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Genome-edited cell lines for high-throughput screening

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Summary

Measurement of gene expression for high-throughput screening is an increasingly used technique that has been developed for not only gene dosage disorders resulting from disease-associated copy number variations, but also for induction/repression of genes modulating the severity of a disease phenotype. Traditional methods have employed transient or stable transfection of reporter constructs in which a single reporter is driven by selected regulatory elements from the candidate gene. However, individual regulatory elements are inherently unable to capture the integrated regulation of multiple enhancers at the endogenous locus, and random reporter insertion can result in neighborhood effects that impact the physiological responsiveness of the reporter. Therefore, we outline a general method of employing genome editing to insert reporters into the 3' UTR of a candidate gene, which has been used successfully in our studies of the *Pmp22* gene associated with Charcot-Marie-Tooth disease. The method employs genome editing to insert two non-homologous reporters that maximize the efficiency of identification of biologically active molecules through concordant responses in small molecule screening. We include a number of aspects of the design and construction of these reporter assays that will be applicable to creation of similar assays in a variety of cell types.

Keywords

genomics; gene expression; HTS; rare disease; reporter gene; transcription

1. Introduction

Since the development of luciferase assays that enabled rapid readout of gene expression with a high dynamic range [1], they have been used in a wide variety of screening experiments. When employed in small molecule screening assays, luciferase constructs were

often generated with fusions to candidate gene promoters and/or regulatory elements [2,3]. While this strategy is informative, it is common for genes to be regulated by multiple enhancers [4], which are often generally dispersed at locations quite far away from the promoter, either upstream, downstream or within introns [5–9]. Given that selection of candidate regulatory elements is often based on incomplete information, it would be advantageous to embed reporter genes within the candidate locus. Figure 1 depicts the nucleosomal structure and transcription factor binding to a typical gene. Insertion of a reporter within the native locus allows for co-transcription and co-translation of the reporter along with the endogenous gene. This type of assay will not only capture transcriptional and epigenomic mechanisms of gene regulation, but can also be used to identify compounds that affect gene regulation at the level of RNA stability and translation initiation, both of which are subject to multiple control mechanisms including microRNA-mediated regulation.

Charcot-Marie-Tooth disease (CMT) is a class of inherited neuropathies affecting the peripheral nervous system. Over fifty percent of CMT cases fall within subtype 1A, which is caused by a 1.4 Mb duplication encompassing the *Pmp22* (peripheral myelin protein 22) gene. In the interest of identifying modulators of *PMP22* expression, we have developed methods for reporter based screening at this locus. In these studies, we recently found that use of randomly integrated reporters in a high-throughput screen [10] utilizing a single enhancer [6] fused to a luciferase reporter were incapable of detecting some pharmacologically relevant compounds that were subsequently identified through a genome editing-enabled screen using reporters targeted to the *Pmp22* gene locus [11]. While reporters have often been inserted in endogenous loci by traditional homologous recombination techniques in mouse embryonic stem cells, the advent of genome-editing techniques [12,13] enabled facile insertion of reporters at desired loci in a wide range of cell types [11,14,15]. Recent studies have used similar methods for development of other screening lines [14,15].

Here, we outline a method to develop genome-edited reporters that are placed before the 3' UTR of the desired, candidate gene [11,14]. This design includes several key features. First, the 'ribosome stuttering' 2a sequence [16,17] is used between the candidate gene ORF and the reporter gene(s) to ensure that two separate proteins are coded by the same transcript (Figure 2). Second, we employ the previously described coincidence reporter design [18], in which two unrelated luciferase reporters are placed in series behind the candidate gene ORF with an intervening 2a sequence. Incorporation of the coincidence reporter design eliminates biased selection of luciferase enzymatic modulators. Since insertion of two reporters reduces the efficiency of homologous recombination, we discuss options to enhance the efficiency of screening larger numbers of clones to obtain the desired reporter lines.

Our application of this technology has been based on a copy number variant (CNV) disorder, and the dramatic increase in human genome sequencing has uncovered a large number of CNV-associated disorders where an under- or over-expression of a gene causes a genetic disorder [19,20]. This approach is therefore widely applicable. Moreover, the development of projects to identify genetic modifiers of disease progression [21] provides additional targets that could be the substrate of reporter assay development for therapeutic discovery. Therefore, application of this technology where specific druggable targets have not been

identified can provide a means to explore the diverse chemical libraries that are available for HTS.

2. Materials

2.1. qHTS Materials

1. 1536-well white solid bottom microtiter plates (Greiner BioOne) for luminescence assays.
2. Weighted metal lids with gas exchange holes (Wako Automation).

2.2. Liquid dispensers

1. Multidrop combi dispenser (Thermo Fisher Scientific), a peristaltic pump dispenser for cell dispense.
2. Pintool dispenser (Kalypsis, Wako Automation), a 1536-well pin-based compound transfer tool compatible with DMSO solvent, or equivalent dispenser capable of 20–25 nl compound transfer.
3. BioRaprtr flying reagent dispenser (FRD) (Beckman Coulter), a piezoelectric liquid dispenser, or multidrop combi peristaltic pump dispenser for addition of luminescence reagents.

2.3. Luminescence reader

1. ViewLux (PerkinElmer) CCD camera based plate reader.

2.4. Assay medium

1. DMEM high glucose non phenol red media with L-Glutamine, 10% FBS, 1% penicillin-streptomycin in 0.9% NaCl antibiotic reagent.

2.5. Control Compounds

1. PTC124 dissolved in 100% DMSO to a stock concentration of 10 mM, Stock solution snap frozen and stored at –20 °C.
2. Cilnidipine dissolved in 100% DMSO to a stock concentration of 20 mM. Stock solution snap frozen and stored at –20 °C.
3. Digitonin dissolved in 100% DMSO to a stock concentration of 20 mM. Stock solution snap frozen and stored at –20 °C.

2.6. Luminescence reagents

1. Nano-Glo Dual-Luciferase Reporter Assay System (Promega): One-Glo Ex Luciferase reagent for FLuc read, NanoDLR Stop & Glo reagent for NLuc read. Reagents prepared according to manufacturer's protocol and protected from light. One-Glo reagent aliquoted into 10 ml tubes and stored at –20 °C. NanoDLR Stop & Glo reagent prepared fresh before each experiment. Both

reagents should be filtered through 0.22 μ m filter before being dispensed through a piezoelectric dispenser.

2. CellTiter-Glo (Promega) cytotoxicity reagent. Reagent prepared according to manufacturer's protocol and protected from light. CellTiter-Glo should be filtered through 0.22 μ m filter before being dispensed through a piezoelectric dispenser.

3. Methods

3.1. Construction of Transgene

1. The transgene consists of two arms of homology flanking the insertion site at the 3' end of the candidate gene's ORF (Figure 2). One arm of homology will be derived in part from the last coding exon of the transcript and the downstream homology arm will be in the 3' UTR so that it retains potential microRNA targeting sites. Amplify the two homology arms of approximately 800 nucleotides each. Ideally, amplification should use genomic DNA from the cell type of interest, since cell lines may contain SNP's that differ from the reference genome. It is recommended to introduce a few silent mutations within the guide RNA recognition sequence in the recombination donor construct to prevent re-cleavage by the CRISPR-Cas9 system after recombination.
2. The two homology arms are then assembled with the reporters (Fig. 2) using standard or Gibson cloning. Multiple reporter configurations are possible, and Table 1 includes a list of reporter cassettes that we have developed. Further description of these configurations are included below in Notes 2–5. In all cases, the reporter cassette contains the 2a ribosome stuttering sequence (See Note 1) cloned in frame between the native ORF and the first firefly luciferase reporter (Figure 2). If multiple reporters are used, another 2a sequence is cloned in frame between the firefly luciferase ORF and the second reporter (e.g. nanoluciferase, see Note 2). It is important that the constructs be cloned such that the 2a sequences replace the stop codon of the candidate gene, as well as the first reporter stop codon. Cloning software can be used to verify that there is an

²-A number of luciferase reporters have been identified, and even individual subtypes are available with varying half-lives, as well as secreted versions. Both firefly (FLuc) and nanoluciferase (NLuc) reporters are available in shorter half-life versions that have altered kinetics of induction and/or repression. However, it should be noted that even a highly active candidate gene will express the reporter from a single copy insertion, and our experience is that the more stable luciferase versions may be required to have adequate signal for high-throughput screening in plate luminometer formats where cell numbers per well are low (e.g. 1536 well screening) [11].

⁵-Another design consideration is selection of a secreted luciferase, such as Gaussia luciferase (GLuc) [27] or secreted nanoluciferase (sNLuc) [28]. Since secreted enzymes are quite stable, this design feature provides a significant boost in signal compared to non-secreted versions. However, the accumulation of stable reporter in the medium is also a problem, particularly in screening for compounds that reduce gene expression. In practical terms, this can be circumvented by washing the cells at some point after compound addition, and then allowing cells to secrete enzyme 30–60 mins prior to measurement in order to assess accumulated enzyme levels from addition of the compound. Since this is not practical for high-throughput screening, compounds can also be administered within 1–2 hours of plating to minimize reporter accumulation in the absence of compounds. Another important advantage of secreted reporters is that candidate clones can be screened without having to replicate clones, which can be quite time-consuming, as a relatively small amount of medium can be removed for reporter measurement. Therefore, for many situations, a combination of non-secreted reporter (e.g. firefly) and a secreted reporter will have a number of advantages in identifying candidate clones.

¹-It is important to note that the 2a stuttering sequence does introduce several amino acids, and in some cases, an additional furin cleavage site cloned just upstream of the 2a sequence [17] can be used to remove the extra 2a sequence (Figure 2).

uninterrupted ORF from the start codon of the candidate gene to the stop codon of the second reporter. Since the genome editing insertion of a transgene into the locus can be inefficient depending on the target cell type, a third gene (with another preceding 2a sequence) is cloned in series (see Notes 3–4). This can be a fluorescent or selectable marker (e.g. GFP or Neomycin resistance)

3. Upon cloning of the transgene, sequence the construct thoroughly to avoid cloning of PCR-induced mutations particularly in homology arms. Simultaneous sequencing of PCR fragments from the target cell line will determine if changes from the reference genome are SNP's or PCR-induced errors.

3.2. Transfection of Cell Line

1. Since there are a variety of transfection methods as well as genome editing techniques, they will not be specified here, but is important to use a high efficiency transfection method (unless a good selection marker is employed). In our case, the reporter transgene was co-transfected with DNA plasmids for either TALENs, or CRISPR-Cas9 [11]. RNA transfection of either genome editing reagent is also possible. It is generally recommended to target a double strand break to as close as possible to the native stop codon, although it should be noted that the availability of high quality target sites for CRISPR-Cas9 may affect design of the desired recombination.
2. At 1–4 days after transfection, cells can be prepared for FACS sorting of fluorescent marker (See Note 3) or antibiotic selection can be applied (See Note 4). For antibiotics, ensure that the appropriate level of antibiotic has been established for the cell line. For antibiotics that take more time for cell death (e.g. G418), ensure that cells do not become prematurely confluent during selection. For FACS, fluorescent cells can be sorted as single cells into a 96-well plate. Some cell lines do not grow well under such conditions, so conditioned medium can be used to enhance survival of single cells.

3.3. Identification and Evaluation of Clones

1. Evaluation of candidate clones should include use of known modulators of the candidate gene (either small molecule or siRNA) to show that the reporter assays are similarly regulated as the candidate gene. For example, we employed siRNA for a known transcriptional regulator of Pmp22, Sox10 [6], to determine that the reporter activity responded in a similar manner to endogenous gene [11]. Known pharmacological modulators can be similarly employed. If a secreted reporter is

³If the candidate gene is already expressed at a certain level in the target cell line, one can clone the selectable marker in series with the reporters (with an intervening 2a sequence) so that the marker is expressed from the same regulatory elements that drive the endogenous gene and inserted reporters. We have employed fluorescent reporters to allow isolation of candidate clones by FACS sorting and selectable markers such as neomycin and puromycin for antibiotic selection [11]. In either case, use of this method is advantageous since many of the homology arms are likely to lack regulatory elements. Therefore, random insertion of the transgene rather than insertion by homologous recombination will likely not provide adequate expression levels of either fluorescent or antibiotic selection markers.

⁴In some cases the candidate gene may not be expressed at very high levels, and therefore, an independent cassette can be cloned into the construct. One such example is the commonly used PGK-Neo cassette, which can be flanked by loxP sites. A selection marker can be used to identify desired clones and cre-mediated recombination can then be used to remove the marker as needed.

used, it is important to wash the medium at 24–48 hours after siRNA transfection, and then allow reporter accumulation for 30–60 mins prior to reporter measurement (See Note 5).

2. Isolate genomic DNA from the clones and perform PCR with at least one primer outside of the homology arm and another in the 2a sequence (or reporter) to test that the reporters are indeed inserted at endogenous, candidate gene locus. Due to the inherent inefficiency in inserting reporter cassettes, it is anticipated that most positive lines will have a single modified allele, but this can be tested by PCR for retention of the wildtype allele in the reporter clones.
3. Another caveat is that the cloning process may uncover clones that differ substantially from the parental cell line due to selection pressures in the cloning process. Therefore, some evaluation of morphology and limited gene profiling of cell type-specific genes by qRT-PCR should be used to assess how well the reporter line resembles the parental cell line. For diploid cell lines, it is prudent to also establish that karyotype is unaltered.
4. The 2a stuttering sequence is generally quite efficient, but a Western blot for the protein of the candidate gene can be used to validate that the original protein is not fused to one or more of the reporters.

3.4. Miniaturization and qHTS Compatibility Validation of Clones

1. Optimization and validation of individual clones for evaluation in 1536-well microtiter plate format is necessary prior to any small molecule or biologic screening effort. Optimal cell density should be determined by plating various numbers of cell per well across a 1536-well plate. To determine the minimal number of cells required to obtain an acceptable and cost-effective assay output when scaling up for HTS, 500–1500 cells/well is a recommended cell density range to test. Four different cell densities are plated in quadrants at 4 μ l per well across white solid-bottom 1536-well plates in fresh assay medium with a multidrop combi dispenser (see Note 6).
2. In addition to determining optimal cell density for basal reporter signal, each cell density for individual clones should be tested across pharmacological controls, if available, as well as reporter control compounds (see ref 14 for examples). Each compound should be tested in at least 32 replicates (one column of 1536-well plate) at the concentration that achieves a maximum response (e.g., 3–10 fold above the EC/IC₅₀) to allow for the determination of the response range and assay statistics [22,23], and tested in a 16-pt, 1:2 or 1:3 titration in duplicate per cell density. DMSO vehicle control should also be included in at least 32 replicates (Figure 3). Twenty three nL of respective control compound are added to each assay well by pintoole transfer resulting in a final 174-fold dilution. PTC124 and cilnidipine are useful FLuc and NLuc control compounds,

⁶.When plating cells in 1536-well format the use of a peristaltic pump dispenser is suggested as it is gentler on cells than piezoelectric dispensers and more time efficient and consistent than manual plating. It is also recommended to filter the cells through at least a 40 μ m mesh size strainer prior to dispensing for uniform cell suspensions.

respectively, which directly bind and modulate the reporter enzyme half-life in the cell [6]. PTC124 is tested at a high concentration of 57 μ M and titrated in a 16-pt, 1:3 dilution resulting in a final concentration range of 4 pM–57.5 μ M, while cilnidipine is used at a high concentration of 115 μ M and titrated in a 16-pt, 1:2 dilution resulting in a final concentration range of 3.5 nM–115 μ M. Some cytotoxicity may be observed in certain cell lines at the high concentration of cilnidipine. Digitonin can be used as a cytotoxicity control across most cell lines at a high concentration of 57.5 μ M and titrated in a 16-pt 1:2 dilution resulting in a final concentration range of 1.8 nM–57.5 μ M. Compounds should be added to cells within an hour of plating if a secreted reporter is used to minimize the basal accumulation of reporter present in the media and to maximize signal to background for a given assay. If non-secreted reporters are used, cells can be incubated 16–24 hr at 37 °C, 5% CO₂ and 95% RH prior to compound addition to allow the cells to recover from the stress of plating. Cells should be incubated at 95% RH with weighted metal lids with gas exchange holes to minimize evaporation and edge effects that can occur in 1536-well plates (see Note 7).

3. Depending on the biology of interest, cells should be incubated with compound 8–48 hr at 37 °C, 5% CO₂ and 95% RH. A time course for optimal response can be performed by plating and treating replicate plates above that are assayed at various time points. FLuc and NLuc coincidence luminescence are measured with the Nano-Glo Dual-Luciferase reporter assay system according to the manufacturer's protocol. All reagents should be filtered through a 22 μ m membrane to remove particulates prior to dispensing through a piezoelectric or peristaltic pump to prevent clogging of the tips and ensure even distribution of the reagent. In short, one volume of One-Glo EX luciferase reagent, which can be reconstituted and stored at –30 °C protected from light for long term storage, is added to each well with a BioRAPTR FRD or equivalent dispenser, incubated for 10 min at room temperature, and FLuc luminescence is measured on a ViewLux plate reader. One volume NanoDLR Stop & Glo reagent prepared fresh is added as above, incubated 10 min at room temperature, and NLuc luminescence is measured on a ViewLux plate reader (Table 2). Detector settings on the plate reader should be optimized for individual reporters in each assay so that no well on any given plate is over exposed, while reasonable basal luminescence signal is achieved at the lowest cell density. It is suggested to change exposure and gain rather than binning, if possible, to maintain consistent resolution in CCD camera-based systems.
4. A cytotoxicity counter screen should also be optimized for each clone prior to any significant screening effort to determine the contribution of compound cytotoxicity to the output signal. The initial pharmacological characterization of compounds identified in loss-of-signal assays, as well as bell-shaped dose response curves identified in gain-of-signal assays require an understanding of

⁷·An alternative to weighted metal lids for 1536-well plates is to seal microtiter plates with breath-easy sealing membranes (Sigma-Aldrich) to allow for CO₂ gas exchange while minimizing evaporation from individual wells that can increase well-to-well variability. When breath-easy membranes are used, plates should be centrifuged prior to removing the seals to minimize spill over between wells.

the acute cytotoxic properties of active compounds identified in HTS. For example, bell-shaped concentration-response curves (CRCs) seen in gain-of-signal assays may be due to cytotoxic effects of a given compound at the higher concentrations or a non-cytotoxic consequence of signal quenching or direct reporter enzyme inhibition [24]. Cells should be plated, treated (including a cytotoxicity control compound such as Digitonin) and incubated as above. Cytotoxicity can be measured by either fluorescence or luminescence assays. CellTiter-Glo is a common luminescence based assay that measures cellular ATP which is directly correlated to cell viability. Cells are plated and treated as above, then one volume CellTiter-Glo reagent is added to each assay well with a BioRAPTR FRD, incubated for 10 min at room temperature, and luminescence is measured on a ViewLux plate reader with optimized measurement settings for a given cellular assay system (Figure 3) (see Note 8).

5. Quantified luminescence values for each variable optimized above should be analyzed for basic HTS statistics. First, average signal, standard deviation and coefficient of variation (CV) should be calculated for the high concentration replicates of each of the control compounds, as well as DMSO treated and untreated wells at each cell density and time point.

$$\text{signal to background(S:B)} = \frac{\text{AVE signal neutral control}}{\text{AVE signal positive control}} \quad (\text{eq. 1})$$

$$\text{signal to noise(S:N)} = \frac{\text{AVE positive control} - \text{AVE neutral control}}{\sqrt{(\text{STDEV positive control})^2 + (\text{STDEV neutral control})^2}} \quad (\text{eq. 2})$$

$$\text{Z' factor} = 1 - \frac{(3 \bullet \text{STDEV positive control} + 3 \bullet \text{STDEV neutral control})}{|\text{AVE positive control} - \text{AVE neutral control}|} \quad (\text{eq. 3})$$

Signal to background (S:B) (eq. 1), signal to noise (S:N) (eq. 2) and Z' factor (eq. 3) statistics should be calculated for each control compound compared to

⁸.There are several different methods by which to interrogate compound cytotoxicity in cell based assays. A nonlytic, fluorescence output alternative to the CellTiter-Glo luminescence assay described above is CellTiter-Fluor (Promega). The CellTiter-Fluor cell viability assay measures the activity of a conserved protease, where a cell permeable pro-fluorescent substrate is cleaved by active protease in live cells generating fluorescence in direct correlation with cell number. The assay should be performed according to manufacturer's protocol where one volume of reagent is added per well and fluorescence is measured (excitation 388–400 nm, emission 505 nm) on a plate reader (such as Perkin Elmer's Envision or the Tecan Infinite M1000 Pro).

DMSO vehicle control under each set of assay conditions. General guidelines for application of these statistics in the determination of optimal assay conditions include $CV < 10$ in DMSO vehicle control and untreated wells, and a Z' factor > 0.5 which takes into account S:B and assay signal variation (S:N) between DMSO control wells and the high concentration of a given control compound (Table 2) [22]. Additionally, concentration response curves should be plotted and AC_{50} values determined for each control compound titration. Software such as Graphpad Prism is recommended for curve fitting and AC_{50} determination for the control compound titrations for each of the assay variables tested (Figure 4).

6. Once optimal assay conditions are determined based on control compound performance, a preliminary pilot quantitative high-throughput screen (qHTS) should be performed to validate assay performance. Commercially available libraries such as The Library of Pharmacologically Active Compounds (LOPAC¹²⁸⁰) from Sigma-Aldrich are suggested for this purpose (see Note 9). Library compounds are plated in 384-well plates comprising of seven interplate titrations series (serial dilutions of 1:5 in DMSO) for concentrations ranging from 10 mM to 0.64 μ M. Interplate titrations are then reformatted into 1536-well plates for compound addition to assay wells. Cells are plated at the determined optimal cell density in 4 μ l per well across white solid-bottom 1536-well plates in fresh assay medium with a multidrop combi dispenser as above. Library compounds are transferred in interplate titrations to assay plates, while appropriate biological and reporter controls are added to each assay plate as described above. It is recommended to include one column of DMSO vehicle control, 8–32 replicate wells of high concentration blocks of each control compound, and 8–16-pt titrations in duplicate of each control to the first four columns of every assay plate. Cells are incubated with compounds and assayed as above for both FLuc and NLuc luminescence at the optimal time point to observe the appropriate biological response. It is also recommended to include a DMSO control plate (a control library plate consisting of DMSO in columns 5–48, with control compounds added as with standard library plates) at the beginning and end of the screen for post-screening correction of systematic errors (see Note 10).
7. Statistical analysis and server software will be needed to process qHTS data. Screening results should be corrected for systematic errors using the B-score method and normalized to controls [25]. Normalized qHTS data is typically described as percent inhibition, in a loss of signal assay, or percent activation, in a gain of signal assay. Potency values for active small molecules can be determined in qHTS and presented as EC_{50} , IC_{50} or AC_{50} . Assay performance

⁹There are several commercially available diversity libraries, including the Prestwick Chemical Library and Tocriscreen Plus collection that are comparable in size and composition to the LOPAC library. Any of these chemical libraries can be used to validate a 1536-well qHTS assay. Regardless of the chemical library tested, compounds should be transferred to assay plates from low to high concentration to minimize the effects of carry over that can occur with pipette transfer.

¹⁰DMSO control plates placed at the beginning and end of a library screen can be used to indicate and correct for dispense errors that may occur across a screen during cell or reagent addition, as well as indicate the amount of carryover from high concentration library plates.

should be assessed based on the Z' factor of the controls included on each plate and the minimum significance ratio (MSR) of the intra-plate control titrations, where an MSR <2 is considered a reproducible assay [26].

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Figure 1.

The diagram shows the chromatin structure of the Pmp22 gene including nucleosomes (circles) and transcription factors that determine Pmp22 expression level. Since this structure cannot be recapitulated in episomal or inserted constructs, the endogenous locus provides the best insertion point for reporters. Insertion of a reporter at the 3' end of the ORF allows reporter expression that is subject to multiple levels of regulation, including transcription and chromatin effects. In addition, retention of the 3' UTR and the cotranslation of the reporter from the single native start codon makes the single transcript subject to microRNA and other regulators of RNA processing, stability, and translation.

Figure 2.

A) Reporter insertion is performed by genome editing in which TALEN or CRISPR constructs direct a double strand break at the 3' end of the ORF, prompting recombination with the cotransfected cassette containing homology arms and the desired reporter(s). The 2a ribosome stuttering sequence is included at the junction of the native Pmp22 protein and the reporter, and the site of 2a cleavage is indicated. In some cases, a furin cleavage site can be inserted to prevent addition of the 2a peptide at the end of Pmp22.

B) One potential reporter combination is shown in which two coincidence reporters (firefly and secreted nanoluciferase) are inserted along with a Neomycin resistance gene, which is used to select for the desired recombinants. All four reading frames are separated by 2a ribosome stuttering sequences.

Figure 4.

Concentration response curves (CRCs) for the four control compounds tested across different cell densities for a given genome-edited FLuc-P2A-secNLuc cell line. FLuc CRCs are shown with gray symbols and NLuc CRCs are shown with blue symbols. Data was plotted and curves fit in Prism software (GraphPad). FLuc specific stabilization with PTC124 was observed as bell-shaped curves at the three highest cell densities, while NLuc stabilization was only observed with Cilindipine at the highest cell density. Both Bortezomib, the biological control in this assay, and Digitonin, the cytotoxicity control, demonstrated consistent concordant IC_{50} values at the three highest cell densities. Based on these results 1500 cells/well would be selected as the best cell density with which to use in future qHTS. Error bars represent the standard deviation of two technical replicates.

Table 1.

Reporter Cassette Configurations for genome editing insertion at 3' UTR

Configuration of Reporter Insert	Properties
2a-Gluc *	Secreted Gaussia luciferase reporter
2a-secNLuc-2a-GFP *	Secreted nanoluciferase with GFP for cell sorting
2a-NLuc-2a-GFP	Nanoluciferase with GFP
2a-NLuc-2a-puro	Nanoluciferase with puro for selection
2a-fLuc-2a-NLuc	Coincidence reporter with firefly and nano-luciferase
2a-fLuc-2a-secNLuc	Coincidence reporter with firefly and secreted nanoluciferase
2a-GFP-2a-fLuc-2a-NLuc	Coincidence reporter with GFP for cell sorting
2a-GFP-2a-fLuc-2a-secNLuc	Coincidence reporter with GFP for cell sorting
2a-fLuc-2a-NLuc-2a-Neo	Coincidence reporter with firefly luciferase, nanoluciferase and Neomycin resistance
<u>2a-fLuc-2a-secNLuc-2a-Neo</u>	Coincidence reporter with firefly luciferase, secreted nanoluciferase, and neomycin resistance

The table lists specific combinations of reporters that can be inserted at 3' end of target ORF. Those designated with an asterisk have been published [11], and all cassettes have been assembled in specific clones. In each case, the stop codon at the 3' end of the ORF is designed to be replaced by an in-frame insertion of the 2a ribosome stuttering sequence, followed by one or more reporters, as in Figure 2. Second and third genes in series are always preceded by the 2a sequence. Cassettes include both secreted (Gluc, secNLuc) and nonsecreted reporters, and selection is enabled by GFP by FACS or by selectable markers (puromycin or neomycin resistance). Last two reporter cassette configurations have been used successfully (data not shown).

Table 2:

Protocol table for miniaturization of genome-edited coincidence reporter cell lines and control compound optimization to 1536-well qHTS format.

Protocol Optimziation Table: Generic sequential read reporter cell line			
Step	Parameter	Value	Description
1	Cells	4 μ L	Seed 500,800,1000 or 1500 cells/well in one Greiner white/solid bottom high base, TC plate per genome-edited FLuc-P2A-secNLucclone
2	Compounds	23 nL	Compound transfer by Pintool: Bortezomib (100 μ M high concentration; 16-pt 1:2 titration) used as positive biological control; PTC124 (10 mM high concentration; 16-pt 1:3 titration) and Cilnidipine (20 mM high concentration; 16-pt 1:2 titration) used as reporter controls for FLuc and NLuc respectively; and Digitonin (20 mM high concentration; 16-pt 1:2 titration) used as cytotox control
3	Incubation	24 hr	Incubate at 37°C, 5%CO ₂ , 95%RH for 24 hr
4	Reagent	4 μ L	Add One-Glo EX FLuc luminescence reagent (Promega) with BioRaptr FRD
5	Incubation	10 min	Incubate at room temperature, protected from light; Cell lysis
6	Output 1	Viewlux	Luminescence Read #1 (FLuc): Exposure = 30sec, Gain = High, Speed = Slow, Binning = 2X
7	Reagent	4 μ L	Add NanoDLR Stop & Glo reagent (Promega) with BioRaptr FRD
8	Incubation	10 min	Incubate at room temperature, protected from light
9	Output 2	Viewlux	Luminescence Read #2 (NanoLuc): Exposure = 1 sec, Gain = High, Speed = Slow, Binning = 2X

Step	Notes
1	Cells plated in standard growth media. It is recommended to add 1% Penicillin-Streptomycin (10,000 U/mL) to media prior to plating.
2	Compounds prepared at high concentration stocks in DMSO and plated with vehicle control in source 384-well plate, titrated as specified, and manually transferred in duplicate to a 1536-well polypropylene V-bottom compound plate compatible for use with pintool
4&7	Prepare Nano-Glo DILR Assay Reagents: (a) Reconstitute NanoDLR One-Glo EX Flue substrate by adding 100 ml Dual-Glo Luciferase buffer to one bottle of lyophilized substrate, mix well and protect from light. (May be kept at -30°C for longterm storage); (b) Prepare NanoDLR Stop and Glo Reagent (just enough needed for specific experiment): Dilute the NanoDLRStop & Glo substrate 1:100 in NanoDLR Stop & Glo buffer = Add 60 μ l of Dual-Glo Stop & Glo Substrate to 6 ml Stop & Glo Buffer (per 1536-well plate), mix well and protect from light (Prepare fresh for each experiment and store at room temperature, protected from light); (c) Filter both reagents through a 22 mm membrane to remove particulates

Table 3.

Representative statistical summary of genome-edited FLuc-P2A-secNLuc coincidence reporter cell line tested at four cell densities, each optimized with control compounds in 1536-well qHTS format and presented for the individual reporters. Averages and standard deviations were calculated from 32 (DMSO, PTC124, Cilindipine, Bortezomib, and Digitonin treated) or 96 (untreated) samples per cell density.

Treatment	Untreated Cells				DMSO				62.5 μ M PTC124				125 μ M Cilindipine				625 nM Bortezomib				125 μ M Digitonin			
	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	
Cells/Well	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	
Average	8174	10771	12506	13289	3845	9400	11012	13200	171	698	721	872	892	1875	2235	2706	445	972	1120	1356	104	326	358	405
St. Dev.	722	882	1064	1109	1438	1082	733	969	50	209	126	227	136	260	251	352	96	194	202	189	45	144	167	195
% CV	8.7	8.2	8.5	8.3	37.4	11.5	6.7	7.3	29.5	29.9	17.5	26	15.3	13.9	11.2	13	21.5	20	18	13.9	43	44.1	46.5	48.2
S:B									22.5	13.5	15.3	15.1	4.3	5	4.9	4.9	8.6	9.7	9.8	9.7	36.9	28.9	30.8	32.6
S:N									-2.6	-7.9	-13.8	-12.4					-2.4	-7.7	-13	-12	-2.6	-8.3	-14.2	-12.9
Z									-0.216	0.555	0.75	0.709					-0.353	0.546	0.717	0.707	-0.19	0.595	0.747	0.727
Treatment	Untreated Cells				DMSO				62.5 μ M PTC124				125 μ M Cilindipine				625 nM Bortezomib				125 μ M Digitonin			
	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	
Cells/Well	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	500	800	1000	1200	
Average	5042	3684	4396	5163	1459	3438	4174	5148	996	3734	4626	5635	135	614	617	734	317	697	812	1006	85	171	197	218
St. Dev.	239	352	445	411	504	402	325	376	378	418	364	506	28	159	65	106	55	69	75	88	15	31	38	39
% CV	4.7	9.5	10.1	8	34.6	11.7	7.8	7.3	38	11.2	7.9	9	20.4	25.9	10.6	14.4	17.5	10	9.2	8.8	17.7	17.9	19.2	18
S:B									1.5	0.9	0.9	0.9	10.8	5.6	6.8	7	4.6	4.9	5.1	5.1	17.2	20.1	21.2	23.6
S:N									-2.6	-6.5	-10.7	-11.3	-2.6	-6.5	-10.7	-11.3	-2.3	-6.7	-10.1	-10.7	-2.7	-8.1	-12.2	-13
Z									-0.206	0.404	0.671	0.673	-0.206	0.404	0.671	0.673	-0.47	0.485	0.644	0.664	-0.13	0.603	0.726	0.747