Quantitative High-Throughput Screening Using a Coincidence Reporter Biocircuit

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Reporter-biased artifacts—i.e., compounds that interact directly with the reporter enzyme used in a high-throughput screening (HTS) assay and not the biological process 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 HTS, or quantitative HTS (qHTS), and coincidence reporter technology can be employed to reduce false negatives and false positives, 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 biological or pharmacological question of interest, generation of a stable cell line expressing the CR biocircuit, and qHTS using the CR biocircuit to efficiently identify high-quality biologically active small molecules. © 2017 by John Wiley & Sons, Inc.

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INTRODUCTION

High-throughput screening (HTS) has been used for many years in the pharmaceutical industry to identify therapeutic compounds for the treatment of human disease (Macarron et al., 2011). More recently, it has been broadly applied to identify chemical probes, gain mechanistic insights into complex biological processes via pharmacological profiling, and complement industrial pursuits of novel therapeutics for human disease (Hasson and Inglese, 2013; Dahlin et al., 2015). 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). 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). There have been advancements that minimize these shortcomings, in particular for traditional reporter gene assays that utilize a single reporter, and increase the efficiency of HTS (Auld et al., 2008b; Cheng and Inglese, 2012; Hasson et al.,

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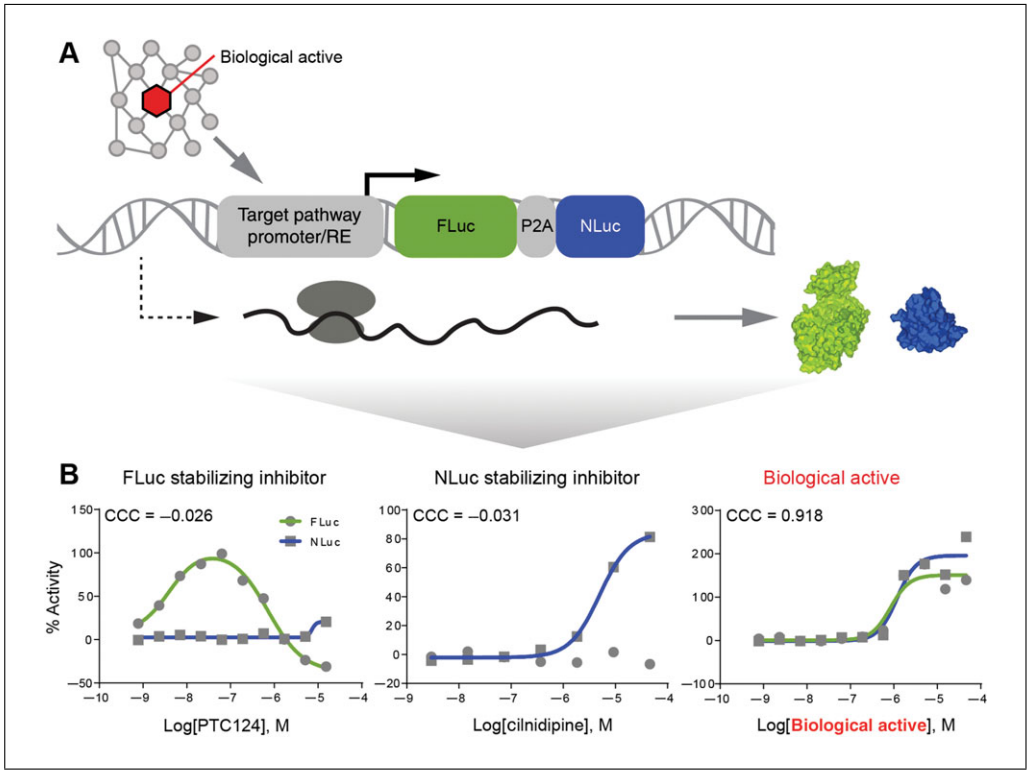

Figure 5.32.1 Coincidence reporter approach and pharmacological response profiles. (**A**) Overview of coincidence reporter technology as it applies to reporter gene assay development and qHTS. A true biologically active compound (red hexagon) has activity within a biological pathway that leads to activation of the targeted pathway promoter or response element (RE) cloned into the coincidence reporter in Basic Protocol 1. This leads to transcription and subsequent translation of two orthogonal luciferase enzymes (FLuc and NLuc) to produce two independent reporters. (**B**) Potential pharmacological response profiles are shown for (left) an FLuc stabilizing inhibitor such as PTC124 that directly interacts with the FLuc enzyme, (middle) an NLuc stabilizing inhibitor such as cilnidipine that directly interacts with the NLuc enzyme, and (right) the biologically active compound (red hexagon in A) that interacts with the biological pathway of interest. Stabilizing inhibitors of either luciferase enzyme display responses in only one reporter-specific channel, whereas true biologically active compounds elicit coincident responses in both reporter channels. Furthermore, stabilizing inhibitors yield concordance correlation coefficients (CCCs) with absolute values well below 1, whereas biologically active compounds yield CCCs very near the ideal CCC of 1.0 (i.e., perfect concordant response of NLuc and FLuc channels). These pharmacological response profiles are used in Basic Protocols 1 and 2 to confirm proper function of the coincidence reporter and guide selection of cell clones, and in Basic Protocol 3 to triage data.

2015). This unit provides a comprehensive procedure for generating a reporter gene assay using coincidence reporter (CR) technology and quantitative high-throughput screening (qHTS) to reduce reporter-biased artifacts and false negatives (Cheng and Inglese, 2012; Hasson et al., 2015). Coincidence reporters are different from dual-reporter assays where one luciferase reporter monitors the biological process of interest and a 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, all cloned downstream of the response element (RE) or promoter, where both reporters monitor the biology of interest. Thus, a "biological active" will elicit a coincident response in both reporter enzyme readouts, whereas a reporter-based artifact will elicit a response in one readout. The experimental principle and example readouts are shown in Figure 5.32.1. Basic Protocol 1 describes construction and validation of the coincidence reporter biocircuit, and Basic Protocol 2 describes generation of stable cell lines expressing the biocircuit. Finally, Basic

Protocol 3 describes qHTS for biologically active compounds with significantly reduced risk of reporter-based artifacts.

STRATEGIC PLANNING

Reporter Design

The success of an assay relies in large part on design of the coincidence reporter biocircuit. The reporter must be grounded in relevant biology and generate a readout that is reproducibly measurable in miniaturized format. Ideally, the RE or promoter region of interest should be genetically and/or pharmacologically tractable, e.g., via mechanisms such as transcription factor silencing or compound-mediated modulation. It should also be sufficiently characterized to permit design of an effective mimic (e.g., with respect to promoter length or RE characteristics). For example, the endoplasmic reticulum stress response element can be activated with tunicamycin and is well characterized (Montminy et al., 1986). Similarly, cAMP response element (CRE) activation downstream of GPCR signaling is well characterized and can be used to monitor pharmacological modulation of GPCR activity, for example, β-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, firefly luciferase (FLuc) and nanoluciferase (NLuc) (Fig. 5.32.1). 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 eliminating artifactual 'hits' attributable to direct stabilizing inhibition of 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 2A (P2A) contributes to the stable stoichiometric expression of the two luciferase enzymes (Kim et al., 2011; Kuzmich et al., 2013).

Vectors

The choice of vector for the reporter should be made prior to beginning the experiment. Maps of the pNLCoI1 (Promega) and pCI 9.0 (available from Addgene, James Inglese Lab Plasmids) vectors are shown in Fig. 5.32.2. Sequence information can be downloaded from the NCBI for pNLCoI1 (accession no. KM359771) or obtained from the Inglese laboratory for pCI 9.0. Both vectors lack a promoter element to allow for easy construction of reporter gene assays. If the RE or promoter signal is weak, a minimal promoter may be needed to enhance expression. For these studies, pNLCoI2 (Promega) can be used, or gene synthesis can be performed to build a RE/promoter + minP insert for pNLCoI1 or pCI 9.0. pCI 9.0 differs from pNLCoI1 in that each reporter contains an *N*-terminal 3×FLAG tag to assess reporter protein expression during monoclone and polyclone generation. In addition, the vectors use different antibiotics for selection: hygromycin B for pNLCoI1 and puromycin for pCI 9.0.

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

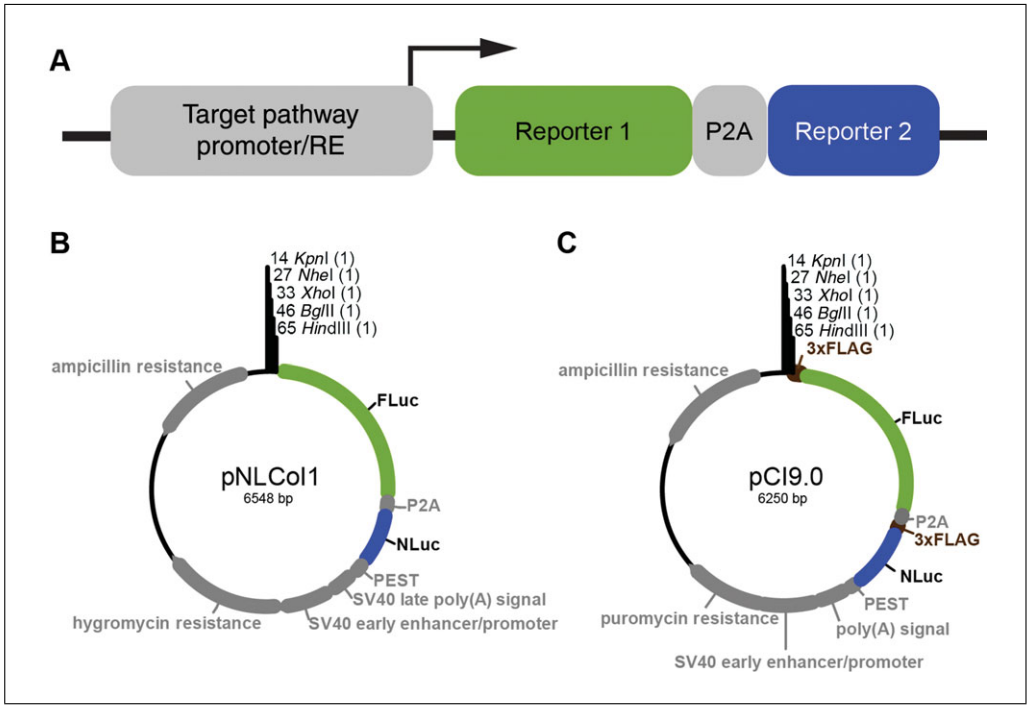

Figure 5.32.2 Generating a coincidence reporter biocircuit using pNLCol1 or pCI 9.0 (**A**) Linear diagram depicting the architecture of the coincidence biocircuit and its relationship to an element driving transcription. (**B**) Circular plasmid maps for pNLCol1 (Promega) and pCI 9.0 (Inglese laboratory or Addgene) showing multiple cloning sites for insertion of the promoter or response element. pCI 9.0 incorporates *N*-terminal FLAG tags on each reporter to allow assessment of reporter expression by western blotting during clonal cell line development.

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.

There are several sources of DNA for the RE/promoter region of interest. Regions of 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 (IDT) or Geneart (Life Technologies), 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 flanking restriction sites added during primer design.

Although this unit 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).

Cells

Before beginning, it is important to be familiar and comfortable with standard cell culture practices for the cell line that will be used (Phelan and May, 2015). Many of the steps rely on cell type–specific knowledge that must be empirically determined (e.g., confluence for passaging, ability of the cell line to grow from single-cell density, optimal transfection conditions) and are assumed to be known prior to starting. The choice of cell line should be fully vetted based on the scope of the project. Cell line parameters for consideration should include the biological or pharmacological process being investigated, ease of use

Table 5.32.1 Instrumentation for qHTS

Step	Instrument	Vendor
Dispensing cells	Multidrop Combi Reagent Dispenser[a]	Thermo Scientific
	EL406 Dispenser	BioTek
	BioRAPTR Flying Reagent Dispenser	Beckman Coulter
Transferring compounds	Hornet PinTool[a]	Wako Automation
	MultiMek Nanoscreen NSX	Beckman Coulter
	Mosquito	TTP LabTech
	Echo Liquid Handler	Labcyte
	CyBi-Well (384 only)	CyBio
Dispensing reagents for NanoDLR	BioRAPTR Flying Reagent Dispenser[a]	Beckman Coulter
	EL406 Dispenser	BioTek
	Multidrop Combi Reagent Dispenser	Thermo Scientific
Reading luminescence	ViewLux[a]	PerkinElmer
(HTS-compatible plate readers)	EnVision with plate stacker	PerkinElmer
	M1000 PRO	Tecan
	Synergy Neo2	BioTek

[a]Preferred instrumentation for the indicated step.

for transfection and scaling to low-volume microtiter plates, and amenability to secondary assays. Coincidence reporter technology has been successfully transfected into several neurologically relevant cell lines, including human neuroblastoma BE(2)M17 (Hasson et al., 2015) and SK-N-BE cells and rat RT4 and S16 Schwann cells (unpublished).

Cell transformants carrying the coincidence reporter are selected using hygromycin B or puromycin, depending on the vector that is used. A kill curve experiment should be completed prior to starting the protocol to determine the optimal antibiotic concentration for the specific cell line used. Life Technologies provides a protocol for performing such an experiment (*http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics/transfection-basics/transfection-methods/stable-transfection.html*).

Compound Libraries

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 available in a 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 is dependent on the scope and goals of the project (Inglese and 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 G protein–coupled receptor (GPCR) activation of a given pathway? Each of these would be begin with different library selections. Some chemical libraries are built around "drug-likeness" and their adherence to Lipinski's rule of 5. 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 (Inglese and 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 that include structurally related analogs and provide structure-activity relationship (SAR) guidance for medicinal chemistry.

The screening format must also be considered. Although the protocols below are outlined for qHTS, the coincidence reporter can also be used for single-concentration screening. Again, coincident responses (in this case, similar activity 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 curves (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 titrations and will depend on initial library plating, liquid handling capabilities, and screening format (384 or 1536 wells) (Yasgar et al., 2008).

qHTS and Instrumentation

Before beginning, it is important to be familiar with assay design and equipment/instrumentation for high-throughput screening. A recommended reference is Assay Guidance Manual. For equipment and instrumentation, refer to the chapter by Jones et al. (2004). In addition, recommended HTS-compatible plate readers and instruments for dispensing cells, reagents, and compounds are listed in Table 5.32.1.

BASIC PROTOCOL 1

CONSTRUCTION AND VALIDATION OF THE COINCIDENCE REPORTER BIOCIRCUIT

This protocol outlines the cloning steps necessary to generate and validate the performance of the coincidence reporter biocircuit to interrogate a given promoter or RE. Validation is performed by transiently transfecting the reporter into cells and testing the response of the reporter to various controls. After validation, confidence should be obtained that the coincidence reporter biocircuit is working properly—that basal FLuc and NLuc are detectable and can be modulated with specific stabilizing inhibitors (PTC124 and cilnidipine, respectively), and the RE/promoter of interest can be modulated with known biological control(s). Importantly, the biological control should elicit a coincident response in both channels and provide a sufficient assay window for screening ($Z' > 0.5$; Zhang et al., 1999).

Materials

Vector plasmid: pNLCoI1 (Promega, cat. no. N1461) or pCI 9.0 (Addgene, James Inglese Lab Plasmids, cat. no. 74229)
Geneblock/Gene synthesis, PCR product, or plasmid vector containing RE or promoter region of interest
DNA restriction enzymes (see Fig. 5.32.2 for multiple cloning sites)
Calf intestinal alkaline phosphatase (CIAP; New England Biolabs, cat. no. M0290)
QIAquick PCR Purification kit (Qiagen, cat. no. 28104) or equivalent
Quick Ligation kit (New England Biolabs, cat. no. M2200) or equivalent
Competent *E. coli*, e.g., DH5α (New England Biolabs) or Top10 (Life Technologies)
Transfection reagent (e.g., Lipofectamine 2000)
SOC medium
LB agar plates containing 100 µg/ml ampicillin
50% (v/v) glycerol
QIAprep Spin Miniprep kit (Qiagen, cat. no. 27104) or equivalent
HiSpeed Plasmid Maxi kit (Qiagen, cat. no. 12662) or equivalent
Dimethyl sulfoxide (DMSO)

10 mM PTC124 (see recipe)
20 mM cilnidipine (see recipe)
Biological control compound(s) specific for RE/promoter (see recipe)

1.5-ml microcentrifuge tubes
Tissue culture hood
37°C, 5% CO_2, 95% humidity incubator
2-ml cryovials
6-well tissue culture–treated plates (Corning, cat. no. 3506)
96-well solid white tissue culture–treated assay plates (Corning, cat. no. 3917)
Nano-Glo Dual-Luciferase Reporter (NanoDLR) Assay System (Promega, cat. no. N1610 or N1620)
Plate reader with luminescence capabilities and amenability for HTS (see Table 5.32.1)
Graphing software (e.g., GraphPad Prism)

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000) and Sanger sequencing (Chapter 7 in Ausubel et al., 2017)

Subclone RE/promoter into coincidence reporter vector

1. Simultaneously digest 1 μg vector (pNLCoI1 or pCI 9.0) and 1 μg RE or promoter region of interest in separate 1.5-ml microcentrifuge tubes using the same DNA restriction enzymes according to the multiple cloning site (Fig. 5.32.2) and manufacturer's protocol.

2. *Optional:* If a single enzyme was used to digest DNA, use CIAP according to manufacturer's protocol to dephosphorylate the linearized vector to ensure proper ligation.

3. Purify linearized vector and RE/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 by gel electrophoresis. DNA can then be purified using a gel DNA extraction kit.

4. Ligate the DNA fragments together using a Quick Ligation kit with a 3:1 molar ratio of purified RE/promoter to vector. Include a separate ligation reaction with digested and purified vector only as a negative control.

   The control is used to inform ligation efficiency and determine the number of colonies to be screened for ligated plasmid.

5. Transform 2 μl of each ligation reaction into competent *E. coli* (DH5α or Top10) according to manufacturer's protocol.

   Single colonies should be observed following transformation. Colonies ideal for growth are isolated from their nearest neighbors and lack satellite colonies (smaller colonies formed around a larger colony).

6. Pick four to eight single colonies and grow each overnight (~16 hr) in 5 ml LB containing 100 μg/ml ampicillin.

7. Mix 500 μl of each bacterial culture with 500 μl of 50% glycerol in a 2-ml cryovial to generate stocks for future use. Store at −80°C.

8. Isolate DNA from 4 ml of each bacterial culture using a DNA miniprep kit.

9. Perform digests with appropriate DNA restriction enzymes and run on an agarose gel to identify colonies that produce DNA with the RE/promoter properly ligated into the vector.

10. Using sequencing technology, verify the correct DNA sequence of each clone identified in step 9.

    Sanger sequencing is required for this step. If sequencing facilities are not available, overnight sequencing services are provided by a variety of vendors (e.g., Eurofins Genomics).

11. Use the glycerol stocks (step 7) to generate fresh overnight cultures of sequence-verified clones with enough volume for large-scale DNA preps.

12. Use a HiSpeed Maxiprep kit to generate large-scale preps of sequence-verified plasmid DNA for validation and Basic Protocol 2.

    Once correct clones have been identified by sequencing, glycerol stocks of unwanted clones should be discarded.

Validate coincidence reporter biocircuit using transient transfection

13. Using an optimized transfection protocol for the cell type in use, transfect sequence-verified high-quality RE/promoter-vector DNA into cells in a 6-well tissue culture–treated plate.

    Cells should be at the density recommended by the transfection reagent protocol (typically, 50-80%). For general transfection protocols, see Hawley-Nelson, 2001.

14. At 24 hr post-transfection, passage cells into a 96-well white solid-bottom plate.

15. Incubate cells with pharmacological control compounds in titration (7-10 concentrations) to validate correct integration and modulation of the coincidence reporter biocircuit.

    PTC124 titration range: 10^{-12} to 10^{-5} M
    Cilnidipine titration range: 10^{-9} to 10^{-4} M
    Biological control: three log units on either side of the EC_{50}/IC_{50}

    PTC124 and cilnidipine are FLuc and NLuc stabilizing inhibitors, respectively. They should be administered 18-24 hr prior to the luminescence read.

    The biological control is an activator or inhibitor of the RE/promoter. It is assay-dependent and determined by the RE/promoter under investigation. Examples include tunicamycin to activate the endoplasmic stress response element (ERSE) or forskolin to activate the cAMP response element (CRE). The incubation time is also assay-dependent based on the promoter and/or biological process being interrogated.

    DMSO and blank wells are also necessary controls. Figure 5.32.3A shows a representative control plate layout for a 96-well plate with 9-pt titrations of reporter and biological controls. For 10-pt titrations, concentrations can be extended into column 10, leaving twelve wells for the single concentration of the biological control for Z′ calculations.

16. Evaluate the luminescent signal for each luminescent reporter in each condition using the Nano-Glo Dual Luciferase Reporter Assay System according to manufacturer's protocol.

    This is an easy-to-use add-read-add-read protocol and the manufacturer's instructions should be followed.

17. Use appropriate graphing software (e.g., GraphPad Prism) to analyze the data to confirm proper function of the coincidence reporter biocircuit.

    Before proceeding to Basic Protocol 2, it is imperative that the expected response profiles are observed, as outlined in Figure 5.32.1B. PTC124 should elicit an FLuc-specific response, cilnidipine an NLuc-specific response, and the biological control a coincident response (similar potency) for FLuc and NLuc. [NOTE: Cilnidipine is a calcium channel antagonist of the dihydropyridine class, and care should be taken to ensure that the action

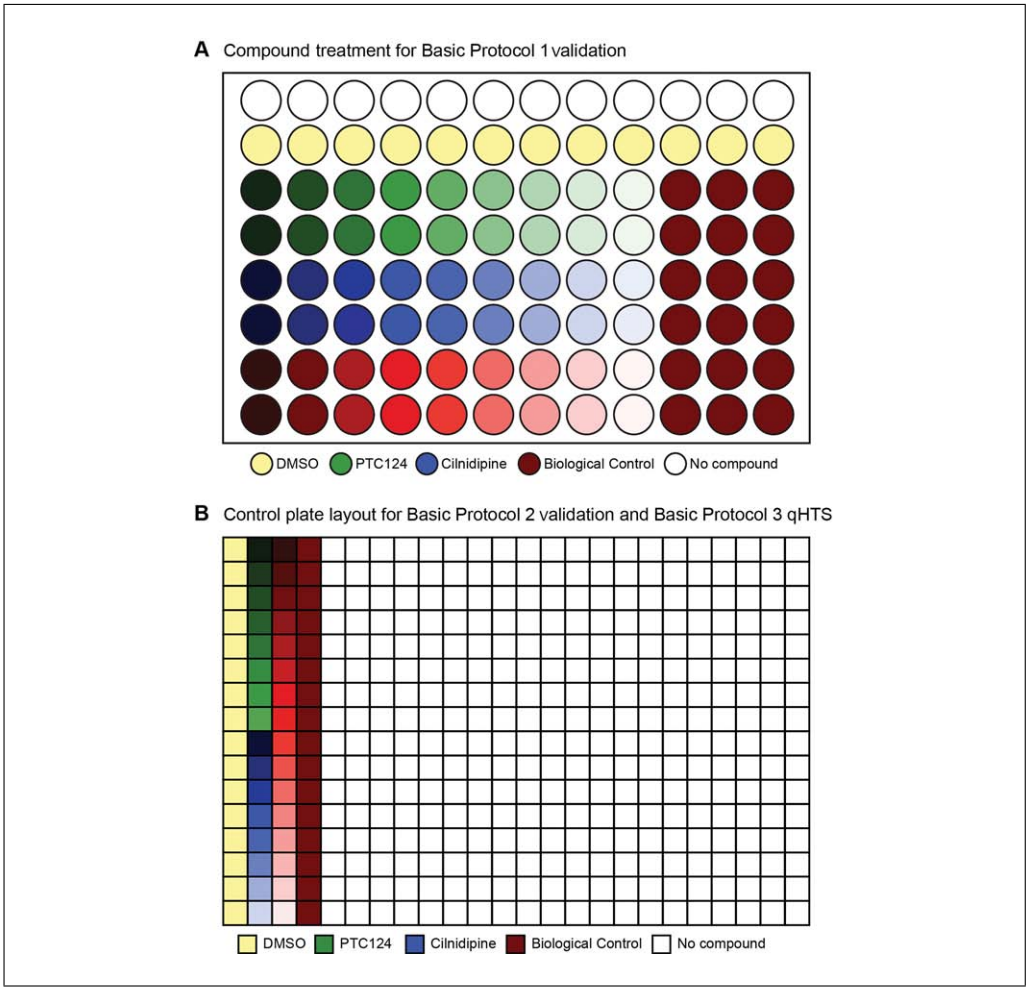

Figure 5.32.3 Plate layouts for control compound treatment. (**A**) Layout for validation of coincidence reporter biocircuit (Basic Protocol 1). Row 1 serves as an untreated control to determine if DMSO has an effect on cell type or assay conditions. Row 2 is a DMSO control to which all other treatments can be normalized during data analysis. DMSO concentration should be kept as low as possible. Rows 3-8, columns 1-9, are titrations of PTC124 (10^{-5} to 10^{-12} M), cilnidipine (10^{-4} to 10^{-9} M), and the biological control (including $\geq$3 log units on either side of the EC_{50} /IC_{50}). Rows 3-8, columns 10-12, are a single high concentration of the biological control to be used for calculating Z′ to assess the potential signal window of the assay. (**B**) Suggested layout for a 384-well plate for Basic Protocols 2 and 3. Column 1 is DMSO. Column 2 is 8-point titrations of PTC124 (10^{-5} to 10^{-12} M) and cilnidipine (10^{-4} to 10^{-9} M). Column 3 is a titration of the biological control (including at least 3 log units on either side of the EC_{50} /IC_{50}). Column 4 is a single high concentration of the control to be used for Z′ calculation and for normalization during data analysis. The number and location of control compounds may vary when collaborating with a screening center that has a predefined preference. For 1536-well format, each well of this 384-well parent control plate is transferred twice into columns of a 1536-well daughter compound plate, giving 32 wells of DMSO and 2 wells of each titration point.

of this compound as a channel blocker is not pharmacologically relevant to the assay biology under study. Other NLuc inhibitors that can be used can be found in Ho et al. (2013).] 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 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 five-parameter, two-sigmoidal-curve equation (Hasson et al., 2015):

$$Y = S_0 + \frac{S_{max} - S_0}{1 + 10^{(\mathrm{LogEC}_{50} - X)}} + \frac{S_{inf} - S_{max}}{1 + 10^{(\mathrm{LogIC}_{50} - X)}}$$

where S_0 is the signal at zero concentration of test compound, S_{max} is the maximum theoretical signal, S_{inf} is the signal at infinite concentration of test compound, EC_{50} is the theoretical concentration at half-maximal stimulated signal, IC_{50} is the concentration at half-minimum inhibited (or cytotoxic) signal, and X is the log of the tested compound concentration in the same units in which EC_{50} and IC_{50} are reported. Note that since the EC_{50} and IC_{50} components often overlap, the effective EC_{50} and IC_{50} values can be estimated numerically from the fitted curves and reported as such.

For systems where basal expression levels of FLuc are very high relative to the amount of FLuc stabilized by the inhibitor, such that no increase in FLuc signal is observed at intermediate inhibitor concentrations, a standard three- or four-parameter equation may be used to characterize inhibition of the FLuc signal. Similarly, cilnidipine should be fit with a four- or five-parameter curve fit, depending on curve shape that is either sigmoidal or bell-shaped, respectively. Biological control(s) should be fit with either a 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 three-parameter sigmoid (with Hill slope = 1) compared to a four-parameter sigmoid (where Hill slope is allowed to vary), or a four-parameter sigmoid compared to a five-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 will produce a screening window large enough to identify modulators. This can be evaluated by calculating the Z′-factor:

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

where σ_p and σ_n are the standard deviations of the positive and neutral (or negative) controls, respectively, and μ_p and μ_n are the averages of the positive and neutral controls, respectively. Here, $Z' > 0.5$ is generally desirable (Zhang et al., 1999; Inglese et al., 2007). 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/RE under investigation.

BASIC PROTOCOL 2

DEVELOPMENT OF CELL LINES FOR qHTS

This protocol outlines the steps necessary to generate a stable cell line expressing the coincidence reporter biocircuit that produces excellent assay performance metrics for qHTS and proper pharmacological response profiles for the reporter and biological control(s).

Materials

Cells of interest (see Strategic Planning)
Growth medium appropriate for cells
Coincidence reporter (see Basic Protocol 1)
Green fluorescent protein (GFP) vector lacking hygromycin B or puromycin resistance gene
Transfection reagent (e.g., Lipofectamine 2000 or Nucleofector kit)
Hygromycin B or puromycin
Dimethyl sulfoxide (DMSO)
10 mM PTC124 (see recipe)
20 mM cilnidipine (see recipe)
Biological control compound(s) specific for RE/promoter (see recipe)
Mouse anti-FLAG antibody (Sigma, cat. no. F1804, RRID: AB_262044; for pCI 9.0 only)

Tissue culture hood
37°C, 5% CO_2, 95% humidity incubator
6-well tissue culture–treated plates (Corning, cat. no. 3506)
96-well clear-bottom tissue culture–treated plates (Corning, cat. no. 3596)
T25, T75, and T175 cell culture flasks with vented caps
96-well white solid-bottom tissue culture–treated plates (Corning, 3917)
Nano-Glo Dual-Luciferase Reporter (NanoDLR) Assay System (Promega, cat. no. N1610 or N1620)
Reagent Dispenser for NanoDLR (see Table 5.32.1)
384- or 1536-well white solid-bottom tissue culture–treated plates (Greiner Bio-One)
Multidrop Combi Reagent Dispenser with small cassette (Thermo Scientific) or equivalent
Stainless-steel lids (Kalypsys) containing pinholes for gas exchange (1536-well plates only)
Liquid handling instrument for transferring compounds to assay plate (e.g., Multimek, PinTool, Mosquito; see Table 5.32.1)
Plate reader with luminescence capabilities and amenability for HTS (see Table 5.32.1)
Graphing software (e.g., GraphPad Prism)

Additional reagents and equipment for western blotting (Ni et al., 2016)

Transfect cells with reporter

1. Plate cells into two wells of a 6-well tissue culture–treated plate.

   Cells should be plated at a density optimal for transfection as indicated in the manufacturer's protocol for the transfection reagent and empirically determined for the cell type being used.

2. Using an optimized transfection protocol, transfect the coincidence reporter into the desired cells in one well of the plate. In the second well, as a transfection control, transfect cells with GFP or any other control DNA lacking the selection marker (hygromycin B for pNLCoI1 or puromycin for pCI 9.0).

3. At 24 to 48 hr post-transfection, passage transfected cells at 1:5 to 1:15 into new wells of a 6-well plate and allow cells to adhere to the surface of the plate (3-24 hr).

   The passage ratio depends on cell type and cell density post-transfection.

4. Replace medium with fresh medium containing the appropriate selection antibiotic (hygromycin B for pNLCoI1 or puromycin for pCI 9.0).

   The antibiotic concentration depends on the cell type and should be determined in advance by performing a kill curve experiment (see Strategic Planning).

5. Monitor until cells transfected with the coincidence reporter are confluent and all cells in the GFP-transfected well are dead.

   This confirms successful transfection of the coincidence reporter and selection via antibiotic.

6. Passage reporter-transfected cells 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 monoclones

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. The number of 96-well plates needed for either method depends on the chosen cell line and the percentage of viable clones expected.

Generation of both monoclones 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 (below) will determine which cell line is best for qHTS. Although polyclones have the potential for greater assay variability, antibiotic selection and limiting dilution apply strong selection pressure that can result in outlier populations with unreliable reporter expression. Thus, it is advisable to generate both polyclonal and monoclonal populations simultaneously, characterize both, and then choose the best population based on pharmacological response profiles and assay performance metrics.

Subculture cells and evaluate reporter expression

Monoclonal population outgrowth from single-cell density can take weeks to months, depending on cell growth rate. Here, characterization of the polyclonal cell line (described in substeps "a" below) can be completed during the monoclonal outgrowth process (substeps "b" below).

7. Subculture cells for expansion.

   a. Expand cells from the 6-well plate into a T75 culture flask to serve as the polyclonal line.

      These cells can be used "as is" for validation experiments, screening, and comparison to monoclones.

   b. Every 3-4 days, exchange medium in the 96-well plates with fresh medium and examine wells to determine which contain a single cell. Mark those wells clearly on the lid so they can be used later, once colonies are established enough to allow expansion.

8. Subculture for cryopreservation.

   a. As soon as there are enough cells for cryopreservation and plating for cell density optimization, evaluate the polyclonal line for coincidence reporter expression and control compound pharmacological response profiles (Fig. 5.32.1B). Expand cells into T175 flasks to generate additional vials for frozen cell stocks.

   b. Once single-cell colonies are confluent enough for expansion, trypsinize the colonies and transfer to individual wells of a 12-well plate for expansion. Continue to expand into larger wells and then culture flasks as appropriate subculturing densities are achieved. Once each monoclone becomes confluent in a 6-well plate, expand into T25 culture flasks for colony screening and into T75 and T175 flasks for cryopreservation.

      For both polyclones and monoclones, at least four to six vials of cells should be cryopreserved at this stage, so that early-passage cells are always available.

9. For preliminary yes-or-no confirmation of coincidence reporter expression, plate at least three wells of the polyclone and each monoclone into a 96-well white solid-bottom tissue culture–treated plate for luminescence screening. Plate cells so they will be ~90% confluent 48 hr after plating. Incubate 48 hr.

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

10. Use the NanoDLR assay system to determine the luminescence signal for each reporter for each monoclone. Determine which monoclones have coincident reporter expression and proceed to step 11 with the most promising three to four clones as well as the polyclone.

Optimize cell density

11. Using a Multidrop Combi Reagent Dispenser and small cassette, dispense a range of cell densities for each monoclone and polyclone into one half of a 384- or 1536-well white solid-bottom plate for each cell density. For 384-well format, plate one cell density in columns 1-12 and a second cell density in columns 13-24. For 1536-well format, plate one cell density in columns 1-24 and a second in columns 25-48. For starting ranges using four cell densities, use two plates.

    A good starting range is 2000, 5000, 10,000 and 15,000 cells/well for 384-well format or 500, 1000, 1500, and 2000 cells/well for 1536-well format. Optimal density is dependent on cell type (size, growth rate) and assay (promoter strength). For example, in the 1536-well format, BE(2)M17 cells typically behave optimally in the range of 1000-1200 cells/well, whereas smaller HEK293 cells behave optimally in the range of 1600-2400 cells/well.

12. Cover plates and incubate 16-24 hr at 37°C, 5% CO_2, 95% humidity.

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

13. Treat each half of the plate with control compounds (see Fig. 5.32.3B for example layout).

    As in Basic Protocol 1, it is important to have reporter controls (PTC124 and cilnidipine) and biological control(s) in titration on the control plate, as well as 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′ for each cell density. The control plate template in Figure 5.32.3 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 pipetter or via a liquid-handling instrument (e.g., Mosquito)—into a 1536-well compound plate for subsequent pinning into a 1536-well assay plate. Because the cell density optimization is plated into half plates (one half plate per cell density), for this step only, the control columns will occupy columns 1-4 and columns 13-16 in 384-well plates, and columns 1-24 and 25-28 in 1536-well plates. The remaining untreated wells should be used to assess variability in plating and basal reporter expression and comparison to DMSO treatment. If there appears to be a DMSO effect (an increase or decrease in signal when treated with DMSO in the first column of each control block), a DMSO titration should be completed to determine the allowable concentration for the assay, and the volume of compound used in the assay should be adjusted accordingly. The CAPP Denmark 16-channel pipetter is recommended for easy manual pipetting of control plates.

    See Table 5.32.1 for instruments suitable for transferring compounds from compound plates to assay plates.

14. Incubate cells at 37°C, 5% CO_2, 95% humidity for 1-24 hr.

    The incubation time is assay-dependent and must be determined empirically. 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 hr prior to the luminescence read.

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

    See Table 5.32.1 for instruments to rapidly dispense NanoDLR reagent to assay plates and high-throughput compatible plate readers.

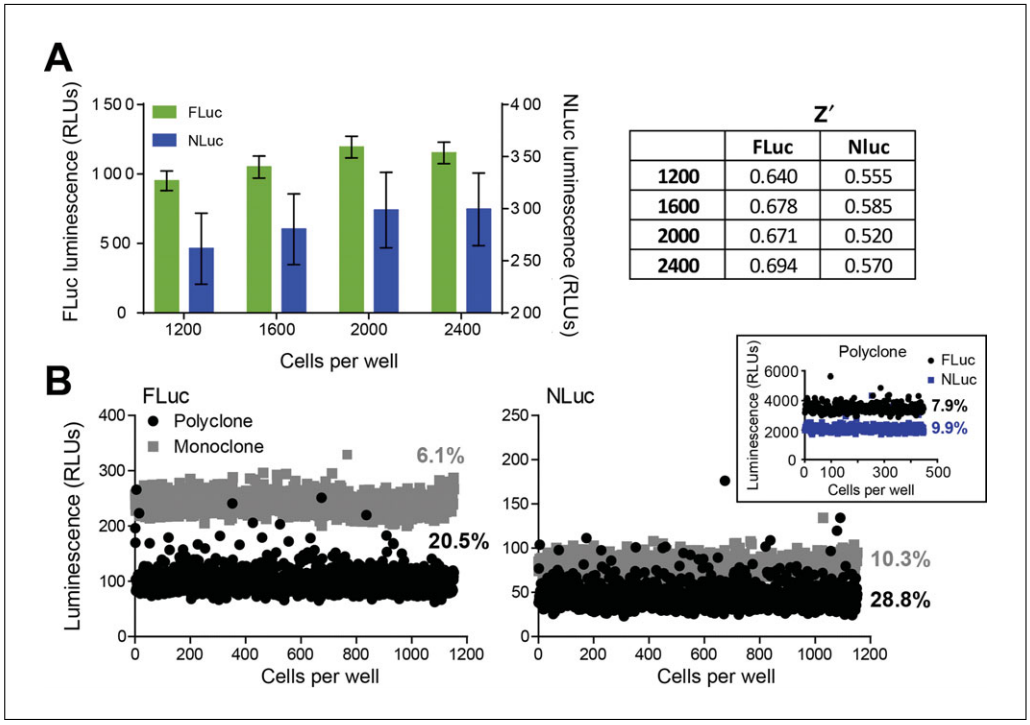

Figure 5.32.4 Cell density and variability evaluations during generation of stable cell lines. (**A**) Cell density optimization of a monoclonal cell line. Left: basal luminescence for each reporter; right: Z′ values for each reporter using a single column of biological control (24 hr incubation) in 1536-well format. For each reporter, basal luminescence increases with cell number and then plateaus. The Z′ is acceptable for all cell densities tested, with the highest Z′ being at 1600 cells/well for NLuc and 2400 cells/well for FLuc. Because the luminescent signal has plateaued at 2000 and 2400 cells/well, 1600 cells/well was chosen for qHTS to maximize Z′ and performance for each reporter with respect to changes in FLuc and NLuc. (**B**) Luminescence values for a polyclone and monoclone comparing CV (%) for each reporter. In this example, the polyclone exhibits unacceptable CVs (>10%) and the monoclone is preferential (CVs ≤ 10%). The inset shows a polyclonal cell line with low variability (CV < 10%). Because this polyclonal line shows reproducible pharmacological response profiles and has low variability, it is acceptable for qHTS.

16. Determine the optimal cell density for the polyclonal line and each monoclone.

The optimal cell density is the one with the highest Z′ and for which neither luciferase signal has plateaued (Fig. 5.32.4A). Freitas et al. report an example of assay optimization for cell density and the parameters to consider (see Table 1 in Freitas et al., 2014), albeit for a single luciferase reporter. For this protocol, cell density should be optimized for both reporters (Fig. 5.32.4A).

Choose optimal cell line

17a. *For pNLCoI1:* Choose the optimal cell line for high-throughput validation and further screening based on assay performance and expected pharmacological response profiles as outlined in Figures 5.32.1 and 5.32.4B.

Reporter activity will vary for each clone generated. Choose the clone (polyclone or monoclone) that exhibits the expected pharmacological response profiles (see Basic Protocol 1, step 17) and has a sufficient signal window (i.e., robust positive control response and low variability, as demonstrated by a Z′ > 0.5) for screening, and proceed to high-throughput screening validation (Fig. 5.32.4B).

For qHTS, where each concentration tested serves as a replicate for the compound, Z′ < 0.5 can be acceptable so long as dose responsivity is observed reliably. See step 20 for calculation of MSR for control compounds. A Z′ < 0.5 and MSR < 3 is considered a good assay for qHTS.

17b. *For pCI 9.0:* Determine FLuc and NLuc protein expression in the polyclone and each monoclone by western blotting, then choose the optimal cell line.

i. Plate cells at an appropriate cell density in a 6-well plate and treat with biological control(s) and DMSO for the empirically determined amount of time for the control compounds.
ii. Use standard western blotting techniques to determine the protein expression of each reporter using anti-FLAG antibody and a loading control such as tubulin or β-actin.
iii. Choose the optimal cell line for high-throughput validation and continued screening based on reporter expression along with expected pharmacological response profiles and excellent assay performance metrics (as for pNLCoI1 in step 17a) (Fig. 5.32.4).

The optimal cell line will exhibit basal reporter expression with DMSO treatment that will increase with biological control compound treatment.

18. Once a clone to be used for screening has been identified, thaw one or two vials of cryopreserved cells (step 8) to expand into many T175 flasks to generate frozen cell stocks that can be used as a reagent during screening.

Cells will be thawed and expanded to the exact number of flasks needed for screening prior to each screening experiment.

Cryopreservation in step 8 will generate four to six vials of early-passage cells. Only one or two vials should be used during this step for additional expansion, so 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.

Validate high-throughput screening

19. Using the optimized cell density parameters, determine assay variability within and across iterations. Run the assay in the chosen format (384 or 1536 wells) with reporter and biological controls (Fig. 5.32.3B) a minimum of three times, each with duplicate plates, and separated in time (ideally days apart to capture variance in handling).

20. Evaluate inter-plate and inter-day variability by calculating control coefficient of variation (CV) and Z' 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 (Haas et al., 2004; Iversen et al., 2004; Eastwood et al., 2006). Additionally, calculate daily intra-plate MSR values 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 < 10% and $Z' > 0.5$ (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 (Haas et al., 2004; Eastwood et al., 2006). MSR is calculated as:

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

where s is defined as the standard deviation of the absolute differences between the log EC_{50} for each compound on duplicate plates or in duplicate titrations, for each channel. MSR < 3 is desired and is indicative of an assay with great reproducibility.

IMPORTANT: Miniaturization and scaling up will 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.

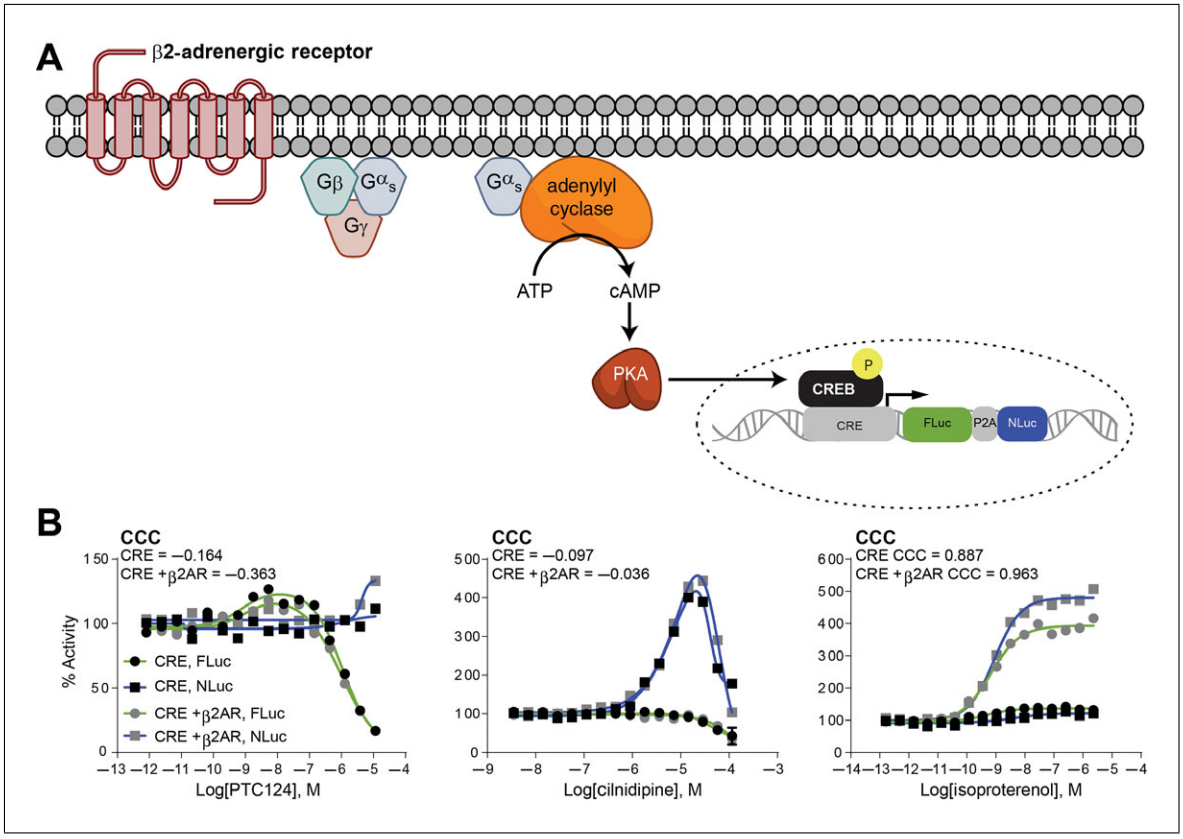

Figure 5.32.5 Pharmacological response profiles for cAMP response element (CRE) coincidence reporter biocircuit. (**A**) Schematic diagram of β2-adrenergic signaling through PKA phosphorylation of CREB, which can be monitored with a CRE coincidence reporter biocircuit. (**B**) Pharmacological response profiles of PTC124, cilnidipine, and isoproterenol in HEK293 cells expressing the CRE coincidence reporter or CRE coincidence reporter plus β2-adrenergic receptor (β2AR). In both cell lines, PTC124 elicits an FLuc-specific response, cilnidipine elicits an NLuc-specific response, and isoproterenol elicits a coincident response. The coincident response for isoproterenol is much greater in β2AR-expressing cells, as expected, because isoproterenol is a β-receptor agonist. Minimal activation is observed in the CRE-only cell line due to endogenous expression of β2AR.

BASIC PROTOCOL 3

qHTS USING A COINCIDENCE REPORTER BIOCIRCUIT

This protocol describes qHTS using the developed coincidence reporter biocircuit to rapidly identify small-molecule modulators of transcriptional activity with significantly reduced risks of reporter-biased artifacts and false negatives. Figure 5.32.5 shows an example of a CRE biocircuit that was generated, optimized, and validated using Basic Protocols 1 and 2, and used for qHTS following the protocol below. Before beginning the protocol, users should be familiar with published examples of qHTS using a coincidence reporter biocircuit (Hasson et al., 2015; Cheng and Inglese, 2012), and should ensure the availability of data analysis software appropriate for compound triage and identification of reporter-biased artifacts.

Materials

Dimethyl sulfoxide (DMSO)
10 mM PTC124 (see recipe)
20 mM cilnidipine (see recipe)
Biological control compound(s) specific for RE/promoter (see recipe)
Coincidence reporter cell line (see Basic Protocol 2)
Growth medium appropriate for cell line
Library of Pharmacologically Active Compounds (LOPAC) titrated in 100% DMSO

Compound libraries titrated in 100% DMSO (7- to 11-point inter- or intra-plate titrations)

384- or 1536-well white solid-bottom tissue culture–treated plates (Greiner Bio-One)

Tissue culture hood

37°C, 5% CO_2, 95% humidity incubator

T175 or T225 cell culture flasks with vented filter caps

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

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

Liquid handling instrument for transferring compounds to assay plate (e.g., Multimek, PinTool, Mosquito; see Table 5.32.1)

Reagent Dispenser for NanoDLR (see Table 5.32.1)

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

Plate reader with luminescence capabilities and amenability for HTS (see Table 5.32.1)

Analysis software for qHTS:

For guided workflow programs with more user-friendly interfaces: Collaborative Drug Discovery Vault, Dotmatics Studies, Genedata Screener, IDBS ActivityBase, or Screenable

For programs that require user ability to pivot incoming plate data: NCGC Curve Fit, scripting in GraphPad software and Tibco Spotfire

Perform screen

1. Prepare control plate template (Fig. 5.32.3B) and protocol table (Table 5.32.2) for reference during the screening process.

   If screening in a 384-well format, the control plate template in Figure 5.32.3B is sufficient for preparing the compound plate. The control plate template is easily prepared in an Excel spreadsheet. If screening in a 1536-well format, each well from the 384-well compound plate should be transferred twice, either manually using a multichannel pipetter or using 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 the 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, the CAPP Denmark 16-channel pipetter is recommended for manual pipetting of control plates in 1536-well format.

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

   To begin, the LOPAC should be screened as a validation library, and it should be screened in duplicate to assess assay reproducibility. After confirmation of reproducibility and dose-dependent relationships, larger libraries can be screened.

4. Plate cells at the optimal density (see Basic Protocol 2) in 384- or 1536-well white solid-bottom tissue culture–treated plates using a Multidrop Combi Reagent Dispenser and small cassette.

5. Cover plates with lids and incubate cells at 37°C, 5% CO_2, 95% humidity incubator for 16-24 hr.

   For 1536-well plates, use stainless-steel lids with pinholes for gas exchange.

Table 5.32.2 Example Sequence Table for qHTS[a]

Step	Parameter	Value	Description	Notes
1	Dispense cells	4 μl	1600 cells/well in 1536-well white solid-bottom tissue culture–treated Greiner plate	Indicate any dispense errors that may have occurred
2	Incubation	24 hr	37°C, 5% CO_2, 95% relative humidity	—
3	Library compounds	23 nl	Compound transfer by PinTool; 11-point, 2-fold titration (57 μM to 56 nM)	Compound plate barcodes with maps saved in C:\\RE_FLUC_NLUC_Date
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.	Control plate map saved 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
5	Incubation	24 hr	37°C, 5% CO_2, 95% relative humidity	—
6	NanoDLR	3.5 μl	Dispense NanoDLR ONE-Glo EX Reagent	ONE-Glo EX buffer + substrate = ONE-Glo EX Reagent, which can be frozen and used in aliquots on day of use; BioRAPTR Tip 1
7	Incubation	10 min	Incubate at room temperature, protected from light	—
8	Detection	ViewLux	Luminescence Read 1 (FLuc)	Exposure = 30 sec; gain = high; 2× binning
9	NanoDLR	3.5 μl	Dispense NanoDLR Stop & Glo Reagent and NLuc substrate	1:100 dilution substrate:Stop&Glo buffer made just before use; BioRAPTR Tip 3
10	Incubation	10 min	Incubate at room temperature, protected from light	—
11	Detection	ViewLux	Luminescence Read 2 (NLuc)	Exposure = 30 sec; gain = high; 2× binning

[a]This information should be followed when screening and reported when publishing. The notes in steps 8 and 11 are examples and will be empirically determined and dependent on the assay being performed.

6. Transfer compounds in 100% DMSO from the library and control compound plates to each assay plate containing cells, cover plates with lids, and return to the incubator for the incubation time for the assay (determined in Basic Protocol 2).

   The incubation time is assay-dependent based on the biological mechanisms being interrogated.

   See Table 5.32.1 for instruments for transferring compounds from compound plates to assay plates.

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

   See Table 5.32.1 for list of instruments to rapidly dispense NanoDLR reagent to assay plates and high-throughput-compatible plate readers.

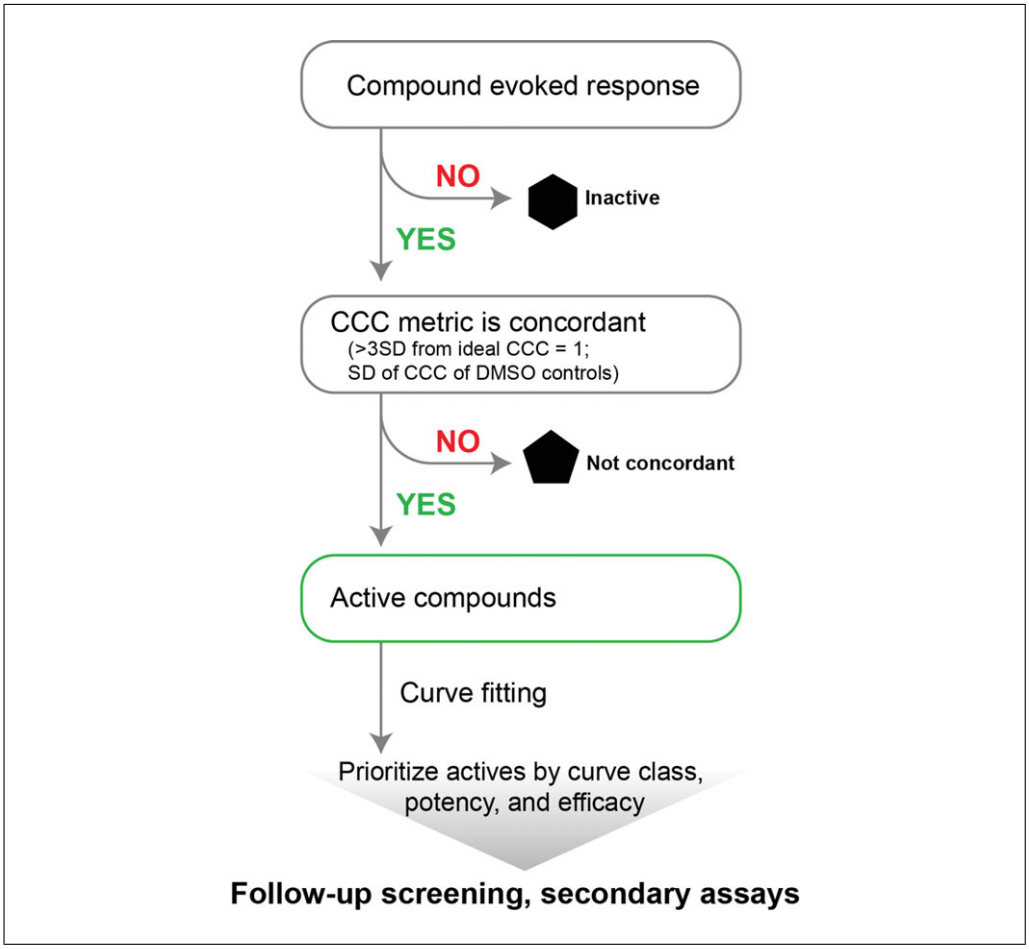

Figure 5.32.6 Compound triage for identifying active compounds from qHTS for follow-up and secondary assays. All data from primary qHTS screening are uploaded into third-party screening software (e.g., Dotmatics, Tibco Spotfire, or Collaborative Drug Discover Vault). Filters are applied to eliminate inactive compounds that do not evoke a response exceeding 3 or 6 SD of the median signal in either reporter channel. From the remaining compounds, the concordant correlation coefficient (CCC) metric is applied to eliminate non-concordant compounds, i.e., those that do not have CCC scores $>$3 SD from 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 is 0.65. Compounds with CCC scores $>$0.65 are considered active, and are fitted using four-parameter curve fits and assigned to curve classes according to Inglese et al. (2006). Compounds are then prioritized for follow-up screening and secondary assays based on curve class, rank order potency, and efficacy (Inglese et al., 2006).

Analyze data

8. Import assay plate data and the 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.

   See Figure 5.32.6 for a flow chart of compound triage in the next steps.

   As an assay performance metric, the Z′ should be assessed for LOPAC 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 (Zhang et al., 1999; Haas et al., 2004). For example, an assay that has a Z′ of 0.3 or 0.4 but shows the anticipated pharmacological response profiles of control compounds and dose-dependent relationships of library compounds would still be considered an excellent assay. Reproducibility can be assessed by plotting duplicate runs in a correlation plot (with logAC_{50} values from run 1

on the x axis and logAC_{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 against their geometric means on a log-log plot.

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):

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

where

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

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

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

at the *i*th concentration for FLuc and NLuc.

The terms 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. These functions are also built into statistics software such as R, which itself has extension packages available such as epiR that contain CCC as a single function. For additional details about CCC, see Hasson et al. (2015).

Actives are defined as compounds that elicit a coincident response in both FLuc and NLuc, with signal on either or both channels exceeding 3 SD of the median signal of all compounds at a given concentration, and have a CCC score not farther than 3 SD from the ideal CCC score of 1.0. For example, in a screen with a CCC SD of 0.15 for the controls, compounds with a CCC score greater than 0.65 would be defined as active. The SD for the signal magnitude of each respective channel is calculated from all neutral DMSO controls. Biological control titrations should be the basis for the SD of the CCCs, but for a library with relatively low activity, the SD of the CCCs can be calculated from the CCCs of all the individual compounds in the library, particularly if control titrations are not available.

11. Apply curve-fitting algorithms and prioritize 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 four-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 five-parameter bell-shaped 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 guided predominantly by curve class and rank order potency (Inglese et al., 2006).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A.

Compound stock solutions

Prepare a 10 mM stock of PTC124 (SelleckChem, cat. no. S6003) and a 20 mM stock of cilnidipine (SelleckChem, cat. no. S1293) in 100% DMSO. Aliquot, snap freeze in a dry ice/ethanol bath, and store up to 2 years at 80°C.

Prepare stocks of biological control compounds similarly, using a concentration determined empirically depending on the compound, cell type, and assay. Prepare stocks according to solubility and storage recommendations for each compound. Choose concentrations based on dilution factor for the plate type (384- or 1536-well) and maximum concentration to be used.

COMMENTARY

Background Information

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 (Luckow and Schutz, 1987; Kalbe et al., 2000), and delineate signaling pathways and cellular pharmacology (Montmayeur and Borrelli, 1991; Himmler et al., 1993). Functional promoter analysis studies were initially performed by transiently transfecting cells with a series of cloned promoter fragments (varying in length or containing 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 1990s and early 2000s, tremendous growth in chemical library size and increasing prevalence of 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 persists today (Fan and Wood, 2007; Michelini et al., 2010). 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 but untraceable assay-dependent artifacts, false positives, and false negatives due to narrow concentration testing. The need to overcome these issues and increase the efficiency and quality of HTS has motivated the development of qHTS and coincidence reporter technology.

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 (Inglese et al., 2006; Hasson et al., 2015). For example, single-concentration screening at 10 μM would inaccurately report as inactive a compound with nanomolar potency that is cytotoxic at 10 μM, or would fail to detect a compound of very modest potency (>10 μM) 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 2000s, an increasing number of studies were demonstrating the propensity of library compounds to interact directly with reporter enzymes, irrespective of assay conditions (Heitman et al., 2008; Auld et al., 2008a,b; Herbst et al., 2009; Auld et al., 2009a,b; Auld et al., 2010). 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 biologically relevant) increase in reporter signal (Auld et al., 2008a,b; Auld et al., 2010). 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 off-target activity, as 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 (RLuc) as the orthogonal enzymatic reporter pair, driven by CRE activation 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 that there are fewer direct inhibitors of NLuc compared to RLuc, and that NLuc inhibitors are generally less potent (Ho et al., 2013). Thus, the second-generation coincidence reporter capitalizes on the enhanced pairing of FLuc and NLuc. 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, CRCs, and assay performance metrics

Known FLuc and NLuc stabilizing inhibitors (reporter controls) should elicit reporter-specific responses and 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 coincidence reporter technology in an efficient qHTS paradigm. The activity of the reporter controls is attributed to a stabilization of the reporter enzymes that increases their half-life. 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 expression will be low and a reporter-biased increase of the CRC, as shown in Figures 5.32.1 and 5.32.5, is expected. In loss-of-signal assays, however, basal expression can be high and reporter controls may primarily result in reporter-biased decreases in output signal due to high levels of reporter expression and an inability of the reporter control to further stabilize the reporter protein. In situations where basal expression is exceedingly low (e.g., when the reporter is targeted to a tightly silenced locus), there may not be sufficient reporter to observe stabilization. Exceedingly low basal expression utilizing random integration of the coincidence reporter, as outlined in these protocols, indicate that reporter and/or transfection optimization and iterative assay design are 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). Nonetheless, in all assays, the distinguishing feature between a reporter-biased artifact and a true biological or pharmacological modulator is single vs. coincident (similar potency) reporter readouts, respectively.

Assay performance metrics such as Z' and MSR should be monitored during development and validation to assess assay window and reproducibility. Values for Z' should generally be ≥ 0.5, and MSR should be <3 for both FLuc and NLuc. In addition to providing a metric by which to guide progression through assay development, Z' and MSR should also be evaluated during qHTS to help identify reproducibility issues (e.g., due to errors in automated dispensing, signal loss, plate-specific cell-death) 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 inform data analysis efforts on the reliability of each plate's data. It is important to note that although Z' 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.

During assay development (Basic Protocols 1 and 2), it is important to remember that assay volume changes (i.e., miniaturization and scale-up to large-scale screening) can diminish assay performance and thus iterative assay optimization may be required. Critical evaluation of pharmacological response profiles, Z', and MSR provides valuable guidance during iterative assay development. However, an advantage of employing qHTS is that the resultant CRCs can be more tolerant of lower assay

performance (e.g., $Z' < 0.5$), as the pharmacological nature of the data (i.e., a response profile generated over four orders of magnitude in compound concentration) is a concentration-dependent relationship obtained from multiple data points.

CCCs: Triage of true biological actives and elimination of reporter-biased artifacts

Although screening a small, focused library of 3,000 compounds may only take 3 days (from plating 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 5.32.4 outlines the broad steps for assessing the data from a compound screen to focus on compounds with coincident responses in the reporter channels. Following elimination of inactive compounds (in which there is no evoked response in either channel), the remaining compounds should be considered active only if responses in the FLuc and NLuc channels are concordant, as determined by curve classes or a CCC within 3 SD of the ideal CCC of 1, with signal exceeding an activity threshold at any concentration point, generally 3 or 6 SD. The SDs for the signal of each channel and the CCC are 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

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 Protocols 1 and 2 to be performed as an iterative process in which first-, second-, and third-generation reporters are developed. If problems arise during 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 an alternative promoter length to include additional enhancer and/or regulatory elements, or inclusion of a minimal promoter for signal amplification (Fig. 5.32.7). 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 was 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 (Sigma, cat. no. L9506) and D-luciferin (Sigma, cat. no. L9504) can be used to assess activity of compounds against FLuc enzyme using a 10-min incubation and substrate (D-luciferin) K_m of 10 μM. Currently, there is no recombinant NLuc enzyme available. The National Center for Advancing Translational Sciences (NCATS) and others have systematically profiled libraries against recombinant FLuc enzyme and secreted NLuc enzyme (obtained from mammalian cells), and can provide information regarding NLuc activity of 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, and so on have not been attempted previously (Fig. 5.32.5).

Anticipated 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

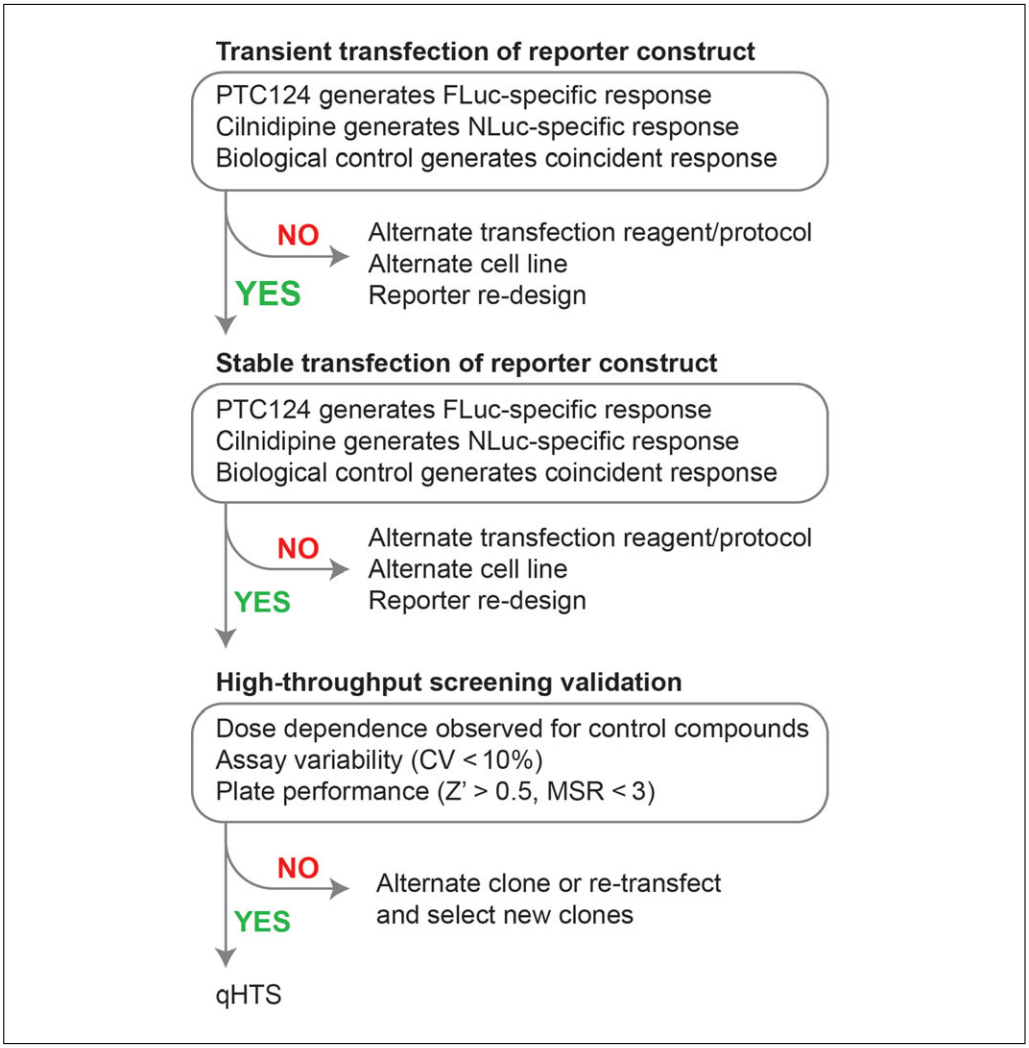

Figure 5.32.7 Troubleshooting guidance for iterative assay development. Criteria for progression through the protocols and suggestions for troubleshooting are outlined. Transient transfection of the reporter construct is completed in Basic Protocol 1 and stable transfection of the reporter construct and high-throughput screening validation is completed in Basic Protocol 2.

be obtained using all three protocols. Basic Protocols 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 HTS 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 5.32.1 and 5.32.5.

The key advantage of using a coincidence reporter biocircuit is to reduce false positives due to direct effects of library compounds on the reporter's cellular half-life and/or enzymatic activity. The probability that a compound will influence the cellular half-life and/or enzymatic activity of two non-homologous reporters simultaneously expressed from a shared response element or gene locus is low, and therefore the most likely explanation for concordant responses is 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. Because relative luminescence modulation from a reporter-biased response can be greater than the desired biological pathway modulation, it can be selected over the biologically relevant compound for follow-up in the absence of a second discriminating coincidence reporter. In this way, the coincidence reporter aids in lowering the occurrence of false negatives.

Time Considerations

If the promoter or RE of interest is well-characterized, Basic Protocol 1 can be completed within 2 to 3 weeks. This time can be significantly increased if the promoter or RE is not well-characterized and several reporter constructs are required in order to interrogate the biological process and identify biological controls for assay development. Basic Protocol 2 can take weeks to months depending on the cell line as well as its amenability to stable transfection protocols (e.g., growth from single-cell density, doubling time). The time needed for Basic Protocol 2 can also be amplified if problems arise during clone characterization or cell density optimization that require iterative clone selection. The time to complete Basic Protocol 3 depends on library size and breadth of the primary screening stages. It can vary from weeks to months or more, with a large portion of the time being devoted to data analysis.

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 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 time 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 needed for Basic Protocol 3.

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Internet Resources

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

Assay Guidance Manual. The entire manual should be read prior to beginning experiments.

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

Nano-Glo Dual-Luciferase Reporter Assay Technical Manual.

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

Provides information on cell culture basics.