected to further secondary screening including the use of a second biochemical test of activity, a cellular measure of pathway activity, cytotoxicity measurements using four human cell lines, and solubility and permeability measurements.

All compounds of interest were subjected to LC/MS/ELSD analysis using a sample from the original screening stock and a sample from the medium remaining after completion of the dose-response study (at the highest concentration). Each compound used for subsequent studies was either repurchased from the original vendor and purified in-house, or synthesized and purified in-house.

Results

Ranking of primary screening actives and of confirmed active compounds can serve as a useful means to describe the outcome of the HTS and analysis, and it is further enhanced with the inclusion of comments on factors that disqualified initial actives and on ranking strategies. Example:

> The structures of all primary HTS and confirmed active compounds are included in the supplementary materials. Compounds considered for additional study, ranked by percentage activity, are shown in Figure 1. Confirmed active compounds are defined as those with a potency that is equal (within five-fold) in both biochemical assays, a similar (within ten-fold) potency in the cellular assay, a minimal dosage window (20-fold) between the cellular activity assay and any single cytotoxicity assay, solubility of greater than 10 µM in water (pH 7.4), and permeability in the PAMPA model (pH 7.4 to 7.4) of greater than $1{,}000 \times 10^6$ cm s^{-1}.

Conclusions

In this commentary, we have proposed an initial set of guidelines for reporting small-molecule HTS data, which is summarized in Table 1. The development of a standardized protocol to describe small-molecule screening projects would aid in the unambiguous interpretation of published results and facilitate transfer of screening data between databases. The screening and data standards communities should develop these guidelines more fully, based on the frameworks of existing minimum information guidelines efforts[6] (http://mibbi.sourceforge.net/), and to the level of detail of the minimum information about a cellular assay (MIACA) data model[7] (http://miaca.sourceforge.net/) and related standards that describe biological experiments.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

1. Davis, R.E. *et al. Assay Drug Dev. Technol.* **5**, 85–103 (2007).
2. Bertelsen, M. *Methods Enzymol.* **414**, 348–363 (2006).
3. Gunter, B., Brideau, C., Pikounis, B. & Liaw, A. *J. Biomol. Screen.* **8**, 624–633 (2003).
4. Zhang, J.H., Chung, T.D. & Oldenburg, K.R. *J. Biomol. Screen.* **4**, 67–73 (1999).
5. Eastwood, B.J. *et al. J. Biomol. Screen.* **11**, 253–261 (2006).
6. Brazma, A., Krestyaninova, M. & Sarkans, U. *Nat. Rev. Genet.* **7**, 593–605 (2006).
7. Orchard, S. *et al. Proteomics* **6**, 4439–4443 (2006).