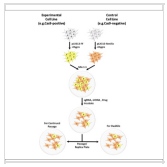

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LucifeRace: A luminescence-based competition assay

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1 *Works for me* This protocol is published without a DOI.

Cancer Dependency Map Target Validation
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ABSTRACT

Fluorescence-based cell competition assays have been used effectively to validate genetic dependencies predicted by the Cancer Dependency Map (Cheung et al. PNAS. 2011, Cowley et al. Scientific Data. 2014). However, these can be labor-intensive and are dependent upon the availability of flow cytometry equipment. To overcome these limitations we developed LucifeRace, a luminescence-based competition assay. It offers several advantages over the traditional competition assay in terms of ease of use, scalability, and application in a wider array of cell biology labs.

Firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferases are evolutionarily distinct enzymes, and have different substrate, cofactor, and pH requirements. When present in a mixture, they can be measured independently using a multiplexed sequential dual luminescence assay. In the LucifeRace assay, two cell populations, one labeled with firefly luciferase and the other with Renilla luciferase, are mixed together and after some incubation period in the presence of a chemical or genetic perturbagen are subjected to a dual luminescence assay to measure the contribution of each cell type to the mixture. Variations of this protocol have been used to assess the effects of small molecules and genetic perturbations in human cancer cell lines (Giacomelli et al. Nat. Genetics. 2018, Takeda et al. Cell. 2018, Chan et al. Nature. 2019, Price et al. Cancer Research. 2019, Shauer et al. Sci. Rep. 2020).

To efficieintly label cell lines with firefly or Renilla luciferase, we created a pair of lentiviral vectors built on the pLX313 backbone. These vectors promote constitutive expression of the luciferases under control of the human EF1α promoter, and a contain a hygromycin resistance cassette to allow for the selection of transduced cells. pLX313-firefly and -Renilla luciferase expression vectors can be obtained from Addgene:
https://www.addgene.org/118016/
https://www.addgene.org/118017/

pLX311-Cas9 and pLX311-LacZ allow for constitutive expression of Cas9 or LacZ under control of the human EF1α promoter, and a contain a blasticidin resistance cassette to allow for the selection of transduced cells:
https://www.addgene.org/118018/
https://www.addgene.org/117731/

This protocol is designed for the use of sgRNA-expressing vectors that contain a puromycin resistance cassette (i.e. lentiGuide-Puro):
https://www.addgene.org/52963/

Lentivirus packaging plasmids developed by the Weinberg lab can also be obtained from Addgene (Stewart et al. RNA. 2003):
https://www.addgene.org/8455/
https://www.addgene.org/8454/

(Protocol Image adapted from Giacomelli et al. Nat. Genet. 2018)

References

Cheung, H.W.*et al.* Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci USA* **108**, 12372-7 (2011).

Cowley, G.S.*et al.* Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of

context-specific genetic dependencies. *Scientific Data* **1** (2014).

Giacomelli, A.O. *et al.* Mutational processes shape the landscape of TP53 mutations in human cancer. *Nat Genet* **50** (2018).

Takeda, D.S. *et al.* A Somatically Acquired Enhancer of the Androgen Receptor Is a Noncoding Driver in Advanced Prostate Cancer. *Cell* **174** (2018).

Chan, E.M. *et al.* WRN helicase is a synthetic lethal target in microsatellite unstable cancers. *Nature* **568** (2019).

Price, C.P. *et al.* Genome-Wide Interrogation of Human Cancers Identifies EGLN1 Dependency in Clear Cell Ovarian Cancers. *Cancer Res* **79** (2019).

Shauer, N.J. *et al.* Selective USP7 inhibition elicits cancer cell killing through a p53-dependent mechanism. *Sci Rep* **10** (2020).

Stewart, S.A. *et al.* Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* **9** (2003).

Dyer, B.W., Ferrer, F.A., Klinedinst, D.K. & Rodriguez, R. A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* **282** (2000).

Hampf, M. and Gossen, M. A protocol for combined Photinus and Renilla luciferase quantification compatible with protein assays. *Anal Biochem* 356 (2006).

PROTOCOL CITATION

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MATERIALS TEXT

Promega™ offers a pre-made, easy-to-use dual luminescence assay reagent called Dual-Glo®. Alternatively, the following recipe can be used to produce homemade FLR and RLR reagents (Dyer et al. *Analytical Biochemistry.* 2000. and Hampf and Gossen. *Analytical Biochemistry.* 2006).

Refer to MSDS for each of the components and take all appropriate safety precautions.

Several components need to be dissolved to create the **stock solutions**, which need to be combined to create the Firefly Luciferase Reagent (**FLR**) and the Renilla Luciferase Reagent (**RLR**).

Reagents should be made in batches, aliquoted, and frozen at -20 to -80°C. The long-term stability of the reagents has not yet been thoroughly tested, and multiple rounds of freeze-thaw should be avoided.

If using the homemade reagents to perform the dual luminescence assay, 5X Passive Lysis Buffer (**PLB** from Promega™) and Phosphate Buffered Saline (**PBS**) must also be purchased and mixed with the **FLR** at a 25:15:10 ratio (FLR:PBS:PLB) prior to performing the assay.

Table 1. Use the following recipes to make high concentration stocks for each component of the firefly luciferase reagent (FLR):

Reagent	Final Conc.	Dissolution
glycylglycine	1 M	Dissolve 1.32 g in 10 mL ddH2O
KxPO4, pH = 8	1 M	Dissolve 13.73 g of KH2PO4 and 156.6 g of K2HPO4 in 1 liter ddH2O
EGTA	0.5 M	Dissolve 1.9 g in 10 mL ddH2O
ATP	200 mM	Dissolve 0.55 g in 5 mL ddH2O
DTT	1 M	Dissolve 1.54 g in 10 mL ddH2O
MgSO4	1 M	Dissolve 1.2 g in 10 mL ddH2O
CoA	10 mM	Dissolve 7.6 mg in 1 mL ddH2O
D-luciferin	10 mM	Dissolve 28 mg in 10 mL ddH2O

Table 2. Combine the stock components together according to the following table to make 50 mL of FLR.

Reagent	Stock	Final Conc.	Volume (in 50 mL total)
glycylglycine	1 M	25 mM	1.25 mL
KxPO4, pH = 8	1 M	15 mM	750 µL
EGTA	0.5 M	4 mM	400 µL
ATP	200 mM	2 mM	500 µL
DTT	1 M	1 mM	50 µL
MgSO4	1 M	15 mM	750 µL
CoA	10 mM	0.1 mM	500 µL
D-luciferin	10 mM	75 µM	375 µL
ddH2O			to 50 mL

Table 3. Use the following recipes to make high concentration stocks for each component of the renilla luciferase reagent (RLR):

Reagent	Final Conc.	Dissolution
NaCl	5 M	
EDTA	0.5 M	
KxPO4, pH = 5.1	1 M	Dissolve 13.73 g of KH2PO4 and 156.6 g of K2HPO4 in 1 liter ddH2O
BSA	10 mg/mL	
NaN3	0.5 M	Dissolve 325 mg in 10 mL ddH2O

coelenterazine	0.5 mM	Dissolve 1 mg in 2.36 mL of methanol and 2.36 mL of ethanol

Table 4. Combine the stock components together using the following table to make 50 mL of RLR.

Reagent	Stock	Final Conc.	Volume (in 50 mL total)
NaCl	5M	1.1 M	11 mL
EDTA	0.5 M	2.2 mM	200 µL
KxPO4, pH = 5.1	1M	220 mM	11 mL
BSA	100 mg/ml	0.44 mg/mL	220 µL
NaN3	0.5 M	1.3 mM	130 µL
coelenterazine	0.5 mM	1.43 µM	143 µL
ddH2O			to 50 mL

SAFETY WARNINGS

Always follow appropriate biosafety protocols for working with human cell lines and lentivirus.

BEFORE STARTING

1) Prior to performing genetic perturbation experiments with a new cell line, you should determine:
   a) The maximum density of these cells in 96-well plates at confluence
   b) The doubling time of these cells in 96-well plates
   c) The linearity of the luminescence signal with respect to cell number

This information will help you identify the optimal seeding density, duration between passages, and dilution ratio at each passage. For example, a cell line with a maximum density of 20,000 cells/well and a doubling time of 1 day can be seeded at ~2500 cells/well (1250 expressing firefly luciferase + 1250 expressing Renilla luciferase) and passaged every 4 days at a 1/16 dilution.

Generate stable cell lines 2w

1 Select an appropriate cell line using the Cancer Dependency Map portal. This protocol has been optimized for adherent cell lines that are propagated in serum-containing medium and are resilient when treated with trypsin. 2w

2 Using lentiviral transduction, engineer 2 stable derivative cell lines from the selected parental cell line. One of these derivatives should express Cas9 and firefly luciferase (i.e. pLX311-Cas9/pLX313-Firefly), and the other should express a control ORF (or empty vector) and Renilla luciferase (i.e. pLX311-LacZ/pLX313-Renilla). When generating these lines, aim for a multiplicity of infection (MOI) of ~0.3 to ensure that most cells contain a single integrant of each expression vector.
*For production of lentivirus and stable cell lines please see:
https://portals.broadinstitute.org/gpp/public/resources/protocols
*Always follow appropriate biosafety protocols for working with human cell lines and lentivirus.

Day 0 - Mix and seed luciferase-labeled cells 10m

3 Trypsinize exponentially-growing stable cell lines (pLX311-Cas9/pLX313-Firefly and pLX311-LacZ/pLX313-Renilla) to generate two single-cell suspensions. 10m

4 Determine the density of each cell suspension using a hemocytometer or other cell counting device. 2m

5 Dilute each cell suspension to an appropriate density (see **Before Starting** note) and thoroughly mix the two cell suspensions together at a 1:1 (cell:cell) ratio in a 50 mL conical vial. 10m

6 Pipette or carefully decant the cell mixture into a multichannel reservoir. 1m

7 Transfer 200 µL of the cell mixture into mutliple wells of a 96-well plate using a multichannel pipette. 5m

As you plan the scale of your experiments, be sure to account for the inclusion of technical replicates as well as the following controls:

1) Negative control sgRNAs that target:
- non-essential genes
- non-coding regions
- non-human genes

*These help control for non-specific effects associated with lentiviral transduction, selection in puromycin, and cell fitness effects associated with DNA cleavage by Cas9.

2) sgRNAs that target firefly luciferase
*These act as internal controls for Cas9 activity.

3) sgRNAs that target essential genes
*These allow you to determine the expected maximal killing effect of lethal sgRNAs.

4) Uninfected cells selected in puromycin

5) Uninfected cells not selected in puromycin
*Controls 4 and 5 are used to determine the extent of puromycin-mediated killing.

6) Wells containing media only or PBS
*These help reduce evaporation from experimental wells, and can be used to determine the level of background luminescence.

8 Allow cells to settle to the bottom of the 96-well plate at room temperature on a level surface (~15 minutes) and then incubate the plate in a 37°C tissue-culture incubator overnight. 15m

9  5m

Optional: You may wish to seed the cell mixture into a few wells of an additional 96-well plate. This plate can be subjected to a dual luminescence assay at an early time point to determine the relative proportions of the two labeled populations prior to infection. Note that raw Renilla luminescence values are typically 10-fold greater than raw firefly luminescence values.
*See **Step 22-26** below for details on how to perform the dual luminescence assay.

Day 1 - Infect cells with sgRNA-expressing lentivirus 30m

10 Using a multichannel pipette, add Polybrene infection reagent to each well of the 96-well plate to acheive a final concentration of 5 µg/mL. Pre-dilute the Polybrene in fresh media, if necessary. 5m

11 Add high-titer lentiviruses that encode the chosen experimental or control sgRNAs (e.g. lentiGuide-Puro, pXPR003 backbones) (5-10 µL of virus per well, aim for 80-100% infection efficiency). 5m

12  2h

Optional: A spinfection protocol may be used to enhance infection efficiency.

13 Incubate infected cells in a 37°C tissue-culture incubator overnight. 18h

Day 2 - Select for infected cells using puromycin 5m

14 Using a multichannel pipette, carefully aspirate all ~200 µL of the supernatant from the 96-well plate and safely dispose of waste. 5m
*Remember, the supernatant contains active lentivirus.

15 Add 200 µL of fresh media containing puromycin (1 µg/mL) to all infected wells and a portion of the uninfected control wells. 5m

Add 200 µL of fresh media lacking puromycin to the remaining uninfected control wells and empty wells.

16 Incubate plates in a 37°C tissue-culture incubator for 48 hours. This will allow the puromycin to kill all cells that did not become infected with an sgRNA-expressing lentivirus. 2d

Day 4 - Replate cells 5m

17 Using a multichannel pipette, carefully aspirate all ~200 µL of the supernatant from the 96-well plate and dispose appropriately. 5m

18 Add 50 µL of 0.25% trypsin-EDTA to each well and incubate cells at 37ºC until all cells lift from plate (~1-10 min). 15m

19 Add 150 µL of fresh (serum-containing) media and mix to resuspend cells. 5m

20 Split the entire mixture into two replica plates: 5m
- a **Dual Luciferase Assay** plate and
- a **Trypsinize and Reseed** plate.

*It is easiest to first load 100 µL of fresh media into each replica plate and then aliquot 100 µL of the cell mixture into those plates.

21 Allow cells to settle to the bottom of the 96-well plates at room temperature on a level surface (~15 minutes) and then incubate the plates in a 37°C tissue-culture incubator overnight. 15m

Day 5 - Perform dual luciferase assay and replate cells 5m

22 For the **Dual Luciferase Assay** plate: 5m
Using a multichannel pipette, carefully aspirate all ~200 µL of the supernatant from the 96-well plate and dispose appropriately.

23 Add 50 µL of the firefly luciferase reagent (FLR) containing passive cell lysis buffer and PBS (see **Materials**), or Dual-Glo® Luciferase Reagent from Promega™, which already contains lysis buffer. 25m

Protect from light, and incubate plate for 20 minutes at room temperature.

24 Obtain raw firefly luminescence readings using a luminometer that is compatible with 96-well plates. 1m 36s

25 Add 25 µL of the Renilla luciferase reagent (RLR) or Promega™ Stop & Glo® reagent. 25m

Protect from light, and incubate plate for 20 minutes at room temperature.

26 Obtain raw Renilla luminescence readings using a luminometer that is compatible with 96-well plates. 1m 36s

27 For the **Trypsinize and Reseed** plate:
Using a multichannel pipette, carefully aspirate all ~200 µL of the supernatant from the 96-well plate and dispose appropriately.

28 Add 50 µL of trypsin-EDTA to each well and incubate cells at 37ºC until all cells lift from plate (~1-10 min). 15m

29 Add 150 µL of fresh (serum-containing) media and mix to resuspend cells. 5m

30 Split the mixture into two **new** replica plates at an appropriate dilution (see **Before Starting** note): 5m
- a **Dual Luciferase Assay** plate and
- a **Trypsinize and Reseed** plate.

*It is easiest to first load a defined amount of fresh media into each replica plate and then aliquot the appropriate dilution of the cell suspension into those plates. For example, for a 1/16 dilution, first load 187.5 µL of fresh media into each new replica plate, and then add 12.5 µL of the mixed cell suspension.

31 Allow cells to settle to the bottom of the 96-well plate at room temperature on a level surface (~15 minutes) and then incubate the plate in a 37°C tissue-culture incubator for the desired amount of time (typically 2-4 days). 15m

Day 9 (and onwards) - Perform dual luciferase assay and replate cells

32 Repeat all steps from Day 5. Continue the process of reading and replating for as long as replicates remain consistent (tested up to 17 days).

Data Analysis

33 We recommend performing the following calculations to arrive at an approximation of the log-ratio of edited:unedited cells:
1) Divide the raw firefly luminescence reading of each experimental well by the mean raw firefly luminescence readings of wells infected with the chosen set of negative control sgRNAs (from **Step 7**). This is the fractional firefly luminescence reading (i.e. the % viability of the edited cells).
2) Divide the raw Renilla luminescence reading of each experimental well by the mean raw Renilla luminescence readings of wells infected with the same set of negative control sgRNAs. This is the fractional Renilla luminescence reading (i.e. the % viability of the unedited cells).
3) Divide the fractional firefly luminescence reading by the fractional Renilla luminescence reading. This is the edited to unedited ratio.
4) Calculate the logarithm of the edited to unedited ratio (or plot the ratio on a log-scale axis).
*Because Cas9 is expressed by cells that express firefly luciferase:
- Log ratios near 0 suggest that the edited and unedited cells had equal fitness.
- Log ratios less than 0 suggest that edited cells had a competitive fitness disadvantage relative to unedited cells.
- Log ratios greater than 0 suggest that edited cells had a competitive fitness advantage relative to unedited cells.