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Quantitative High-Throughput Screening Using a Coincidence Reporter Biocircuit

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Abstract

Reporter-biased artifacts, compounds that interact directly with the reporter enzyme used in a high-throughput screening (HTS) assay, and not the biology or pharmacology being interrogated, are now widely recognized to reduce the efficiency and quality of HTS used for chemical probe and therapeutic development. Furthermore, narrow or single concentration HTS perpetuates false negatives during primary screening campaigns. Titration based or quantitative HTS (qHTS) and coincidence reporter technology can be employed to reduce false positives and false negatives, respectively, thereby increasing the quality and efficiency of primary screening efforts where the number of compounds investigated can range from tens of thousands to millions. The three protocols described here allow for generation of a coincidence reporter (CR) biocircuit to interrogate a biology or pharmacology of interest, generation of a stable cell line expressing the CR biocircuit, and performing qHTS using the CR biocircuit to efficiently identify high-quality biologically active small molecules.

Keywords

quantitative high-throughput screening; coincidence reporter; assay development

Coincidence reporter for small molecule screening

INTERNET RESOURCES (optional)

Assay Guidance Manual. Basics of Assay Equipment and Instrumentation for High Throughput Screening.

<http://www.ncbi.nlm.nih.gov/books/NBK92014/>

Nano-Glo Dual-Luciferase Reporter Assay Technical Manual

<https://www.promega.com/resources/protocols/technical-manuals/101/nanoglo-dual-luciferase-reporter-assay-protocol/>

Cell Culture Basics

<https://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics.html>

Pubchem

<https://pubchem.ncbi.nlm.nih.gov/>

INTRODUCTION

High-throughput screening (HTS) has been used for many years in the pharmaceutical industry to identify therapeutic compounds for the treatment of human diseases (Macarron et al., 2011). More recently, HTS has become a broadly applied approach to identify chemical probes, gain mechanistic insights into complex biological processes via pharmacological profiling, and complement industrial pursuits of novel therapeutics for human disease (Dahlin et al., 2015; Hasson and Inglese, 2013). Despite the increasing use of single concentration HTS and the ability of HTS to canvas compound collections in the millions, many of these screens are plagued by assay-dependent artifacts, false positives, and narrow concentration testing false negatives due to current compound library evaluation strategies and assay design limitations, respectively (Inglese et al., 2006; Thorne et al., 2010). The high false positive and false negative rates lead to wasted time on follow up of compounds that act via non-relevant mechanisms and missed opportunities, respectively (Inglese et al., 2006; Thorne et al., 2010). Accordingly, there have been advancements in the field to minimize the shortcomings, in particular of traditional single reporter gene assays, and to increase the efficiency of HTS (Auld et al., 2008b; Cheng and Inglese, 2012; Hasson et al., 2015). Here, we provide a comprehensive protocol for generating a reporter gene assay using coincidence reporter technology and quantitative high-throughput screening (qHTS) that was recently published (Cheng and Inglese, 2012; Hasson et al., 2015) to reduce reporter-biased artifacts and false negatives. Coincidence reporters are different from dual reporter assays where one luciferase reporter monitors the biology of interest and the second luciferase reporter serves as an internal control for cell viability. Coincident reporters utilize two non-homologous reporter enzymes expressed from a single transcript and separated at the protein level by a ribosomal skipping sequence, cloned downstream of the response element (RE) or promoter, where both luciferase reporters monitor the biology of interest. Thus, a biological active would elicit a coincident response in both reporter enzyme readouts.

STRATEGIC PLANNING

Before beginning, it is important that the experimenter is familiar and comfortable with standard cell culture practices for the cell line that will be used (Phelan, 2007). Many of the steps in the following protocols rely on cell type specific knowledge that is empirically determined such as confluence for passaging, ability of the cell line to grow from single cell density, optimal transfection conditions, etc. and these are assumed to be known prior to starting these protocols. Choice of cell line should be fully vetted before beginning based on the scope of the project. Cell line parameters for consideration should include the biology or pharmacology being investigated, ease of use for transfection and scaling to low volume microtiter plates, and amenability to secondary assays. Step 4 in Basic Protocol 2 requires antibiotic selection with Hygromycin B or Puromycin depending on which coincidence reporter vector is being used. Thus, a kill curve experiment should be completed prior to starting this protocol to determine the concentration of Hygromycin B or Puromycin required for selecting resistant clones for the cell line being used. The choice for using pNLCoI1 (Promega) or pCI9.0 (obtained via request from Inglese laboratory) should be made prior to beginning. pCI 9.0 differs from pNLCoI1 in that each reporter in the pCI 9.0

vector contains an N-terminal 3xFLAG tag to assess reporter protein expression during monoclonal and polyclonal generation (Figure 1).

Furthermore, before beginning, it is advised that the experimenter be familiar with the assay guidance manual on the basics of assay equipment and instrumentation for high-throughput screening (online resource below).

Promoters

Promoters and REs for reporter gene assays are chosen based on the biological pathway or pharmacology being interrogated. Broadly speaking, any promoter can be used in a reporter gene assay so long as some basic characterization is done beforehand. In order to characterize a promoter of interest, promoter analysis experiments should be carried out using the dual reporter construct and transient transfection (Solberg and Krauss, 2013). Various lengths of the promoter region are cloned into the reporter construct and luciferase expression is measured as a readout of transcriptional activity based on biological or pharmacological manipulation to identify regulatory elements, enhancer regions, and other functional core elements within the promoter region (Solberg and Krauss, 2013). These studies are helpful in determining the optimal promoter length, encompassing many of the relevant RE and enhancer regions for maximal transcriptional response, and a sufficient assay window.

Although this protocol outlines the steps necessary to generate a conventional reporter gene assay using insertion of a promoter fragment or RE into the coincidence reporter biocircuit, coincidence reporter technology has also been used successfully, and continues to be used, to monitor endogenous changes in transcription using genome editing and the qHTS paradigm (Hasson et al., 2015).

Library Selection

The Library of Pharmacologically Active Compounds 1280 (LOPAC1280) from Sigma is often used as a validation library. This library should be run in duplicate and evaluated for reproducibility before moving into larger or assay-focused libraries. Screening of LOPAC1280 is outlined in Basic Protocol 3. The library is obtainable from Sigma in single concentration, and can be used as such, but will need to be prepared in titration using automated liquid handlers for qHTS (Yasgar et al., 2008).

Library selection will be dependent on the scope and goals of the project (Auld, 2008). For example, is the goal to pharmacologically profile a RE or a given gene, to find a drug-like compound, or to generate a chemical probe for GPCR activation of a given pathway? Each of these would be begun with different library selections. There are chemical libraries built around “drug-likeness” and their adherence to Lipinski’s rule of 5, if that is desired. For broad pharmacological profiling, a larger and more diverse chemical library would be appropriate. For targeted biology such as GPCR activation, smaller focused libraries can be considered as a starting point (Auld, 2008). Although they seem obvious, they serve as useful starting libraries from which to expand. Expansion can include the aforementioned larger diversity collections or large combinatorial libraries which include structurally related analogs that provide structure-activity relationship (SAR) guidance for medicinal chemistry.

Beyond which library to screen, the format in which to screen must be considered. Although the protocols below are outlined for qHTS, the coincidence reporter can also be used for single concentration screening. Again, coincident responses (similar activity in this case instead of similar potency) are the key to identifying biological actives and eliminating reporter-biased artifacts. It is recommended to screen using the qHTS paradigm as increasing the concentration range and number of data points, replicates of each compound, reduces the false negative rate for the primary screening effort (Malo et al., 2006). Concentration response curve (CRCs) can range from 5 to 11 point titrations with the minimum recommended being 7 points and the ideal being 11—as the number of titration points increases, the probability of false negatives decreases (Malo et al., 2006). Compound plates can be prepared as inter- or intra-plate titration and will depend on initial library plating, liquid handling capabilities, and screening format (384 or 1536) (Yasgar et al., 2008).

BASIC PROTOCOL 1. Design, development, and validation of coincidence reporter biocircuit

The success of an assay relies in large part on reporter design. A reporter must be grounded in relevant biology and generate a readout that is reproducibly measurable in miniaturized format. The RE or promoter region of interest ideally is genetically and/or pharmacologically tractable, e.g., via mechanisms such as transcription factor silencing or compound-mediated modulation. A RE or promoter sequence should also be sufficiently characterized to permit the design of an effective mimic (e.g., with respect to promoter length or RE characteristics). For example, the endoplasmic response element can be activated with Tunicamycin and is well characterized in the literature (Montminy et al., 1986). Similarly, cAMP response element (CRE) activation downstream of G protein coupled receptor (GPCR) signaling is well characterized and can be used to monitor pharmacological modulation of GPCR activity such as beta adrenergic signaling in the presence of agonists such as isoproterenol (Cheng et al., 2010; Samali et al., 2010).

REs and/or promoter regions are cloned into a reporter construct adjacent to two non-homologous luciferase reporters. Coincidence reporter technology builds upon the well-established advantage of standard reporter gene assays — signal amplification of subtle biology using bioluminescent luciferase enzymes — with the additional advantage of elimination of artifactual ‘hits’ (those attributable to direct stabilizing inhibition of the luciferase) through the use of two orthogonal luciferase reporters (Cheng and Inglese, 2012; Hasson et al., 2015). The use of an efficient ribosomal skipping sequence derived from porcine teschovirus-1, P2A, contributes to stable stoichiometric expression of Firefly luciferase (FLuc) and Nanoluciferase (NLuc) (Kim et al., 2011; Kuzmich et al., 2013) (Figure 1 and 2). This Basic Protocol outlines the cloning steps necessary to generate and validate the performance of the coincidence reporter biocircuit to interrogate a given promoter or RE.

Materials

Subcloning:

pNLCoI1 (Promega catalog no. N1461) or pCI9.0 (via request from Inglese laboratory)

DNA restriction enzymes (see multiple cloning site for pNLCoI1 and pCI9.0 in Figure 1)

Geneblock/Gene synthesis, PCR product, or plasmid vector containing RE or promoter region of interest

Calf intestinal alkaline phosphatase (CIAP) (NEB catalog no. M0290)

QIAquick PCR purification kit (Qiagen catalog no. 28104), or equivalent

Quick ligation kit (New England Biolabs catalog no. M2200), or equivalent

Competent *E. coli* strain (DH5alpha or Top10)

SOC medium

LB agar plates containing 100 µg/mL Ampicillin

QIAprep Spin Miniprep kit (Qiagen catalog no. 27104), or equivalent

Access to rapid Sanger sequencing to confirm finalized plasmid design

HiSpeed Plasmid Maxi kit (Qiagen catalog no. 12662), or equivalent

Validation of coincidence reporter:

Growth media

Transfection reagent (ex. Lipofectamine 2000)

PTC124 (SelleckChem catalog no. S6003)

Cilnidipine (SelleckChem catalog no. S1293)

Biological control compound(s)—dependent on RE/promoter chosen

Nano-Glo Dual-Luciferase Reporter (NanoDLR) Assay System (Promega catalog no. N1610 or N1620)

Graphing software (GraphPad Prism, etc.)

Tissue culture hood

37°C, 5% CO₂, 95% humidity incubator

Cell culture flasks (T25 and T75)

6 well and 96 well tissue culture-treated plates, Corning 3506

96 well solid white tissue culture-treated assay plates, Corning 3917

Plate reader with luminescence capabilities and amenability for HTS—See Table 1**

Subclone RE/promoter of interest into coincidence reporter vector

- 1 Digest 1 µg pNLCoII or pCI9.0 using DNA restriction enzymes, and 1.5 mL microcentrifuge tube, according to multiple cloning site and manufacturer's protocol (Figure 1).

pNLCoII and pCI9.0 vectors lack a promoter element to allow for easy construction of reporter gene assays. Sequence information can be downloaded from NCBI for pNLCoII (accession number [KM359771](#)) or obtained from the Inglese laboratory for pCI 9.0. If the RE or promoter signal is weak, a minimal promoter may be needed to enhance expression. For these studies, pNLCoII can be used or gene synthesis can be used to build a RE/promoter + minP insert for pNLCoII or pCI 9.0.

- 2 Simultaneously, digest 1 µg RE or promoter region of interest with the same DNA restriction enzymes in a separate 1.5 mL microcentrifuge tube.

There are several sources of DNA for the RE/promoter region of interest. Regions up to 3 kB, inclusive of REs and most commonly used promoter regions (~ 1 kB most proximal to the first coding exon), can be made easily using G blocks from IDT or Genart from LifeTechnologies, or amplified using standard PCR with the addition of restriction sites during primer design. Alternatively, larger regions (e.g., to include more distal 3' UTR and enhancer regions) can be generated by PCR amplification of genomic DNA, also with the flanking restriction sites added during primer design.

- 3 Optional: If a single enzyme was used to digest DNA, use calf intestinal alkaline phosphatase (CIAP) to dephosphorylate the linearized vector according to the manufacturer's protocol to ensure proper ligation in step 5.

- 4 Purify the linearized vector, and response/promoter DNA, using a QIAquick PCR purification kit.

If PCR has been used to amplify the promoter of interest from genomic or plasmid DNA, confirmation of successful amplification and band size should be obtained using gel electrophoresis. DNA can be purified using a gel DNA extraction kit.

- 5 Use the NEB quick ligation kit and a 3:1 molar ratio of purified RE/promoter to vector to ligate the two DNA fragments together.

- 6 Simultaneously, use the quick ligation kit to perform a ligation using digested and purified vector only (negative control) to inform ligation efficiency and determine the number of colonies to be screened for ligated plasmid.

- 7 Transform 2 µL of each ligation reaction in competent *E. coli* (DH5alpha or Top10 from NEB or LifeTechnologies) following the manufacturer's protocol.

- 8** Pick 4–8 colonies and grow each colony in 5 mL of LB + 100 µg/mL Ampicillin overnight (~16 hours).
- Single colonies should be observed following transformation. Colonies ideal for growth are isolated from its nearest neighbors and are absent of satellite colonies (smaller colonies formed around a larger colony).
- 9** Use DNA miniprep kit (Qiagen, or equivalent) to isolate DNA from 4 mL of each bacterial culture. Use 500 µL of each bacterial culture to generate a glycerol stock for future use.
- To generate glycerol stock, add 500 µL of 50% glycerol to 500 µL of fresh bacterial culture and store at –80°C in a 2 mL cryovial. Once correct clones are identified by sequencing, glycerol stocks of unwanted clones should be discarded. A larger DNA prep should be generated once a colony has been identified as correct via restriction digest and sequence verification (step 12). Glycerol stocks are useful for the inoculation of bacterial cultures for the larger DNA prep.
- 10** Perform digest with appropriate DNA restriction enzymes and run on agarose gel to identify colonies that produced DNA with properly ligated RE/promoter into the vector.
- 11** Sequence verify each clone that was identified as correct via restriction enzyme digest.
- Sanger sequencing is required for this step. If sequencing facilities are not available at the experimenter's institution, there are overnight sequencing services provided through a variety of vendors including Eurofins Genomics.
- 12** Using a HiSpeed Maxiprep kit (Qiagen, or equivalent) and appropriate volume of bacterial culture, generate a large scale prep of the sequence verified plasmid DNA for the validation steps and Basic Protocol 2.

Validate coincidence reporter biocircuit using transient transfection

- 13** Using sequence verified high-quality DNA of RE/promoter-vector, and optimized transfection protocol for cell type in use, transfect DNA into a 6 well tissue culture-treated plate (Hawley-Nelson, 2001).
- Cells should be at the density recommended by transfection reagent protocol (typically 50–80%).
- 14** Twenty four hours post transfection, passage cells into 96-well white solid bottom plate.
- 15** Incubate cells with pharmacological control compounds in titration (7–11 concentrations) to validate correct integration and modulation of the coincidence reporter biocircuit. Figure 3A shows a representative control plate layout for a 96 well plate. PTC124 and Cilnidipine should be administered 18–24 hours prior

to the luminescence read. Incubation time of the biological control(s) will be assay-dependent based on promoter and/or biology being interrogated.

- a. PTC124—FLuc stabilizing inhibitor
 - i. Titration range: 10^{-12} - 10^{-5} M
 - b. Cilnidipine—NLuc stabilizing inhibitor
 - i. Titration range: 10^{-9} - 10^{-4} M
 - c. Biological control (activator or inhibitor of the RE/promoter chosen)—this will be assay dependent and determined by the RE/promoter you have chosen to investigate.
 - i. 3 log units on either side of the EC_{50}/IC_{50}
- 16** Use Nano-Glo Dual Luciferase Reporter Assay (NanoDLR; Promega) system according to manufacturer's protocol to evaluate the luminescent signal for each luminescent reporter in each condition.
- This is an easy-to-use add-read-add-read protocol and manufacturer's instructions should be followed.
- 17** Use an appropriate graphing program such as GraphPad Prism to analyze the data to confirm proper function of the coincidence reporter biocircuit (See Figure 2).

Prior to proceeding to Basic Protocol 2, it is imperative that the expected response profiles outlined in Figure 2 are observed. PTC124 should elicit an FLuc-specific response, Cilnidipine an NLuc-specific response¹, and the biological control a coincident response (similar potency) for FLuc and NLuc. Data from the reporter control titrations may yield different response profiles depending on the expression levels of the reporter, and should be analyzed appropriately. For most expression regimes where PTC124 stabilization of FLuc reporter manifests as an increase in signal at intermediate concentrations followed by a decrease in signal at high concentrations as a result of FLuc inhibition, PTC124 should be fit with the following, 5-parameter, two sigmoidal curve equation (Hasson et al., 2015):

$$Y = S_0 + \frac{S_{max} - S_0}{1 + 10^{(Log EC_{50} - X)}} + \frac{S_{inf} - S_{max}}{1 + 10^{(Log IC_{50} - X)}}$$

For systems where basal expression levels of the FLuc reporter are very high relative to the amount of FLuc stabilized by the inhibitor, such that no increase in FLuc signal is observed at intermediate concentrations of

¹NOTE: Cilnidipine is a calcium channel antagonist of the dihydropyridine class and care should be taken to ensure that the action of this compound as a channel blocker is not pharmacologically relevant to the assay biology under study. Other NLuc inhibitors that can be used are can be found in Ho et al. (2013).

inhibitor, a standard 3- or 4-parameter equation may be used to characterize the inhibition of the FLuc signal. Similarly, Cilnidipine should be fit with a 4- or 5-parameter curve fit depending on curve shape that is either sigmoidal or bell-shaped, respectively. Biological control(s) should be fit with either the user-defined two sigmoidal curve fit or a four parameter fit depending on shape (Beck et al., 2004). For comprehensive determination of the correct model to use, pairwise F-tests of the curve fit models in order of increasing variable parameters should be performed, e.g. a 3-parameter sigmoid (with Hill slope = 1) compared to a 4-parameter sigmoid (where the Hill slope is allowed to vary), or a 4-parameter sigmoid compared to a 5-parameter two sigmoid model. Of course, the number of variable parameters cannot exceed $n-1$ where n is the number of data points comprising the titration.

Additionally, it is important to evaluate the assay window potential during this step to ensure that the response from the RE/promoter being used will produce a screening window large enough to identify modulators—this can be evaluated by calculating Z-factor:

$$Z = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where Z greater than 0.5 is generally desirable (Inglese et al., 2007; Zhang et al., 1999). If the signal window from the initial design is not sufficient, consider addition of the minimal promoter (minP) that may amplify the signal from the promoter or RE under investigation.

BASIC PROTOCOL 2. Cell Line Development for qHTS

In Basic Protocol 1, confidence should be obtained that the coincidence reporter biocircuit is working properly—basal FLuc is detectable and can be modulated with PTC124, basal NLuc is detectable and can be modulated with Cilnidipine, and the RE/promoter of interest can be modulated with the known biological control(s). Importantly, the biological control should elicit a coincident response in both channels and provide a sufficient assay window for screening (Z -factor > 0.5) (Zhang et al., 1999). This protocol outlines the necessary steps to generate a stable cell line expressing the coincidence reporter biocircuit that produces excellent assay performance metrics to be used in qHTS (Basic Protocol 3).

Materials

Coincidence reporter biocircuit (Basic Protocol 1)

Growth media—dependent on cell line

Transfection reagent (ex. Lipofectamine 2000 or appropriate Nucleofector kit)

Hygromycin B or Puromycin selection antibiotic (concentration to be determined by kill curve for cell line of interest)

PTC124 (SelleckChem catalog no. S6003)

Cilnidipine (SelleckChem catalog no. S1293)

Biological control compound(s)—dependent on RE/promoter chosen

Nano-Glo Dual-Luciferase Reporter (NanoDLR) Assay System (Promega catalog no. N1610 or N1620)

Green fluorescent protein (GFP) vector that lacks Hygromycin B or Puromycin resistance genes (transfection control)

Graphing software (GraphPad Prism, etc.)

If using pCI 9.0, mouse anti-FLAG antibody can be used to assess protein expression (Sigma catalog no. F1804, RRID: AB_262044)

Tissue culture hood

37°C, 5% CO₂, 95% humidity incubator

Cell culture flasks with vented cap (T25, T75 and T175)

6-well tissue culture-treated plates, Corning 3506

96-well clear bottom tissue culture-treated plates, Corning 3596

96-well solid white tissue culture-treated assay plates, Corning 3917

Solid white tissue culture-treated assay plates (384 or 1536), greiner bio-one

Stainless-steel lids containing pinholes for gas exchange (1536-well plates only)

Multidrop Combi Reagent Dispenser with small cassette (Thermo Scientific), or equivalent

Liquid handling (Multimek, PinTool, Mosquito, etc.) for transfer of compounds to assay plate—see Table 1

Reagent Dispenser for NanoDLR—see Table 1

Plate reader with luminescence capabilities and amenability for HTS—See Table 1

- 1 Plate cells into two wells of a 6-well tissue culture-treated plate.
- 2 Using the validated coincidence reporter biocircuit and optimized transfection protocol, transfect the coincidence reporter into one well of a 6-well plate. Simultaneously, transfect green fluorescent protein (GFP), or any other control DNA lacking a Hygromycin B (pNLCoI1) or Puromycin (pCI 9.0) selection marker, as a transfection control.

Cells should be plated at a density optimal for transfection as indicated in the manufacturer's protocol for transfection reagent and empirically determined for cell type being used. The coincidence reporter technology has been successfully transfected into several

neurologically relevant cell lines including human neuroblastoma BE(2)M17 and SK-N-BE cells and rat Schwann cells—RT4 and S16 (unpublished).

- 3 Twenty-four to forty-eight hours after transfection, passage transfected cells 1:5 to 1:15 (coincidence reporter biocircuit and GFP) into a new well of a 6-well plate depending on cell type and cell density post transfection. Once cells have adhered to surface (3–24 hours after plating), replace growth media with growth media + Hygromycin B selection antibiotic (pNLCoI1) or grown media + Puromycin (pCI 9.0).

Concentration of Hygromycin B or Puromycin will be dependent on cell type and should be determined prior to the start of Basic Protocol 2 by performing a kill curve experiment. Lifetechnologies provides a protocol for performing a kill curve to determine appropriate concentration necessary to select resistant clones: <http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics/transfection-basics/transfection-methods/stable-transfection.html>

- 4 Monitor cells until cells containing the coincidence reporter are confluent for expansion and all cells in the GFP transfected well are dead (confirms successful transfection of coincidence reporter and selection via antibiotic).
- 5 Passage coincidence reporter biocircuit transfection into:
 - a. one well of a 6-well tissue culture-treated plate for expansion
 - b. Four to ten 96-well clear bottom tissue culture-treated plates for limiting dilution cloning, or single cell sorting, to obtain monoclonal.

If a cell sorter is easily accessible, single cells can be dispensed into each well of a 96-well plate rather than using limiting dilution cloning. Number of 96-well plates needed for limiting dilution or signal cell sorting will be cell type specific and dependent on the percentage of viable clones expected for the chosen cell line.

Generation of both monoclonal and a polyclonal line at this stage is important, and either can be used for qHTS in Basic Protocol 3. Pharmacological response profiles obtained during cell density optimization (steps 11–17) will determine which cell line will be best for qHTS. Although polyclones have the potential for greater assay variability, antibiotic selection and limiting dilution applies strong selection pressure that can result in outlier populations with unreliable reporter expression. Thus, we suggest generating both polyclonal and monoclonal populations simultaneously, characterize both, and choose the best population based on pharmacological response profiles and assay performance metrics. Because monoclonal cell population outgrowth from single cell density can take weeks to months depending on cell growth rate, characterization following steps denoted as “a” of

the polyclonal cell line can be completed during the monoclonal outgrowth process denoted as steps “b”.

- 6 Subculture cells for expansion.
 - a. *Expand cells from the 6-well plate into a T75 culture flask to be cryopreserved and used for validation experiments—these cells serve as the polyclonal coincident reporter biocircuit cell line and can be used “as is” for either screening purposes or comparison to monoclonal (Figure 4A).*
 - b. Every 3–4 days exchange media with fresh growth media and examine wells of the 96-well plates to determine which wells contain a single cell—mark those wells clearly on the lid so that they can be used later once colonies are established enough to allow for expansion.
- 7 Subculture for cryopreservation.
 - a. *As soon as there are enough cells for cryopreservation and plating for a cell density optimization experiment (see step 11 below), the polyclonal line should be evaluated for coincidence reporter expression and control compound pharmacological response profiles (Figure 2).* Expansion of cells into T175 flasks should be continued to generate additional vials of frozen cell stocks. At least 4–6 vials of cells should be cryopreserved at this stage so that early passage cells are always available.
 - b. Once single cell colonies are confluent enough for expansion, trypsinize the colony and transfer to a 12-well plate to expand each colony. Continue to expand each colony into larger wells and subsequent culture flasks as appropriate subculturing densities are achieved. Once each monoclonal becomes confluent in a 6-well plate, expand cells into a T25 and T75 culture flask for colony screening and cryopreservation, respectively. A minimum of 4–6 vials of cells should be cryopreserved at this stage so further expansion from T75 to T175 will most likely be necessary.
- 8 For preliminary yes or no confirmation of coincidence reporter expression, plate at least 3 wells of the polyclonal and each monoclonal into a 96-well white solid bottom tissue culture-treated plate for luminescence screening. Cells in the 96-well plate should be plated such that they will be ~90% confluent 48 hours after plating.

Colony screening is a tedious process and a sufficient number of clones should be obtained for analysis. We recommend screening at least 10 clones per transfection as a starting point. Bear in mind that it is possible to obtain clones that are antibiotic resistant but do not express the reporter.

- 9 Incubate cells for 48 hours then use the NanoDLR assay system to determine luminescent signal for each reporter for each monoclonal.
- 10 Determine which monoclonal have coincidence reporter biocircuit expression and proceed to step 15 with the most promising 3–4 clones and the polyclonal from step 7.

Cell density optimization

- 11 Using a Multidrop Combi Reagent Dispenser and small cassette, dispense a range of cell densities into one ½ of a white, solid bottom plate for each cell density.

For 384-well cell density optimization, a suggested starting range is 2,000, 5,000, 10,000 and 15,000 cells per well. For 1536-well cell density optimization, a suggested starting range is 500, 1000, 1500, and 2000 cells per well. This will be cell type (size, growth rate) and assay-dependent (promoter strength). For example, our previous data indicate that BE(2)M17 cell lines typically behave optimally in the range of 1000–1200 cells per well in 1536-well format; however, smaller HEK293 cells behave optimally in the range of 1600–2400 cells per well in 1536-well format.

Alternatives to the Multidrop Combi Reagent Dispenser for plating cells are listed in Table 1.

- 12 Cover plates with stainless-steel lids with pinholes for gas exchange and incubate cells for 16–24 hours at 37°C, 5% CO₂, 95% humidity for 16–24 hours.

Stainless-steel lids with pinholes are only required for 1536-well plates. For 384-well screening, the lids provided with 384-well plates can be used.

- 13 Treat each ½ plate with control compounds (see Figure 3 for control compound plate layout example).

See Table 1 for list of instruments for transferring compounds from compound plates to assay plates.

As in Step 17 of Basic Protocol 1, it is important to have reporter controls, PTC124 for FLuc and Cilnidipine for NLuc, as well as biological control(s) in titration on the control plate and 8–16 wells of a single concentration of the biological control that elicits a reliable increase (or decrease for a loss-of-signal assay) in reporter expression to assess Z-factor for each cell density. The control plate template in Figure 3 can be used “as-is” for preparing the compound plate for use in 384-well plates. If screening in 1536-well format, each well of the 384-well plate will be transferred twice, either manually using a multichannel pipette or via an instrument such as a mosquito liquid handler, into 1536-well compound plate for subsequent pinning into a

1536-well assay plate. Because the cell density optimization is plated into ½ plates, for this step only, the control columns will occupy columns 1–4 and columns 12–15 in 384-well plates, and columns 1–4 and 25–28 in 1536-well plates. We recommend the CAPP Denmark 16-channel pipette for easily pipetting control plates manually.

- 14** Incubate cells at 37°C, 5% CO₂, 95% humidity for 1–24 hours.

This incubation is assay-dependent and must be empirically determined. Depending upon biological mechanism(s) and pharmacology being investigated, time courses may vary between 1 hr and many days. PTC124 and Cilnidipine should always be added to the assay plate 18–24 hours prior to the luminescence read.

- 15** Determine the luminescent signal for each reporter enzyme using the NanoDLR assay and appropriate plate reader.

Again, see Table 1 for list of instruments to rapidly dispense NanoDLR reagent to assay plates and high-throughput compatible plate readers.

- 16** Determine the optimal cell density for the polyclonal cell line and each monoclonal.

The optimal cell density should be the one in which the signal for each channel is not plateaued and the Z-factor is highest (Figure 4A). Table 1 in Freitas et al. reports an example of assay optimization for cell density and the parameters to consider (Freitas et al., 2014), albeit for a single luciferase reporter—cell density should be optimized for both reporters for this protocol (Figure 4A).

- 17** If using pNLCoII, choose the optimal cell line for high-throughput validation and further screening based on assay performance and expected pharmacological response profiles as outlined in Figure 2 and Figure 4B. If using pCI 9.0, continue to Step 18 prior to choosing the clone for high-throughput validation and continued screening (Figure 4A–D).

Reporter activity will vary for each of the clones generated. Choose the clone (polyclone or monoclonal) that exhibits the expected pharmacological response profiles outlined in Basic Protocol 1, step 17 and has a sufficient signal window—robust positive control response and low variability ($Z\text{-factor} > 0.5$)²—for screening and proceed to high-throughput screening validation (Figure 4B).

- 18** Determine FLuc and NLuc protein expression of the polyclonal and each monoclonal using Western blotting and choose optimal cell line for high-throughput validation and continued screening.

²NOTE: Using qHTS, where each concentration tested serves as a replicate for the compound, Z-factor values less than 0.5 can be acceptable so long as dose responsiveness is observed reliably. See Step 22 for calculation of MSR for control compounds. A Z-factor value lower than 0.5 and MSR < 3 is considered a good assay for qHTS.

- a. Plate cells at appropriate cell density for a 6-well plate and treat cells with biological control(s) and DMSO for the empirically determined amount of time for the control compounds.
 - b. Use standard Western blotting technique to determine protein expression of each reporter using FLAG antibody (Sigma, catalog no. F1804, RRID: AB_262044) and a loading control such as tubulin or beta-actin.
 - c. The optimal cell line will exhibit basal reporter expression, FLuc and NLuc, with DMSO treatment that will increase with biological control compound treatment. For the optimal cell line, proper reporter protein expression should be consistent with expected pharmacological response profiles and excellent assay performance metrics.
- 19 Once the clone that will be used for screening has been identified, thaw 1–2 vials of cells that were cryopreserved in Step 7 to expand into many T175 flasks to generate frozen cell stocks that will be used like a reagent during screening. That is, cells will be thawed and expanded to the exact number of flasks needed for screening prior to each screening experiment.

Cryopreservation in Step 7 will generate 4–6 vials of early passage cells. Only 1–2 vials should be used during this step for additional expansion such that early passage cells will always be in storage if needed. The goal for this expansion step should be 30–50 vials of cells to be used during screening (Basic Protocol 3) to facilitate consistency.

High-throughput screening validation

- 21 Using the optimized cell density parameters, determine assay variability within and across iterations. Run the assay in the chosen 384- or 1536-well format with reporter and biological controls (Figure 3B) a minimum of three times, each in duplicate, and separated in time—ideally days apart to capture variance in handling.
- 22 Evaluate inter-plate and inter-day variability by calculating control CV and Z-factor for each plate, generating curve fits for each compound from each plate, and calculating minimum significant ratio (MSR) using paired values from duplicate plates for each day (Eastwood et al., 2006; Haas et al., 2004; Iversen et al., 2004). Additionally, daily intra-plate MSR values can be calculated if duplicate control titrations are present on each plate (Shukla et al., 2009).

For standard HTS, it is widely recognized that excellent assay performance will produce a CV less than 10% and Z-factor values greater than 0.5 (Zhang et al., 1999). MSR, an assay metric that assesses reproducibility of potency calculations of concentration response curves (CRCs), should be calculated for each reporter using the duplicate plates on each day of HTS validation (Eastwood et al., 2006; Haas et al., 2004). In the MSR equation below “s” is defined as the

standard deviation of the absolute differences between the log EC₅₀ for each compound on duplicate plates, for each channel.

$$MSR=10^{2\sqrt{2}s}$$

A MSR less than 3 is desired and is indicative of an assay with great reproducibility.

It is paramount to note that miniaturization and scale-up degrade assay performance significantly. An assay that is merely adequate under ideal conditions (e.g., small numbers of plates, idealized timing and liquid handling procedures) is unlikely to translate to higher throughput. Iterative assay optimization is most efficient at this stage.

BASIC PROTOCOL 3. QHTS USING COINCIDENCE REPORTER BIOCIRCUIT

Completion of Basic Protocol 2 will generate a stable cell line expressing the coincidence reporter biocircuit with assay performance metrics suitable for qHTS and proper pharmacological response profiles for the reporter and biological controls. Before beginning Basic Protocol 3, ensure the availability of data analysis software appropriate for compound triage and the identification of reporter-biased artifacts from the primary screen. Basic Protocol 3 enables qHTS using the developed coincidence reporter biocircuit from Basic Protocols 1 and 2 to rapidly identify small molecule modulators of transcriptional activity with significantly reduced risks of reporter-biased artifacts and false negatives. Figure 5 shows an example of a cAMP response element (CRE) biocircuit that was generated, optimized, and validated using Basic Protocol 2, and used further for qHTS following the protocol below. Furthermore, published examples of qHTS using a coincidence reporter biocircuit, Hasson et al. (2015) and Cheng et al. (2012), should be referenced and familiar to the experimenter prior to beginning Basic Protocol 3.

Materials

Coincidence Reporter cell line (Basic Protocol 2)
PTC124 (SelleckChem catalog no. S6003)
Cilnidipine (SelleckChem catalog no. S1293)
Biological control compound(s)—dependent on RE/promoter chosen
Growth media—dependent on cell line
Trypsin
Dimethyl sulfoxide (DMSO)
White solid bottom tissue culture-treated assay plates (384 or 1536), greiner bio-one
Stainless-steel lids containing pinholes for gas exchange (1536-well plates only)
Multidrop Combi Reagent Dispenser with small cassette (Thermo Scientific), or equivalent

Liquid handling (Multimek, PinTool, Mosquito, etc.) for transfer of compounds to assay plate—see Table 1

Library of Pharmacologically Active Compounds (LOPAC) prepared in 100% DMSO in titration

Compound libraries prepared in 100% DMSO in titration, 7–11 point titrations, inter- or intra-plate titrations

Reagent Dispenser for NanoDLR—see Table 1

Plate reader with luminescence capabilities and amenability for HTS—See Table 1

Analysis software for quantitative high-throughput screening analysis such as:

Collaborative Drug Discovery Vault, Dotmatics Studies, Genedata Screener, IDBS ActivityBase or Screenable (these are guided workflow programs with more user-friendly interfaces)

NCGC Curve Fit, scripting in Graphpad software and Tibco Spotfire (these programs require user's ability to pivot incoming plate data)

Tissue culture hood

37°C, 5% CO₂, 95% humidity incubator

Cell culture flasks (T175 with vented filter cap)

Plate reader with luminescence capabilities and amenability for HTS—See Table 1

1. Prepare control plate template and protocol table for referencing during the screening process (see Figure 3 and Table 2).

If screening in 384-well format, the control plate template in Figure 3 is sufficient for preparation of the compound plate. The control plate template is easily prepared in an Excel spreadsheet. If screening in 1536-well format, each well from the 384-well compound plate will be transferred twice, either manually using a multichannel pipette, or via an instrument such as a mosquito liquid handler, into a 1536-well compound plate for subsequent pinning into a 1536-well assay plate. As such, a corresponding control plate template should be generated.

2. Prepare control plate according to control plate template.

If screening in 384-well format, control plate titrations are made directly in the final plate. If screening in 1536-well format, compounds are titrated in a 384-well control plate and transferred in duplicate to a 1536-well daughter plate. Again, we recommend the CAPP Denmark 16-channel pipette for manual pipetting of control plates in 1536-well format.

3. Grow cells from Basic Protocol 2 to appropriate confluence for plating in T175 or T225 flasks, ensuring there are enough cells to plate at the optimized cell density for the number of plates required for the chosen library for screening.

To begin, the LOPAC library should be screened as a validation library. The library should be screened in duplicate to further assess reproducibility of the assay. After confirmation of reproducibility and dose-dependent relationships, larger libraries can be screened. Assay performance metric, Z-factor, should be assessed for LOPAC library screening; however, reproducibility between the duplicate runs and observation of dose-dependent relationships (visual quality control and assessment of $MSR < 3$) are more reliable indicators of a successful assay when using the qHTS paradigm (Haas et al., 2004; Zhang et al., 1999). For example, an assay with a Z-factor of 0.3 or 0.4 that shows the anticipated pharmacological response profiles of control compounds and dose-dependent relationships of compounds within the library would still be considered an excellent assay. Reproducibility can be assessed by plotting duplicate runs in a correlation plot ($\log AC_{50}$ values from run 1 on the x axis and $\log AC_{50}$ values from run 2 on the y axis) and evaluating the r^2 value of a line fit to the data with slope=1 and intercept = 0. Alternately, a Bland-Altman style plot can be created as illustrated in Eastwood et al. (2006), plotting the ratio of the AC_{50} values vs. their geometric means on a log-log plot.

4. Plate cells at the appropriate cell density determined in Basic Protocol 2 in white solid bottom tissue-culture treated plates using a Multidrop Combi Reagent Dispenser and small cassette.
5. Cover plates with stainless-steel lids with pinholes for gas exchange (1536-well) or provided plate lids (384-well) and incubate cells at 37°C, 5% CO₂, 95% humidity incubator for 16–24 hours.
6. Transfer compounds in 100% DMSO from the library and control compound plates to each assay plate containing cells, re-cover plates with lids, and return the plates to the incubator for the determined incubation time for the assay (Basic Protocol 2).

See Table 1 for list of instruments for transferring compounds from compound plates to assay plates. This incubation is assay-dependent based on the biological mechanisms being interrogated.

7. Remove plates from the incubator and use the NanoDLR assay system according to the manufacturer's protocol and appropriate plate reader to quantify the luminescent signal for each reporter in each well of each plate.

See Table 1 for list of instruments to rapidly dispense NanoDLR reagent to assay plates and high-throughput compatible plate readers.

8. Import data from plate reader (assay plate data), compound plate map, and control plate map into third-party data analysis software compatible with qHTS data.
9. Generate plate statistics to assess assay performance using on-plate controls.

10. Using qHTS data analysis software, apply filters to eliminate inactive compounds, compounds that did not elicit a response in either channel. Next, determine if the response(s) are concordant using curve class and/or the concordance correlation coefficient (CCC). See (Hasson et al., 2015) for additional detail regarding the CCC:

$$CCC = \frac{2S_{NLucFLuc}}{S_{NLuc}^2 + S_{NLuc}^2 + (\bar{Y}_{NLuc} - \bar{Y}_{FLuc})^2}$$

where

$$\bar{Y}_j = \frac{1}{n} \sum_{i=0}^n Y_{ij}$$

$$S_j^2 = \frac{1}{n} \sum_{i=0}^n (Y_i - \bar{Y}_j)^2; \quad j = NLuc, FLuc$$

$$S_{ij} = \frac{1}{n} \sum_{i=0}^n (Y_{iNLuc} - \bar{Y}_{NLuc})(Y_{iFLuc} - \bar{Y}_{FLuc})$$

at the *i*th concentration for FLuc and NLuc

The terms $\bar{Y}_j$, S_j^2 and S_{ij} are also known as the average, variance, and covariance respectively, and are usually built-in functions within spreadsheet software such as Openoffice Calc and Microsoft Excel. Additionally, these functions are built into statistics software such as R, which itself has extension packages available such as epiR that contain CCC as a single function.

Actives are defined as compounds that elicit a coincident response in both FLuc and NLuc reporters with signal on either or both channels exceeding 3SD of the median signal of all compounds at a given concentration and have a CCC score greater than 3SD from the ideal CCC score of 1.0. The SD is calculated from all neutral, DMSO controls from the primary screening effort or all compounds for relatively low hit-rate screens. For example a screen with a CCC SD of 0.15 for the DMSO controls, compounds with a CCC score greater than 0.65 would be defined as active.

11. Apply curve fitting algorithms and prioritize the active compounds according to curve class, rank order potency, and efficacy.

For gain and loss of signal assays, the majority of compounds will be fit with a 4-parameter curve fit (Inglese et al., 2006). In gain of signal assays, it is possible to obtain bell-shaped curves in which there is a dose-dependent increase in signal at moderate concentrations and cytotoxicity at high concentrations resulting in a loss of signal. These compounds can be fit with the 5-parameter bell shape curve fit defined in Basic Protocol 1, step 17 if appropriate. Prioritization of compounds will vary depending on the scope and biology of the project but in general should be predominantly guided by curve class and rank order potency (Inglese et al., 2006).

REAGENTS AND SOLUTIONS

10 mM PTC124 stock solution

Dissolve 10 mg PTC124 (Selleckchem S6003) in 3.518 mLs DMSO

Aliquot, snap freeze with a dry ice ethanol bath, and store at -20°C

20 mM Cilnidipine stock solution

Dissolve 10 mg Cilnidipine (Selleckchem S1293) in 1.015 mLs DMSO

Aliquot, snap freeze with a dry ice ethanol bath, and store at -20°C

Similarly, prepare biological control stock solutions, snap freeze with a dry ice ethanol bath, and store at -20°C until day of use.

COMMENTARY

Background Information

Reporter Genes in High-throughput Screening—Reporter genes amplify subtle biological events and were first used in molecular biology to facilitate the cloning of genes (Casadaban et al., 1980; Yanisch-Perron et al., 1985), study transcriptional regulation by mapping promoter and enhancer regions (Kalbe et al., 2000; Luckow and Schutz, 1987), and in cell biology to delineate signaling pathways and cellular pharmacology (Himmler et al., 1993; Montmayeur and Borrelli, 1991). Functional promoter analysis studies were initially performed by transiently transfecting cells with a series of cloned promoter fragments, which varied in length or contained mutations and/or deletions, in a reporter vector (most commonly green fluorescent protein, β -galactosidase, or Firefly luciferase) in order to identify all necessary and sufficient regulatory (enhancer and repressor) elements for a given gene (Cheng et al., 2004; Michelini et al., 2010; Solberg and Krauss, 2013). Similarly, early cellular pharmacology studies utilized cloned REs in reporter vectors to monitor signaling pathways, and pharmacological modulation of those pathways, in transiently and stably transfected cell lines in low-throughput experiments (Himmler et al., 1993).

In the 1990's and early 2000's, tremendous growth in chemical library size and the increasing prevalence of high-throughput screening (HTS) compatible instrumentation facilitated the transition of reporter gene assays into miniaturized format and their extensive use in HTS to identify pharmacological modulators of a broad range of cellular processes that still persists today (Fan and Wood, 2007; Michelini et al., 2010). These early HTS assays used a single concentration of the library compound and a single reporter. However, this assay format is now appreciated to be prone to a high degree of reproducible, though non-traceable, assay-dependent artifacts, false positives, and false negatives due to narrow concentration testing. The need to overcome these issues, and increase efficiency and quality of HTS, has motivated the development of quantitative high-throughput screening and coincidence reporter technology.

Quantitative high-throughput screening (qHTS), introduced in 2006, is a titration-based screening paradigm that efficiently identifies subtle pharmacology that would otherwise be

missed when screening with single concentration HTS (Hasson et al., 2015; Inglese et al., 2006). For example, single concentration screening at 10 μ M would inaccurately report a compound with nanomolar potency that is cytotoxic at 10 μ M as inactive, or fail to detect a compound of very modest potency (>10 μ M) or low efficacy (e.g. below the cutoff determined for retesting). Medicinal chemistry efforts to remove cytotoxicity and maintain or improve potency, enhance efficacy or increase solubility, respectively, could afford a very promising biologically active compound.

By the late 2000's, an increasing number of studies were demonstrating library compounds' propensity for direct interaction with reporter enzymes, irrespective of assay conditions (Auld et al., 2010; Auld et al., 2008a; Auld et al., 2009a; Auld et al., 2008b; Auld et al., 2009b; Heitman et al., 2008; Herbst et al., 2009). In fact, several lead compounds initially reported as active were subsequently shown to directly inhibit the reporter used in the assay, resulting in a promoter-independent – rather than a biologically-relevant – increase in reporter signal (Auld et al., 2010; Auld et al., 2008a; Auld et al., 2008b). Far from being a rare occurrence, such reporter-biased activity was found to account for 40–95% of preliminary actives from screens employing reporter genes (Ho et al., 2013). To minimize the time, and money, wasted pursuing assay-dependent artifacts, the first generation 'coincidence reporter' was introduced in 2012 (Cheng and Inglese, 2012). The coincidence reporter encodes two non-homologous reporters with orthogonal enzymology, stoichiometrically expressed and separated by a highly efficient P2A ribosomal skipping sequence (Kim et al., 2011; Kuzmich et al., 2013). This system significantly reduces the off-target activity since only a small number of compounds will interact with both reporters. Thus, only coincident responses, response profiles with similar potencies, are considered "hits" or biological actives. The first generation reporter employed Firefly luciferase (FLuc) and Renilla luciferase as the orthogonal enzymatic reporter pair driven by cAMP response element activation (CRE) in a proof-of-principle qHTS experiment (Cheng and Inglese, 2012).

Introduction of the smaller and brighter nanoluciferase (NLuc; Promega) subsequently improved the orthogonal enzymatic pair. A systematic characterization of luciferase reporter-biased artifacts demonstrates fewer direct inhibitors of NLuc compared to RLuc. Furthermore, NLuc inhibitors are generally less potent (Ho et al., 2013). The second generation coincidence reporter capitalizes on the enhanced pairing of FLuc and NLuc as an orthogonal pair. When used together with qHTS, the coincidence reporter facilitates more rapid identification of true biologically active compounds and minimizes distracting reporter-biased artifacts. (Hasson et al., 2015).

Critical Parameters

Pharmacological response profiles, concentration response curves (CRCs) and assay performance metrics—Known Firefly luciferase stabilizing inhibitors should elicit an FLuc-specific response, known Nanoluciferase stabilizing inhibitors should elicit an NLuc-specific response (reporter controls), and the biological control(s) should elicit coincidence responses at all assay validation stages and on each plate (on plate controls) during qHTS. These pharmacological profiles confirm a properly functioning

coincidence reporter biocircuit and are paramount for employing the coincidence reporter technology in an efficient qHTS paradigm. It should be noted that reporter control responses require a basal level of reporter expression, which is usually present. In gain-of-signal assays the basal will be low and a reporter-biased increase, attributed to stabilization of the enzyme that increases the half-life, of the concentration response curve, as shown in Figure 2 and Figure 5, would be observed. However, in loss-of-signal assays the basal can be high and reporter controls may primarily result in reporter-biased decreases in output signal. In situations where basal reporter expression is exceedingly low, for example due to reporter targeting to a tightly silenced locus, sufficient reporter may not be present to observe stabilization. Exceedingly low basal expression utilizing random integration of the coincidence reporter, as outlined in these protocols, would suggest reporter and/or transfection optimization and iterative assay design is necessary to obtain an assay with sufficient signal. If the coincidence reporter is being adapted for use in genome editing, where targeting to a tightly silenced locus may be the intended design, exceedingly low basal reporter expression would be anticipated and a well-characterized biological control would be required to confirm coincident increases in reporter expression as a result of unsilencing (for example, epigenetic modulators). Yet, in all assays, the distinguishing feature for a reporter-biased artifact and a true biological pharmacological modulator during screening is single vs coincident (similar potency) reporter readouts, respectively. Additionally, assay performance metrics such as Z-factor and MSR should be monitored during development and validation to assess assay window and reproducibility. Values for Z-factor should generally be ≥ 0.5 and MSR should be < 3 for both FLuc and NLuc. During assay development (Basic Protocol 1 and 2), it is important to remember that assay volume miniaturization and scale-up to large-scale screening can diminish assay performance and iterative assay optimization may be required. Critical evaluation of pharmacological response profiles, Z-factor, and MSR during this stage provides valuable guidance during the iterative assay development process. However, an advantage of employing qHTS is that the resultant concentration response curves (CRC) can be more tolerant of lower assay performance (e.g. Z-factor < 0.5), as the pharmacological nature of the data (i.e., a response profile generated over 4 orders of magnitude in compound concentration) is a concentration-dependent relationship obtained from multiple data points.

In addition to providing a metric by which to guide progression through assay development, Z-factor and MSR should also be evaluated during qHTS to help identify reproducibility issues—due to automation dispense errors, signal loss, plate-specific cell-death, etc.—that may have occurred during the primary screening stages. Similarly, visual quality control checks of proper pharmacological response profiles of all on-plate controls for each plate will also inform data analysis efforts on the reliability of each plate's data. It is important to note that although Z-factor and MSR are well-characterized assay performance metrics for high-throughput screening, the reliability of observing concentration-dependent relationships is critical as each concentration serves as a technical replicate and a measure of reproducibility for that compound.

Concordance correlation coefficient (CCC): Triage of true biological actives and elimination of reporter-biased artifacts—Although screening a small, focused

library of 3,000 compounds takes only 3 days (from plating the cells into assay plates to quantifying luminescence), analysis of titration-based qHTS can take much longer depending on the type of informatics support available and the curve fitting software being used. Figure 4 outlines the broad steps for assessing the data from a compound screen and focusing on compounds with coincident responses in the FLuc and NLuc reporter channels. Following elimination of inactive compounds—compounds in which there is no evoked response in either channel—remaining compounds should be considered active only if the responses in the FLuc and NLuc channel are concordant as determined by curve classes or a CCC greater than 3 standard deviations (SD) from the ideal CCC of 1 with signal exceeding an activity threshold at any concentration point, generally 3 or 6 SD. The SD for the signal of each respective channel, and the CCC, is calculated from all neutral DMSO controls. For a library with relatively low activity, the SD of the CCC can be calculated from all the individual compounds in the library. Prioritization of compounds for follow up screening and secondary assays will be dependent on the scope of the project and prior knowledge of the assay biology but should be predominantly guided by curve class and rank order potency of the active compounds.

Troubleshooting

Assessment of pharmacological profiles of reporter and biological control compounds will provide insight into the success of each protocol. If the expected pharmacological response profiles are not observed, caution should be taken prior to proceeding and second generation assays should be considered. It is not uncommon for Basic Protocol 1 and 2 to be an iterative process in which there are first, second, and third generation reporters developed. If problems arise during Basic Protocol 1 (transient transfection validation), reporter design is most practical at this stage. If lack of signal or insufficient signal is the concern, reporter re-design options include alternative promoter length to include additional enhancer and/or regulatory elements or inclusion of minimal promoter for signal amplification (Figure 7). If appropriate pharmacological response profiles to control compounds are not observed, reporter re-design may rectify the issue if lack of signal is the suspected cause of inadequate or skewed pharmacological responses. It is also possible that the transfection may have been unsuccessful so it is always advisable to try alternative transfection protocols. If non-coincident responses are observed with the known biological controls, careful evaluation of the DNA and amino acid sequence should be performed to ensure proper expression of the reporters and P2A skipping sequence. Evaluation of the biological control(s) using recombinant enzymes for each reporter should be performed to determine if the non-coincident response is due to contributing activity against the reporter enzyme (i.e. although uncommon, the biological control can have activity in the pathway of interest and interact directly with the reporter). Recombinant FLuc enzyme (catalog #L9506) and D-luciferin (catalog #L9504) can be purchased from Sigma to assess activity of compounds against FLuc enzyme using a 10 minute incubation and substrate (D-luciferin) K_m of 10 μM . However, there is no recombinant NLuc enzyme currently available. NCATS and others have systematically profiled libraries against the recombinant FLuc enzyme and secreted NLuc enzyme, obtained from mammalian cells, and can provide information regarding NLuc activity of your biological control compound(s) if necessary.

Unexpected pharmacological responses in Basic Protocol 2 may indicate incomplete integration during transfection, issues during antibiotic selection, and/or lack of retention of the reporter construct during cell outgrowth. A second transfection attempt should be made prior to reporter re-design or consideration of an alternative cell line. If a second selection process is unsuccessful, alternative transfection protocols should be considered as well as alternative cell lines. In addition, a second generation coincidence reporter biocircuit can be considered if manipulations to the promoter length, addition of minimal promoter, etc. have not been attempted previously (Figure 5).

Understanding Results

A reporter gene assay using coincidence reporter technology suitable for qHTS that reliably reduces false negatives and reporter-biased artifacts (false positives), and increases detection of subtle pharmacology compared to a single luciferase reporter gene assay, will be obtained using all three protocols. Basic Protocol 1 and 2 can be completed independently of Basic Protocol 3 to generate a stable cell line expressing a coincidence reporter biocircuit that can be used for single concentration high-throughput screening if accessibility to chemical libraries in titration is limited. Anticipated pharmacological response profiles for reporter controls, PTC124 and Cilnidipine, are highlighted in each protocol and exemplified in Figures 2 and 5. The troubleshooting section above and troubleshooting workflow (Figure 7) should be consulted if unanticipated pharmacological response profiles are not observed. Additionally, iterative assay design and optimization should be considered if assay performance metrics, explained in each protocol and the commentary, are not within the specified limits.

The key advantage of using a coincidence reporter biocircuit is to reduce false positives due to interactions of library compounds on the expressed reporter's cellular half-life and/or enzymatic activity. By the simultaneous expression of two non-homologous reporters (e.g., FLuc and NLuc) from a shared response element or gene locus, the probability that a compound will influence the cellular half-life and/or enzymatic activity of both reporters is unlikely and therefore the most likely rationale for concordant responses is the modulation of cellular processes impinging upon the response element or gene locus. The larger the chemical library tested, the more significant this becomes. This is because compounds advanced for further study from HTS will be selected from potentially numerous actives of which coincident activity may represent a significant minority. Relative luminescence modulation from a reporter-biased response can be greater than the desired biological pathway modulation and, without a second discriminating coincidence reporter channel, selected over the more relevant compound for follow-up. In this case the coincidence reporter would aid in lowering the false negative occurrence.

Time Considerations

If the promoter or RE of interest is well-characterized, Basic Protocol 1 can be completed within two to three weeks. The time commitment of Basic Protocol 1 can be significantly increased if the promoter or RE is not well-characterized and several reporter constructs are required in order to interrogate the biology and identify biological controls for assay development. Basic Protocol 2 can take weeks to months depending on the cell line being

used and the amenability of the cell line to stable transfection protocols (growth from single cell density, doubling time, etc.). Time commitment for Basic Protocol 2 can be amplified if problems arise during the clone characterization or cell density optimization process (see Figure 6) and iterative clone selection must be done. Time to complete Basic Protocol 3 will be dependent on library size and breadth of the primary screening stages and can vary from weeks to months or more with a large portion of the time commitment being devoted to data analysis.

As demonstrated above, qHTS using a coincidence reporter biocircuit is a lengthy process and can take weeks to months of dedicated effort depending on the complexity of the assay, the size of the library, and level of automation. There are over 120 academic screening centers registered with the academic drug discovery consortium (<http://addconsortium.org/>) and screening facilities at the National Center for Advancing Translational Sciences (NCATS) with experts in high-throughput screening that participate in collaborative projects. If assay development is an interest but resources (robotics, compound libraries, data analysis infrastructure) are limited, and/or the time commitment is prohibitive, collaborating with a screening facility is recommended. Furthermore, cell lines expressing a coincident reporter biocircuit can be used in single concentration HTS to reduce the time commitment of Basic Protocol 3.

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Significance Statement

High-throughput screening (HTS) is commonly used to identify chemical probes and compounds with therapeutic potential for drug development. Reporter gene assays are largely used in HTS to identify transcriptional and response element modulators that are associated with a biology or pharmacology of interest. Traditional reporter gene assays use a single reporter that is susceptible to reporter-biased artifacts, caused by compounds interacting directly with the reporter enzyme and not the biology or pharmacology of interest, that elicit false positive responses. The coincidence reporter is employed to increase the efficiency and quality of primary HTS where a biological active elicits a coincident response in two non-homologous reporter enzyme readouts, distinguishing it from reporter-biased artifacts that elicit a response in only one reporter readout.

Figure 1.

Generating a coincidence reporter biocircuit using pNLCoI1 or pCI9.0. **(A)** Linear diagram depicting the architecture of the coincidence biocircuit and the relationship to an element driving transcription. **(B)** Circular plasmid maps for pNLCoI1 (Promega) and pCI9.0 (Inglese laboratory) identifies the multiple cloning site for insertion of promoter or response element during Basic Protocol 1. pCI9.0 incorporates N-terminal FLAG tags on each reporter to allow assessment of reporter expression by Western blot analysis during clonal cell line development.

Figure 2.

Coincidence reporter pharmacological response profiles. **(A)** Overview of coincidence reporter technology as it applies to reporter gene assay development and qHTS. A true biologically active compound is represented as a red hexagon and would have activity within a biological pathway that would lead to activation of the targeted pathway promoter or response element cloned into the coincidence reporter in Basic Protocol 1. This would lead to transcription and subsequent translation of the two orthogonal luciferase enzymes, FLuc and NLuc, to produce two independent reporter enzymes. There are three potential pharmacological response profiles as shown in **(B)**. FLuc stabilizing inhibitors such as PTC124 that directly interact with the FLuc enzyme, NLuc stabilizing inhibitors such as Cilnidipine that directly interact with the NLuc enzyme or the biological active compound (represented as the red hexagon in **(A)**) that interacts with the biology of interest. Stabilizing inhibitors of either luciferase enzyme display responses in only one channel, specific to the luciferase to which they inhibit while true biologically active compounds elicit coincidence responses in both FLuc and NLuc channels. Furthermore, stabilizing inhibitors yield concordance correlation coefficient's (CCC) with absolute values well below 1 while biological active compounds yield CCC's very near the ideal CCC (perfect concordant response of the NLuc and FLuc channels) of 1.0. These pharmacological response profiles are used in Basic Protocol 1 and 2 to confirm proper function of the coincidence reporter and guide selection of cell line clones and in Basic Protocol 3 to triage data.

A Compound Treatment for Basic Protocol 1 Validation **B** Control plate layout for Basic Protocol 2 Validation and Basic Protocol 3 qHTS **Figure 3.**

Plate layouts for control compound treatment. **(A)** Control compound treatment for Basic Protocol 1 validation. Row 1 serves as an untreated control to determine if DMSO has an effect on cell type or assay conditions. Row 2 is the DMSO control to which all other treatments can be normalized during data analysis. DMSO concentration should be kept as low as possible. Rows 3 and 4, columns 1–9, are PTC124 in titration, ranging from 10^{-5} to 10^{-12} M. Rows 5 and 6, columns 1–6, are Cilnidipine in titration, ranging from 10^{-4} to 10^{-9} M. Rows 7 and 8, columns 1–9, are the biological control in titration, ranging in concentration to include at least 3 log units on either side of the EC_{50}/IC_{50} . Rows 3–8, columns 9–12, are a single high concentration of the biological control to be used for calculating Z-factor to assess the potential signal window of the assay. **(B)** Suggested control plate layout for 384-well and 1536-well plates in Basic Protocol 2 and 3. Column one is DMSO, column 2, rows 1–8 is an 8 pt titration of PTC124 ranging from 10^{-5} to 10^{-12} M,

column 2, rows 9–16 is Cilnidipine in titration, ranging from 10^{-4} to 10^{-9} M, column 4 is the biological control in titration ranging in concentration to include at least 3 log units on either side of the EC_{50}/IC_{50} , and column 4 is a single high concentration of the control to be used for Z-factor calculation and for normalization during data analysis. Note: For 384-well screening, the exact layout for the assay plate control compound treatment can be used. Although the number and location of control compounds may vary if collaborating with a screening center that has a predefined preference. For 1536-well screening, each well of this 384-well parent control plate would be transferred twice, using a manual pipette, into columns of a 1536-well daughter compound plate, thus there would be 32 wells of DMSO and 2 wells of each titration point.

Figure 4.

Cell density and variability evaluations during stable cell line generation. **(A)** Cell density optimization of a monoclonal cell line where the left panel shows basal luminescence for each reporter and the right panel shows Z-factor values for each reporter using a single column of the biological control (24 h incubation) in 1536-well format. Basal luminescence increases with cell number and then plateaus for each reporter. The Z-factor is acceptable for all cell densities tested with the highest NLuc Z-factor at 1600 cells per well and the highest Z-factor for FLuc at 2400 cells per well. Because the luminescent signal has plateaued at 2000 and 2400 cells per well, 1600 cells per well was chosen for qHTS to maximize Z-factor and performance for each reporter with respect to changes in FLuc and NLuc (left panel). **(B)** Luminescence values for a polyclone and monoclonal comparing CV (%) for each reporter where the left panel is basal FLuc luminescence and the right panel is basal NLuc luminescence. In this example, the polyclone exhibits unacceptable CV's (>10%) and the monoclonal is preferential with CV's <10%. The inset exemplifies a cell line where the variability is low (CV<10%) after generation of a polyclonal cell line with a coincidence reporter biocircuit. Because this cell line showed reproducible pharmacological response profiles and had low variability at the polyclonal stage, the polyclone would be acceptable to use for qHTS.

Figure 5.

Pharmacological response profiles for cAMP response element coincidence biocircuit. **(A)** Schematic depicting β -adrenergic signaling through PKA phosphorylation of CREB which can be monitored with a cAMP response element (CRE) coincidence reporter biocircuit. **(B)** Pharmacological response profiles of PTC124, Cilnidipine, and Isoproterenol in HEK293 cells expressing the CRE coincidence reporter or the CRE coincidence reporter and the $\beta 2$ Adrenergic Receptor ($\beta 2$ AR). PTC124 elicits an FLuc-specific response in both cell lines, Cilnidipine elicits an NLuc-specific response in both cell lines, and Isoproterenol elicits a coincident response in both cell lines. The coincident response for Isoproterenol is much greater in the $\beta 2$ AR expressing cells, as expected, because Isoproterenol is a beta receptor agonist. Minimal activation is observed in the CRE only cell line due to endogenous expression of $\beta 2$ AR.

Follow-up screening, secondary assays

Figure 6.

Compound triage for identifying active compounds from qHTS for follow up and secondary assays. All data from primary qHTS screening should be uploaded into a third-party screening software such as Dotmatics, Tibco Spotfire, or Collaborative Drug Discover Vault. Filters should be applied to eliminate inactive compounds that do not evoke a response exceeding 3 or 6 SD of the median signal in either reporter channel from the analysis. From the remaining compounds, apply the concordant correlation coefficient (CCC) metric to eliminate compounds that do not have CCC scores greater than 3 SD from a the ideal CCC of 1.0 with SD calculated from all neutral DMSO controls or all compounds where library activity in the assay is relatively low. For example, for a screen with a CCC SD for DMSO of 0.15, the CCC cutoff would be greater than 0.65. Compounds with CCC scores greater than 0.65 would be considered active and those compounds would be fitted using 4-parameter curve fits and assigned to curve classes according to Inglese et al (Inglese et al., 2006). Compounds are then prioritized for follow up screening and secondary assays based on curve classes, rank order potency and efficacy (Inglese et al., 2006).

Figure 7.

Troubleshooting guidance for iterative assay development. Criteria for progression through each basic protocol and suggestions for troubleshooting for each basic protocol are outlined in a flow chart diagram. Transient transfection of the reporter construct is completed in Basic Protocol 1 and stable transfection of the reporter construct high-throughput screening validation is completed in Basic Protocol 2.

Table 1

Instrumentation for qHTS.

Step	Vendor	Instrument
Dispense Cells	Thermo Scientific	Multidrp Combi Reagent Dispenser *
	BioTek	EL406 Dispenser
	Beckman Coulter	BioRAPTR Flying Reagent Dispenser
Compound Transfer	Wako Automation	Hornet PinTool *
	Beckman Coulter	MultiMek Nanoscreen NSX
	TTP LabTech	Mosquito
	Labcyte	Echo Liquid Handler
	CyBio	CyBi-Well (384 only)
NanoDLR Reagent	Beckman Coulter	BioRAPTR Flying Reagent Dispenser *
	BioTek	EL406 Dispenser
	Thermo Scientific	Multidrp Combi Reagent Dispenser
HTS compatible luminescence plate reader	PerkinElmer	ViewLux *
	PerkinElmer	EnVision with plate stacker
	Tecan	M1000 PRO
	BioTek	Synergy Neo2

* Preferred instrumentation for each step

Table 2

Sequence table example for qHTS to be followed when screening and reported when publishing.

Step	Parameter	Value	Description
1	Dispense Cells	4 μ L	1600 cell/well in 1536-well white solid bottom tissue-culture treated grenier plate
2	Incubation	24 hr	37°C, 5% CO ₂ , 95% relative humidity
3	Library compounds	23 nL	Compound transfer by PinTool; 57 μ M to 56 nM; 11 point, 2 fold titrations
4	Control compounds	23 nL	Compound transfer by PinTool; PTC124 and Cilnidipine used as reporter controls for FLuc and NLuc, respectively. Biological control used to induce reporter expression in titration and at a single high concentration.
5	Incubation	24 hr	37°C, 5% CO ₂ , 95% relative humidity
6	NanoDLR	3.5 μ L	Dispense NanoDLR ONE-Glo EX Reagent
7	Incubation	10 min	Incubate at room temperature, protected from light
8	Detection	ViewLux	Luminescence Read 1 (FLuc)
9	NanoDLR	3.5 μ L	Dispense NanoDLR Stop & Glo Reagent and NLuc substrate
10	Incubation	10 min	Incubate at room temperature, protected from light
11	Detection	ViewLux	Luminescence Read 2 (NLuc)

Step	Notes
1	Solid white tissue culture-treated plates from Grenier; indicate any dispense errors that may have occurred
3	Compound plate barcodes with maps saved in C:\\RE_FLUC_NLUC_Date
4	Control plate map saved in in C:\\RE_FLUC_NLUC_Date; DMSO column 1, PTC124 titration column 2, rows 1–16, Cilnidipine titration column 3, rows 17–32, Biological control titration column 3, biological control high concentration column 4
6	ONE-Glo EX buffer + substrate = ONE-Glo EX Reagent which can be frozen and used in aliquots on day of use; BioRAPTR Tip 1
8	Exposure=30 sec, Gain=High; 2X binning
9	1:100 dilution substrate:Stop& Glo buffer made just before use; BioRAPTR Tip 3
11	Exposure=30 sec; Gain=High; 2X binning