

Brief Guide: Experimental Strategies for High-Quality Hit Selection from Small-Molecule Screening Campaigns

SLAS Discovery

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Abstract

Small-molecule screening is a powerful approach to identify modulators of either specific biological targets or cellular pathways with phenotypic endpoints. A myriad of assay technologies are available to assess the activity of enzymes, monitor protein–protein interactions, measure transcription factor activity in reporter assays, or detect cellular features and activities using high-content imaging. A common challenge during small-molecule screening is, however, the presence of hit compounds generating assay interference, thereby producing false-positive hits. Thus, efforts are needed to uncover such interferences to prioritize high-quality hits for further analysis. This process encompasses (1) computational approaches to flag undesirable compounds, and (2) the use of experimental approaches like counter, orthogonal, and cellular fitness screens to identify and eliminate artifacts. In this brief guide, we provide an overview for first-time users, highlighting experimental screening strategies to prioritize high-quality bioactive hits from high-throughput screening/high-content screening (HTS/HCS) campaigns.

Keywords

high-throughput screening, high-content screening, hit triaging, false-positive exclusion, orthogonal assay, counter assay, cellular health

High-throughput screening (HTS) and high-content screening (HCS) are used to identify small-molecule modulators of either precise targets in target-based screenings or distinct pathways and phenotypes in phenotypic screenings.^{1,2} Screening campaigns initially generate a large set of active compounds (primary hits). The most challenging task during the early hit selection phase is to discard false-positive hits and at the same time score the most active and specific compounds.^{3,4} Therefore, a cascade of computational and experimental approaches should be used to select the most promising hits.^{5,6} This brief guide aims at presenting a short overview of essential procedures to experimentally triage primary hit sets toward specific and high-quality hits, while eliminating artifacts.

One important prerequisite for successful screening is the rigorous development of an optimized screening assay (biochemical or cell-based) in terms of robustness, reproducibility, signal window, as well as adjustment to automated robotic platforms. Positive and negative controls should be available to check the quality of the assay and the generated data.⁷ It is safe to say, “Quality in, quality out.” Using such optimized assays to screen a large number of small-molecule modulators will generate a first list of active compounds (hits), which need to be confirmed in independent experiments to pursue reliable hits. The primary

screening is usually done at a single compound concentration. Thus, in a next step, the primary hit compounds are tested in a broad concentration range to generate dose–response curves, from which IC_{50} values can be calculated.⁵ Thereby, the shape of the dose–response curves can give important information. Dose–response curves with steep, shallow, or bell-shaped curves may indicate toxicity, poor solubility, or aggregation of the compound. Hence, these hits are usually removed. Compounds not generating dose–response curves are generally discarded because of lack of data reproducibility. Notably, compounds with artifact nature can also provide convincing-looking dose–response curves. Therefore, to further reduce the number of false-positive hits, computational and experimental strategies are

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needed to effectively select specific compounds. In the following, we will briefly touch on computational approaches and mainly focus on experimental approaches.

Computational analysis of historic data from other screening campaigns can be useful to flag compounds with frequent-hitter potential.⁸ Such frequent-hitter activity may arise from general assay interference or from target or pathway promiscuous molecules (e.g., pan-kinase activity). Importantly, larger datasets of historic screenings can be used to develop cheminformatics filters [e.g., a pan-assay interference compounds (PAINS) filter] to spot and flag promiscuous and undesirable compounds or chemotypes causing general assay interference.^{9–13} Besides, structure–activity relationship (SAR) analyses, which look at the relationship between the molecule structure and its biological activity, can help identifying truly active compound clusters. SAR interpretations also need caution, however, because assay-interfering compounds can actually display a convincing SAR, which is referred to as a structure–interference relationship. Importantly, a genuine SAR provides confidence and potential for future lead optimization.¹⁴ In contrast, a flat SAR suggests nonselective or no binding, and can be a criterion for exclusion of such hits.

Experimental efforts to follow up on HTS/HCS results should include counter, orthogonal, and cellular fitness screens (see **Fig. 1**, upper right), which can be conducted either in parallel or in a consecutive manner. These types of follow-up testing assays do not necessarily need to be high-throughput, because the number of selected primary hits is markedly reduced compared to the size of the screening compound collection.

Counter screens are needed to assess the specificity of hit compounds and thus eliminate false-positive hits (artifacts).⁵ This process is critical to classify and eliminate compounds that interfere with the readout technology used in the screening assay (assay technology interference). Effects such as autofluorescence, signal quenching or enhancing, singlet oxygen quenching, light scattering, and reporter enzyme modulation can cause compound-mediated assay readout interference. In this case, counter screens can be designed in a way that bypasses the actual reaction or interaction, and solely measure the compound action on the detection technology. Also, hit compounds may disrupt affinity capture components; hence, a distinct tag exchange (e.g., His-tag versus StrepTagII) can generate hit confidence. Buffer conditions can help reduce assay interference by adding, for example, bovine serum albumin (BSA) or detergents to counteract unspecific binding or aggregation, respectively. In cell-based assays, absorbance and emission tests can be performed in control cells. Moreover, nonselective inhibition of target-independent activity can originate from nonspecific protein reactivity, aggregation, chelation, or redox interference, which should be analyzed in independent counter assays, usually in designed cell-free counter assays.

The purpose of performing orthogonal screens is to confirm the bioactivity of compounds found to be effective in the primary screen, with additional readout technologies or assay conditions to guarantee specificity.¹⁵ Orthogonal assays analyze the same biological outcome (e.g., biochemical reaction or cellular phenotype) as tested in the primary assay, but use independent assay readouts.⁵ Also, biophysical assays are implemented in target-based approaches to characterize compound actions and affinity. Examples of complementary readout technologies to be used in validation experiments are:

1. Fluorescence-based readout during primary screening assays (biochemical or cell-based assays) can be backed up by luminescence- or absorbance-based readouts in follow-up analysis.
2. Biophysical assays, including surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), microscale thermophoresis (MST), thermal shift assay (TSA), and nuclear magnetic resonance (NMR), should be used in biochemical target-based approaches to validate hit compounds as well as generate affinity data.
3. Bulk-readout assays (plate reader based, one readout value per well) in primary screening should be replaced by microscopy imaging and high-content analysis in follow-up testing to allow the inspection of single-cell effects of hit compounds rather than population-averaged outcomes. Thereby, high-content analyses (e.g., morphology, texture, translocation, or intensity) will provide a more comprehensive picture of the compound's effects on the cellular phenotype.
4. In cell-based phenotypic screening, the use of different cell models (2D vs. 3D cultures; fixed vs. live cells) or disease-relevant (primary) cells can be productive tools to validate screening hits in biologically relevant settings.

Cellular fitness screens are necessary to exclude compounds exhibiting general toxicity or harm to cells. This step is important to classify bioactive molecules that maintain global nontoxicity in a cellular context.¹⁶ Cellular fitness can be assessed using assays with bulk readout representing the health state of the treated cell population as a whole. Examples are investigations of cell viability (e.g., CellTiter-Glo and the MTT assay), cytotoxicity [e.g., the lactate dehydrogenase (LDH) assay, CytoTox-Glo, and CellTox Green], or apoptosis (e.g., the caspase assay).¹⁷ In addition, microscopy-based techniques can be used to test cellular health, which can give a more detailed analysis of the proportion of damaged versus healthy cells on a single-cell level. High-content analysis can evaluate cellular fitness using nuclear staining [DAPI (4',6-diamidino-2-phenylindole) and Hoechst] and counting, staining of mitochondria

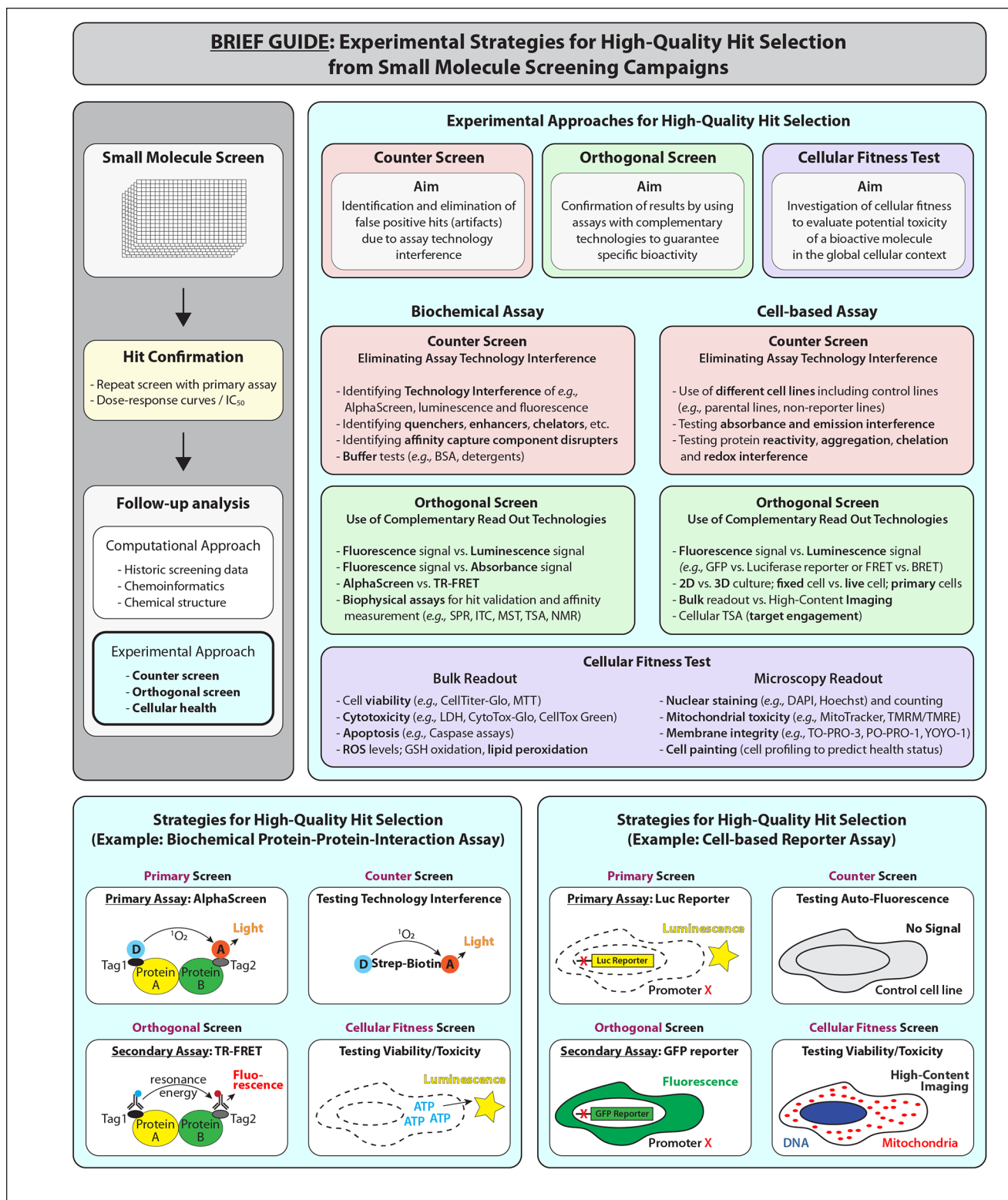


Figure 1. Experimental approaches for high-quality hit selection.

[e.g., MitoTracker and TMRM/TMRE (tetramethylrhodamine methyl ester and tetramethylrhodamine ethyl ester, respectively)], or analysis of membrane integrity (e.g., TO-PRO-3, PO-PRO-1, and YOYO-1).¹⁸ In addition, cell painting is a high-content image-based format for morphological profiling using multiplexed fluorescent staining of eight broadly relevant cellular components or organelles.¹⁹ This extensive staining and subsequent machine learning analysis can provide a comprehensive picture of the cellular state on compound treatment.²⁰ Profiles based on cell painting can be used to predict and label healthy cells versus compound-mediated cellular toxicity.^{21,22}

During early drug discovery, we advise running counter, orthogonal, and cellular fitness screens to develop a detailed picture of a compound's effects. This will aid the decision as to whether a compound can be optimized to an effective, specific, and non-cytotoxic lead. Here, we provide concepts for experimental approaches to select high-quality hits, which we illustrate with two examples (see Fig. 1).

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