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Apparent Activity in High-Throughput Screening: Origins of Compound-Dependent Assay Interference

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Summary of recent advances

Expansive compound collections made up of structurally heterogeneous chemicals, the activities of which are largely undefined, present challenging problems for high-throughput screening (HTS). Foremost is differentiating whether the activity for a given compound in an assay is directed against the targeted biology, or is the result of surreptitious compound activity involving the assay detection system. Such compound interference can be especially difficult to identify if it is reproducible and concentration-dependent – characteristics generally attributed to compounds with genuine activity. While reactive chemical groups on compounds were once thought to be the primary source of compound interference in assays used in HTS, recent work suggests that other factors, such as compound aggregation, may play a more significant role in many assay formats. Considerable progress has been made to profile representative compound libraries in an effort to identify chemical classes susceptible to producing compound interference, such as compounds commonly found to inhibit the reporter enzyme firefly luciferase. Such work has also led to the development of practices that have the potential to significantly reduce compound interference, for example, through the addition of non-ionic detergent to assay buffer to reduce aggregation-based inhibition.

Introduction

High-throughput screening (HTS) involves testing hundreds of thousands, if not millions, of chemical compounds for activity in a biological assay. Originally developed as a drug discovery methodology by pharmaceutical companies for identification of chemical entities with potential therapeutic value, HTS has branched into the academic sector as a technique to discover chemical probes, which are applied as research tools to study a biological target or pathway [1–3].

Libraries used for HTS consist of chemicals with a high degree of structural heterogeneity (compared to nucleic acid or peptide libraries, for example) that often have unknown and undefined properties and biological activities. One challenge in HTS is to successfully differentiate between compounds that demonstrate genuine activity against the biological target, target family, or pathway of interest from compounds that interfere with elements of the assay format or technique. Generally, specific activity of a compound against its target is characterized by a high affinity, non-covalent interaction that is reversible [4]. Activity in an

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assay due to compound interference can result in a “false positive” (see Box 1) [5], and as compounds that are genuinely active against the target are rare (~0.01–0.1% of library), they are easily obscured by a high incidence of false-positives in a screen (Fig. 1A) [6–8]. In this review we will focus on a discussion of compound activity that reproducibly interferes with HTS assays. Such compound interference can arise solely from the compounds themselves [5,9], as is the case for fluorescent compounds (Fig. 1B), or can be the result of their interaction with biological components of the assay system. The latter is the case for compounds that directly inhibit a biological reporter, such as firefly luciferase (Fig. 1C), or compounds that indirectly inhibit or activate proteins in an assay, for example, by undergoing redox cycling to generate hydrogen peroxide. Non-specific chemical reactivity against target proteins, such as covalent binding or metal ion chelation [4,10], is another source of false positives in HTS [11–13], but is out of the scope of this Review and thus will not be discussed in detail here except in the context of redox reactivity.

Introduction to artifacts typical of light-based detection methods in HTS

Current HTS technologies rely heavily on sensitive light-based detection methods, such as fluorescence or luminescence, to quantify the effect of a compound on a target molecule or signaling pathway [5]. While advantages to light-based detection technologies include a desirable balance between sensitivity and ease of automation for HTS, they are also susceptible to a wide-range of different types of assay interference (see Table 1) [9]. Whereas most light-based assay interference is due to spurious events or occurs only at a high(er) compound concentration(s), compound fluorescence and luciferase inhibition show reproducible concentration dependence, making their identification initially more challenging.

Compound Fluorescence

Whether or not a compound fluoresces in an assay depends upon its structural properties and the excitation and emission wavelengths used in the experiment. Generally, conjugated bonds within the compound confer fluorescent character, and the greater the degree of conjugation within a compound, the longer the wavelength at which it fluoresces [14]. Compound libraries tend to contain a greater percentage of heterocyclic compounds and compounds with low levels of conjugation [5,15,16], and thus assays that rely on excitation at relatively short wavelengths (λ_{ex} ~ 350 nm) with detection of fluorescence in the blue spectral region (λ_{em} = 450–495nm), have a greater likelihood of false positives due to compound fluorescence [14]. An additional consideration is compound purity, as samples with minor, albeit highly fluorescent, impurities are also a source of assay interference.

The prevalence of fluorescence interference also depends on the concentration of compound used in the assay relative to the assay fluorophore. This is especially a problem in HTS if fluorophores are used at low concentrations, for example, 1nM or less, in screens with compounds that are commonly tested at concentrations of 10 μ M or greater, so that if a compound sample is fluorescent, it has a high likelihood of interfering with genuine assay signal [15]. Efforts to profile a representative compound collection (<http://pubchem.ncbi.nlm.nih.gov>; PubChem Assay IDs – AIDs – 587–594, 709) indicated that ~2–5% of the compounds in the library fluoresced in the blue spectral region (e.g. AlexFluor 350, coumarin-like fluorescence λ_{ex} ~350nm/ λ_{em} ~440nm), whereas with excitation at ~560nm and detection at ~585nm (typical of resorufin, an orange fluorophore), only 0.004–0.01% of the library fluoresced (Fig. 2A and Table 1) [11,14]. Additionally, Simeonov *et al.* (2008) found that for several fluorescence-based assays involving excitation/emission in the blue spectral region, up to 50% of the actives identified in the screen were actually fluorescent [14].

Detection of fluorescence in the blue spectral range is also plagued by spurious “activity”, which is often due to fluorescence of lint or other particulate matter (Fig. 1B, grey dots) [14, 17] or scattering of light by precipitates/assay components. Both of these phenomena occur with greater incidence at shorter wavelengths of light [14,15,18,19]. Obtaining fluorescence intensity data at a range of compound concentrations can help identify if the activity seen is spurious or due to compound fluorescence [14,20]. Compound fluorescence is reproducible and concentration dependent, with the degree of fluorescence detected proportional to compound concentration (Fig. 1B, blue dots) [14]. If activity follows a concentration-response, it is important to use an orthogonal assay (ideally using a different output reporter, like bio-, chemi-, or electrochemi-luminescence) to determine if the activity is independent of the assay format/technique [14]. There are a number of ways to significantly reduce the occurrence of fluorescence-based artifacts in an assay, as indicated in Table 1.

Compounds that inhibit firefly luciferase: concentration-dependent inhibition or activation in cell-based assays

Firefly luciferase (FLuc)-based bioluminescence assays are highly favored in HTS due to their sensitivity (extremely low endogenous background signal leads to superior signal to background ratio relative to fluorescence methods) [21] and dynamic response in cell-based reporter gene assays, due to a relatively short FLuc protein half-life [22]. FLuc itself, however, is an enzyme, and is thus susceptible to inhibition by small molecules used in screening. A profiling effort of a 70K compound library determined that at least 3% of the library inhibited the enzymatic activity of FLuc in a concentration-dependent manner (Fig. 1C) [6]. This is actually thought to be an underestimate of the true number of FLuc-active compounds in the library, as weakly competitive FLuc inhibitors were likely not identified using this assay format. Chemical series that include compounds found to inhibit FLuc include quinolines, 1,2,4-oxadiazoles, and benzthiazoles (also benzimidazoles and benzoxazoles) - the latter being structurally similar to the natural FLuc substrate, D-luciferin [6].

Although the short protein half-life of FLuc in cells allows FLuc reporter gene assays to be exquisitely responsive to signal modulation by compounds, it also predisposes the FLuc protein to stabilization by compounds. This phenomenon was originally described by Thompson *et al.* (1991), in which it was found that incubating cells with certain compounds led to an accumulation of FLuc protein, by interaction with and stabilization of the FLuc protein by the compound, which ultimately led to increased bioluminescent output upon detection (Fig 2B and Supplementary Figure S1) [22]. This apparent activation is due entirely to off-target activity of the compound against the FLuc reporter protein, and also shows concentration-dependence. Retrospective analysis of cell-based FLuc reporter gene assays indicates that as many as 60% of the actives identified for a given screen are actually compounds that act as FLuc inhibitors [23]. In addition, cell-based assays that require long incubation times with compound, or that measure an increase in FLuc activity from an initially low basal level of activity, are especially prone to a significant enrichment of FLuc inhibitors amongst active compounds [23,24]. Further complicating the identification of FLuc inhibitors in cell-based assays is that these compounds can either appear as activators or inhibitors of the cell-based assay depending upon the affinity of the compound for FLuc, the basal FLuc concentration, whether the compound acts as a competitive or non-competitive inhibitor, and what type of detection reagent is used [24,25].

Determining whether a given active is a FLuc inhibitor is simple and highly recommended: test the compound in a purified FLuc assay using K_M concentrations of substrates (ATP and D-luciferin). Results from profiling efforts carried out by Auld *et al.* (2008) are publically available (PubChem AID 411) [6] and have already assisted investigators in the identification of compounds that inhibit FLuc [26]. Additionally, in order to confirm genuine activity against

the target, compounds identified in a FLuc assay should be re-tested in an orthogonal assay using an alternate reporter before being tested in secondary assays. It should also be mentioned that it is possible for a compound that inhibits an enzymatic reporter to also have relevant biological activity [27,28].

Promiscuous enzymatic inhibition in biochemical assays: compound aggregation

Compound aggregation was recently discovered to be one of the main causes for promiscuous enzyme inhibition [12,29–32]. Under certain conditions, above certain concentrations, some compounds self-associate to form an aggregate structure, which, at 50–400nm in size, can be visualized by transmission electron microscopy (TEM) [31]. Evidence suggests that enzymes are sequestered on the surface of the aggregate particles, where their function is non-specifically inhibited [30,33]. Prevention of compound aggregation through addition of nonionic detergents, such as Triton X-100, effectively relieves enzyme inhibition by this mechanism [30].

Whether or not a compound is prone to aggregation is dependent upon properties of the compound itself, the assay conditions [11,12] and the protein target [34]. For this reason, and because compounds that aggregate are structurally diverse [31], interference due to aggregate inhibition must be empirically determined for a given assay [5,11]. Generally, though, compounds tend to aggregate at micromolar concentrations, and a compound that aggregates at a higher concentration may have legitimate biological activity at lower concentrations [12, 30]. Compound interference by aggregation is relatively easy to identify with a little work, largely because aggregation-based inhibition has hallmark characteristics, as shown in Table 1. While the exact mechanism of how compound aggregates inhibit enzymes is unclear [35], it has been found that addition of 0.01–0.1% Triton X-100 to assay reagents generally allows for significant relief of aggregation-based inhibition (Fig. 2C) [12,30–32], thus making it possible to design a biochemical assay that is less sensitive to this form of inhibition. Significantly, Jadhav *et al.* (2010) found that a screen run without detergent resulted in 15-times more actives than a screen run in the presence of detergent [11]. A protocol to detect aggregation-based inhibition is published and available [36].

In an effort to estimate the prevalence of aggregation-based inhibition for a typical HTS involving a biochemical assay, investigators have tested various small molecule libraries for enzyme inhibition sensitive to Triton X-100 [12,32]. Interestingly, though the same enzyme (AmpC β -lactamase) was used in two of the assays screened, the percentage of compounds that appeared to be sensitive to detergent-dependent inhibition varied between the two screens, highlighting that compound aggregation is conditional and assay-specific. In a 96-well format assay, Feng *et al.* (2005) found that 19% of the 1,030 drug-like compounds tested demonstrated detergent-dependent inhibition when screened at 30 μ M [32]. For a 1536-well assay format, performed against a titration (3nM–30 μ M) of each of ~70K compounds (quantitative HTS-qHTS), Feng *et al.* (2007) found that 95% of the actives identified in the screen were detergent-sensitive inhibitors, and consisted of 1.7% of the total library screened (PubChem AIDs 584, 585) [12]. A screen of ~200K compounds targeting the cysteine protease cruzain (PubChem AID 2249) revealed that approximately 1.9% of the library were detergent-sensitive inhibitors, indicating that the prevalence of this type of assay interference is not library specific [11]. In addition, compounds found to be aggregation-based inhibitors of AmpC were not necessarily found to inhibit cruzain, and vice versa, again underscoring the context-dependence of this phenomenon [11]. Results from these screens indicate that aggregation-based inhibition has the potential to significantly inflate the number of apparent actives identified from a screen.

Compounds Capable of Redox Cycling and Direct Oxidation

Some compounds, such as certain quinones, undergo redox dependent cycling (redox cycling compounds, or RCCs) in the presence of strong reducing agents such as dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP), which results in generation of reactive oxygen species (ROS) [37–40] (Supplemental Figure S2). DTT and other reducing agents are commonly added to buffer systems of enzymatic assays as a means to keep catalytic and key structural amino acids of enzymes in a reduced state, characteristic of a functional protein [40]. The ROS generated by redox-dependent cycling in the presence of DTT or TCEP is hydrogen peroxide (H_2O_2) [38,40,41], which can, in turn, oxidize cysteine residues of proteins, thus non-specifically and promiscuously inhibiting protein activity [38,39]. In this way, RCC-based interference can lead to exaggerated numbers of apparent actives in HTS assays - Smith *et al.* [38] noted this as the main artifact responsible for >85% of the active compounds identified in a HTS against caspase-8. Hallmarks of RCC interference can be found in Table 1.

A robust absorbance-based method for H_2O_2 detection, and thus identification of RCCs, suitable for HTS has been recently described [40]. This assay can detect H_2O_2 generation (in the 1–100 μ M range) by RCCs using H_2O_2 -dependent horseradish peroxidase and monitoring oxidation-dependent absorbance changes of phenol red. This assay was used to profile ~200K compounds available in PubChem (AID: 878) [42] for their ability to generate H_2O_2 . Several common scaffolds, including the pyrimidotriazinedione shown in Figure 2D, were found to be among the compounds that generated H_2O_2 in the presence of 0.8 mM DTT (peroxide generation AC_{50} =0.9 μ M). Not surprisingly, these compounds were promiscuously active against a number of target proteins [42]. RCC-interference is not just restricted to biochemical assays that employ enzymes where important cysteine, tryptophan or methionine residues can be modified, it also can produce promiscuous effects in cell-based assays [42], since H_2O_2 is a common cellular messenger [37,39,43]. It is interesting to note that the acute toxicity associated with some RCCs, such as the quinones, has not prevented their use as anti-cancer agents [44].

Compounds can also act directly as an oxidizing agent, without the production of H_2O_2 . In this case, the potency of such a compound is weakened in the presence of reducing reagents (e.g. $\geq$ 10mM DTT), which act to protect target proteins from either oxidation (as can occur with certain amino-thiophenes) or compound adduct formation (e.g. with *p*-catechols)[45]. A diagnostic test of target oxidation by a compound is to measure the activity of the protease caspase (which requires a thiol for catalytic activity, thus making it sensitive to oxidation) in the presence of the compound. Increased inhibition of the caspase enzyme in the absence of DTT may be indicative of non-specific cysteine oxidation [46]. It should be noted, however, that the activity of some known drugs is actually through such reactive mechanisms, examples being the sulfur rich disulfiram as well as ethacrynic acid - a drug that contains a Michael acceptor. It is thus essential that reactivity be carefully considered with respect to the desired mechanism of the compound.

Conclusion

Although compound-dependent assay interference in HTS cannot, at this time, be entirely eliminated, it is possible to significantly reduce the probability of its occurrence, in addition to making off-target activity significantly easier to differentiate from activity that is target- and pathway-specific. Acknowledging the potential interference that a given assay may be susceptible to and designing appropriate orthogonal assays to confirm compound activity are the best means of identifying artifactual activity early in the probe or drug discovery process. While assay interference adds an additional challenge to the process of HTS, fortunately, at

this time, information on the various types of artifactual activity described in this Review is becoming increasingly available to aid investigators in their chemical biology experiments. Further expansion of publically available data from profiling efforts of representative compound libraries screened for various types of artifactual activity should help identify compounds that may generate false positives in future screens, as well as aid in the design of better assay strategies and systems. The challenge in dealing with assay interference will continue to evolve - as novel assays are developed for HTS (e.g. AlphaScreen®), so, too, will new artifacts specific to these assay systems become evident – and it is the hope that library profiling efforts and cheminformatics will keep pace [13], allowing researchers to focus on genuine chemical probes and avoid diversions caused by artifacts.

Box 1

Definition of terms

Compound interference: For this Review, the activity of a compound in an assay that is reproducible, but may not be related to the biological target of interest. Compound interference contributes to the number of “false positives” in an assay. Common types of compound interference discussed in this Review include compound fluorescence, aggregation, luciferase inhibition, and redox reactivity.

Counter-screen: A screen performed in parallel with or after the primary screen. The assay used in the counter-screen is developed to identify compounds that have the potential to interfere with the assay used in the primary screen (the primary assay). An example of an assay used in a counter-screen would be a biochemical assay to identify compounds that inhibit firefly luciferase. This assay could be used as a counter-screen to a primary screen that utilized the firefly luciferase as a reporter to identify compounds that modulated a biological target/signaling pathway. Counter-screens can also be used to eliminate compounds that possess undesirable properties, for example, a counter-screen for cytotoxicity.

False positive: Generally related to the “specificity” of an assay. In screening, a compound may be active in an assay but inactive toward the biological target of interest. For this Review, this does not include activity due to spurious, non-reproducible activity (such as lint in a sample that causes light-scatter or fluorescence). Compound interference that is reproducible is a common cause of false positives, or target-independent activity.

High-throughput screen (HTS): A large-scale experiment in which collections of compounds are tested for activity against a biological target or pathway. “Screen” for short.

Hits: Slang for putative activity observed during the primary high-throughput screen, usually defined by percent activity relative to control compounds.

Library: A collection of compounds.

Off-target activity: Compound activity that is not directed toward the biological target of interest but can give a positive read-out, and thus be classified as an active in the assay.

Orthogonal assay: An assay performed following the primary assay to differentiate between compounds that generate false positives from those compounds that are genuinely active against the target. Conducted on compounds found active in the primary assay, this assay uses a different reporter or assay format in an effort to confirm that activity of the compound is directed toward the biological target of interest. Compounds inactive in an orthogonal assay are removed from further consideration, as a negative result indicates that the original compound activity was most likely assay format-dependent and not specific to the biology of interest.

Primary assay: The assay used for a high-throughput screen.

Secondary assay: An assay used to test the activity of compounds found active in the primary screen (and orthogonal assay) using robust assays of relevant biology. Ideally, these are of at least medium-throughput to allow establishment of structure-activity relationships between the primary and secondary assays and establish a biologically plausible mechanism of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Assay interference by compounds can be reproducible and demonstrate concentration dependence, producing false positives in a high-throughput screen (HTS)

(A) False-positives in the primary assay used for HTS are generally eliminated upon testing in an orthogonal assay (see Box 1) conducted on all compounds with greater than average activity. (i and ii) Each dot in the scatter plot represents a compound, plotted based on its percent activity in an assay, in this case, an assay to identify inhibitors. In the example shown, a 3 standard deviation (s.d.) threshold value (dotted line) was used to qualify a compound as active. (i) Results from a primary assay to identify compounds with inhibitor activity, with the percentage of active compounds at 5% of the total screened (found below 3s.d. line). (ii) Following an orthogonal assay, 95% of the active compounds are found to be false positives due to assay interference, leaving only a few genuine actives (red dots; 0.25% of compounds screened). (B) Fluorescent compounds (an example of which is shown) behave much like genuine actives, demonstrating concentration dependence (blue dots in scatter plot) that is reproducible, as shown here for results of a fluorescence-based HTS run in the blue spectral range. Sporadic fluorescence interference is also seen (grey dots), but is not reproducible and does not show concentration dependence (adapted from Simeonov *et al.*, 2008 [14]). (C) Compound interference that directly interacts with an enzymatic reporter, such as firefly luciferase, is shown here. This type of compound interference also demonstrates compound concentration

dependence (orange concentration response curve – CRC), and in this specific case, the compound is a competitive inhibitor of firefly luciferase (green CRCs).

Figure 2. Different types of assay interference: compound fluorescence, inhibition of the reporter enzyme firefly luciferase (FLuc), aggregation-based inhibition, and redox cycling compounds (RCC)

(A) Self-organizing map (SOM) depicting the relationship between chemical structure and fluorescence for compounds in a representative library whose fluorescence profile overlaps with AlexaFluor 350 or resorufin [14]. Each hexagon represents a cluster of compounds that are structurally related. Clusters shaded red/orange indicate an enrichment of compounds in the structural class that fluoresce (compared to the average), whereas clusters shaded blue indicate a structural class with fewer than average fluorescent compounds. As can be seen, significantly more compound classes fluoresce in the blue-spectral range, compared to those that fluoresce in the orange region. Additionally, compound classes that fluoresce tend to be related, as demonstrated by an enrichment in fluorescent compounds in adjacent clusters [14]. (B) Some compounds that inhibit the FLuc enzyme (red CRC for biochemical assay) appear to be active in cell-based FLuc reporter gene assays (green and black CRCs) due to the interaction of the compound with the FLuc enzyme. Apparent activation in cell-based assays due to inhibitor-based FLuc stabilization can generate different types of CRCs, with two example CRCs shown here. If the inhibitory compound is competed off the enzyme by excess FLuc substrates found in the detection mix, or by washing of the cells prior to addition of the detection mix, a CRC similar to the one shown by the black-dotted line can be generated. If inhibition is not relieved at high compound concentrations, then a CRC similar to the one indicated by the green hatched-line is commonly seen. (C) Addition of Triton X-100 to the assay buffer significantly relieves inhibition of a compound that forms aggregates. Aggregation-based

inhibition shows concentration dependence and the tendency for a compound to form aggregates is assay-specific. **(D)** Enzyme assay behavior of an example redox cycling compound (RCC). Data shown are from caspase-1 assays performed in the presence of 10 mM DTT (PubChem AID: 896) or 10 mM Cysteine (PubChem AID: 929). Changing the reduction potential of the buffer from a strong (DTT) to a weak (Cys) redox potential alleviates the apparent inhibition by the compound shown (PubChem CID: 647501), a RCC that generates peroxide upon exposure to DTT (PubChem AIDs: 672, 878, 787, 1234, 2035).

Common types of assay interference

Table 1

Assay interference described in detail in this Review are underlined. Missing fields in the Table indicate that a substantial amount of work remains to be done to characterize compounds used in HTS and determine the impact of various types of assay interference. Also not included in this Table, due to lack of available information, are the effects of metal ion interference on assays [4,5] and assay interference by compounds that affect cell membrane permeabilization [47].

Assay Interference	Effect on Assay:	Characteristics of note	Prevention	Prevalence in Library	Percent Enrichment of Actives	Ref
<u>Aggregation</u>	-Biochemical: Non-specific enzyme inhibition; protein sequestration -Cell-based: Protein sequestration	-Concentration-dependent -IC ₅₀ is sensitive to [E] -Efficacy is sensitive to detergent -Time-dependent inhibition -Inhibition curves have steep Hill slopes -Reversible by diluting compound	-Include 0.01–0.1% Triton X-100 in assay buffer	- 1.7–1.9%	-As high as 90–95% of actives in some biochemical assays	[11,12,30,31,33,35,48]
<u>Compound Fluorescence</u>	-Biochemical: Increase in light detected may affect apparent potency; Bleed-through fluorescence in adjacent wells -Cell-based: May affect apparent potency; General increase in light detected; Increase in light detected due to association of compound with cell/cellular structures	-Reproducible -Concentration-dependent	-Use orange/red-shifted fluorophores -Include a pre-read after compound addition but prior to fluorophore addition -Include time delay after excitation of fluorophore (time-resolved) -Use a ratiometric fluorescence output -Use previous profiling efforts to identify fluorescent compounds	-EX340nm/ EM450nm ~2–5% -EX480nm/ EM540nm ~0.01–0.2% -EX525nm/ EM598nm ~0.004–0.01% -EX547nm/ EM618nm ~0.001–.01% -EX570nm/ EM670m ~0%	-Up to 50% of actives in certain assays involving blue-shifted spectral range	[9,11,14,18–20,49–52]

Assay Interference	Effect on Assay:	Characteristics of note	Prevention	Prevalence in Library	Percent Enrichment of Actives	Ref
<u>Firefly Luciferase Inhibitors</u>	-Biochemical: Inhibition -Cell-based: Inhibition <i>or</i> Activation	-In purified (biochemical) firefly luciferase assay with K_M substrate, concentration-dependent inhibition of luciferase	-Test actives against purified firefly luciferase using K_M levels of substrate -Use orthogonal assay with an alternate reporter to confirm activity -Use previous profiling efforts to identify FLuc inhibitors	-At least 3%	-As high as 60% of actives in some cell-based assays	[6,23]
<u>Redox Cycling Compounds</u>	-Biochemical: Inhibition -Cell-based: Inhibition or Activation	-Concentration-dependent -Potency depends on concentration of RCC and reducing reagent -Time dependent enzyme inactivation -pH sensitivity -Effect generally depends on strength of reducing reagent -If H_2O_2 is generated, elimination of activity upon catalase addition	-Replace DTT and TCEP in buffers with weaker reducing agents such as cysteine and glutathione -Use [DTT] $\geq$ 10mM -Use previous profiling efforts to identify RCCs	-Compounds that generate H_2O_2 at appreciable levels: ~0.03%	-Enrichment for a given assay found to be as high as 85%	[37-40,42]
<u>Oxidizers</u>	-Biochemical: Inhibition -Cell-based: Inhibition or Activation	-Concentration-dependent -Potency lessened in the presence of reducing reagent -pH sensitivity -Time dependent enzyme inactivation -Reversible	-Measure caspase activity +/- DTT in presence of compound -Measure reactivity against glutathione		~20-50% enrichment for some assays	[45,46,53]
Covalent reactivity	-Biochemical and Cell-based: Inhibition or activation	-Generally, irreversible modification of enzymes/proteins	-Identify compounds with reactive functionalities	-Less than 0.65% (0.65% of compounds had reactive functional groups and inhibited the		[4,10,11,13,54]

Assay Interference	Effect on Assay:	Characteristics of note	Prevention	Prevalence in Library enzyme target- cruzan)	Percent Enrichment of Actives	Ref
Cytotoxicity	-Biochemical: N/A -Cell-based: Apparent inhibition due to cell death	-Occurs more commonly at higher compound concentrations -May occur more commonly in assays with longer compound incubation times	-Test compounds for cytotoxicity using commercially available assays, dye uptake, or colorimetric assays -Use previous profiling efforts to identify potential candidates (PubChem AID 463)			[55-59]
Inner filter effect (absorption of light)	-Biochemical & Cell-based: Decrease in light detected may affect apparent potency	-Components of assay system are colored	-Check for colored compounds -Conduct excitation/ emission at longer wavelengths			[9,19]
Light scattering	-Biochemical & Cell-Based: Decrease in light detected; Increase in fluorescent ratio for ratiometric assays	-Generally occurs at lower wavelengths of light (blue spectral region) and at higher concentrations of compound	-Conduct excitation/ emission at longer wavelengths			[19]
Quenching	-Biochemical & Cell-Based: Decrease in light detected may affect apparent potency	-Reduction in the excited state of the fluorophore results in decreased light emission				[9,19,60]
Surfactant-like compounds	-Biochemical: Promiscuous activation of enzymes -Cell-based: Cell lysis and	-Concentration-dependent -Non-specific activation of enzymes	-Include 0.1% CHAPS or Triton X-100 in assay buffer			[11,61,62]

Assay Interference	Effect on Assay: cytotoxic at high concentrations	Characteristics of note	Prevention	Prevalence in Library	Percent Enrichment of Actives	Ref