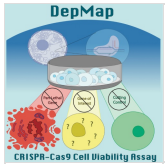

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Single-gene short-term CRISPR ko viability assay

Benjamin Gaeta[1], Tsukasa Shibue[2], Brenton Paolella[1], Francisca Vazquez[1]

[1]Broad Institute; [2]Cancer Program, Broad Institute of MIT and Harvard

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

Cancer Dependency Map Target Validation
Tech. support email: **depmap-tda@broadinstitute.org**

Tsukasa Shibue

ABSTRACT

The Cancer Dependency Map Project is using genome-scale CRISPR loss-of-function screens on a large number of cancer models to systematically identify selective genetic dependencies, which in turn represent potential therapeutic targets. As we identify potential targets through this project, it is becoming increasingly important to have a robust validation strategy to de-risk individual targets before embarking on costly therapeutic projects.

Here we describe an experimental strategy for assessing the **short-term viability effect** after knocking out a gene of interest via CRISPR/Cas9 using a commercially-available luminescent cell viability assay reagent (Cell-titer Glo [Promega]) in a 96-well format. The robustness and reproducibility of this assay are critically dependent on two experimental parameters: seeding density of the cells and the lentivirus infection efficiency. We provide standardized protocols for the rigorous determination of both of these parameters.

In addition, given that CRISPR-mediated gene knockout penetrance differ across cell lines, the use of standardized controls is necessary for the comparative assessment of the strength of the dependency across cell lines. Moreover, there is a non-specific toxicity effect resulting from the DNA double-strand breaks caused by the CRISPR/Cas9 system. It is therefore important to use sgRNAs targeting intragenic regions, which serve as negative controls to account for this non-specific toxicity. We have tested and identified the sequences of negative and positive sgRNAs controls (pan-essential genes) that can be used to scale the cell viability read-outs.

Finally, we also provide a series of templates to record and normalize the data produced throughout this assay.

ATTACHMENTS

CTG Normalization Template.xlsx

Viability Assay Optimization Template.xlsx

PROTOCOL CITATION

Benjamin Gaeta, Tsukasa Shibue, Brenton Paolella, Francisca Vazquez 2020. Single-gene short-term CRISPR ko viability assay. **protocols.io** https://protocols.io/view/single-gene-short-term-crispr-ko-viability-assay-bc6jizcn

KEYWORDS

cancer therapeutic target, target validation, 7 Day Validation, CTG, CRISPR-Cas9 Knock Out, 96 Well, Constitutive Expressing Cas9, sgRNA

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GUIDELINES

Our protocol for the cell-titer-glo assay used to quantify the viability of your cells is nearly the same as that of the provided Promega CTG kit (**ref# G7573**).

- One notable exception is that we use 25ul of undiluted CTG reagent per 100ul of media in 96 well format.
- Additionally, we recommend changing the media to 100ul per well for each plate that will be read out. This will ensure uniformity of CTG density as media evaporates at variables rates across different wells.

MATERIALS

NAME	CATALOG #	VENDOR
CellTiter-Glo(R) Luminescent Cell Viability, 10 x 100ml	G7573	Promega
RPMI 1640 Medium	21875042	Thermo Fisher
Corning® 96-well Flat Clear Bottom White Polystyrene TC-treated Microplates 20 per Bag with Lid S	3903	Corning
15-Dimethyl-15-diazaundecamethylene polymethobromide Polybrene	H9268	Millipore Sigma

SAFETY WARNINGS

- Use BL2+ facility for lentivirus infection and subsequent propagation of infected cells.

BEFORE STARTING

- Obtain and propagate 5+ models (i.e., cell lines) that are putatively sensitive on the genetic perturbation to be tested as well as 5+ lineage-matched control models that are supposed to be insensitive to the same perturbation
- (Optional) Adapt all the cell line models to growth in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS)

Assay Development

1 (Day 0) **Template/Planning**: create a copy of the "Viability Assay Optimization" template and fill out the information.

2 (Day 0) **Make Viral Master Plate**: prepare (a) master plate(s) of BRD003 lenti-virus that contains the following volume of virus per 40 uL of total volume

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0	1.25	1.25	1.25	**1.25**	**1.25**	**1.25**	1.25	1.25	1.25	
C		0	2.5	2.5	2.5	**2.5**	**2.5**	**2.5**	2.5	2.5	2.5	
D		0	5	5	5	**5**	**5**	**5**	5	5	5	
E		0	10	10	10	**10**	**10**	**10**	10	10	10	
F		0	20	20	20	**20**	**20**	**20**	20	20	20	
G		0	40	40	40	**40**	**40**	**40**	40	40	40	
H												

1-12, A-H indicate columns and rows of a 96-well plate, respectively. The number indicates the volume (uL) of BRD003 virus in each well.

* NOTE- up to 4 cell types/one master plate; if seeding > 4 cell types, make multiple master plates

2.1 Prepare (# of cell types) x 400 uL of BRD003 virus

2.2 Fill column 2, rows B-G and columns 3-11, rows B-F (wells w. black numbers) with (# of cell types) x 50 uL of R10 medium

2.3 Aliquot (# of cell types) x 100 uL of BRD003 virus into columns 3-11, row G (wells w. red numbers)

2.4 Transfer (# of cell types) x 50 uL of virus-containing medium from columns 3-11, row G to columns 3-11, row F and mix well (not well itself but its content); change chips of multichannel pipetter

2.5 Transfer (# of cell types) x 50 uL of virus-containing medium from columns 3-11, row F to columns 3-11, row E and mix well (not well itself but its content); change chips of multichannel pipetter

2.6 Transfer (# of cell types) x 50 uL of virus-containing medium from columns 3-11, row C to columns 3-11, row B and mix well (not well itself but its content)

3 (Day 0) **Cell Seeding**: seed the numbers of cells in 100 uL medium (containing 5.6 ug/mL polybrebne [H9268, SIgma-Aldrich]) indicated in this table in two 96 well plates (per cell line):

(Plate I)	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
C		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
D		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
E		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
F		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
G		1000	1000	1000	1000	**1000**	**1000**	**1000**	2000	2000	2000	
H												

(Plate II)	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		2000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
C		2000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
D		2000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
E		4000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
F		4000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
G		4000	2000	2000	2000	**4000**	**4000**	**4000**	4000	4000	4000	
H												

3.1 make 6 x (# of cell type) mL medium containing 5.6 ug/mL polybrene (this is 'A').

3.2 make a suspension of 40K cell/10 mL medium (w/ 5.6 ug/mL polybrene) (this is 'B').

3.3 Aliquot 75 uL of A into Plate I, columns 2-8 .

3.4 Aliquot 50 uL of A into Plate I, columns 9-11; Plate II, columns 2-5 (except for 2E, 2F, 2G).

3.5 Aliquot 25 uL of B into Plate I, columns 2-8.

3.6 Aliquot 50 uL of B into Plate I, columns 9-11; Plate II, columns 2-5 (except for 2E, 2F, 2G).

3.7 Aliquot 100 uL of B into Plate II, columns 6-11 and 2E, 2F, 2G.

4 (Day 0) **Infection**: transfer 40 uL of (virus-containing) medium from the virus master plate to the corresponding well of cell-seeded plate; centrifuge at 900 x g, 1.5h at 37°C.

5 (Day 1) Medium change/Puro selection: change medium with 200 uL of fresh medium
- Puro (+): For only Plate 1, columns 6-8 and Plate II, columns 3-5, 9-11, use 2 ug/mL Puromycin-containing medium.

6 (Day 3) change medium (Puro (-) or (+)).

7 (Day 5) change medium (Puro (-) or (+)).

8 (Day 7) **CTG measurement**: Perform the CellTiter-Glo protocol provided by Promega and paste the CTG values for each cell line under the "Assay Dev" tab of the "Viability Assay Optimization" template.

Determining optimal seeding density

9 For each of the seeding densities – i.e., 1000, 2000, 4000 cells/well --, determine the minimum volume of virus that yields (mean CTG value of Puro (+) wells) > 0.8 x (mean CTG value of Puro (-) wells): Set the mean Puro (-) CTG value for this volume of virus as C_{1000} (or C_{2000}, C_{4000}); check if the C value determined here is larger than '0.7 x (corresponding mean CTG value of infection/Puro (-) sample)'.

10 If $C_{4000} \geq 1.8 \times C_{2000}$, use 4000 cells/well (if not, go to 11).

11 If $1.5 \times C_{2000} \leq C_{4000} < 1.8 \times C_{2000}$, use 3000 cell//well (if not, go to 12).

12 If $C_{2000} \geq 1.8 \times C_{1000}$, use 2000 cells/well (if not, go to 13).

Determining relative infection sensitivity

13 Determine the minimum volume of virus that gives (mean CTG value of Puro (+) wells) > 0.8 x (mean CTG value of Puro (-) wells) for the detemined seeding density of corresponding cells. If an interpolated number of cells is used (3000 or 1500 cells/ well), take the mean virus volume for the "4000" and "2000" values. Based on this virus volume (which reflects sensitivity to viral infection/viral toxicity), **categorize cell lines used for a single project** (usually around 10 cell lines of varying lineages) **into 3 classes**: HIGH/MEDIUM/LOW infection sensitivity. When you prepare a set of virus for viability assay, pick one cell line from each class and titrate all the virus against these cell lines (see an example below):

sensitivity	Example cell lines
LOW	SNU503_colon, JIMT1_breast, HCC95_lung, KYSE410_esophagus
MED	SW837_colon, HCC15_lung, KYSE450_esophagus
HIGH	HCC202_breast, AU565_breast, SKBR3_breast, ZR751_breast

Titration of Virus for Viability Assay

14 Measure the titers of 9 types of virus prepared/aliquoted as below, using 3 representative cells lines -- 1 from HIGH, MEDIUM, and LOW infection sensitivity (as identified by the Week1 'Assay Dev' [above].

15 Prepare virus for each of the:

- 3 negative controls (BRD003-sgLacZ/-sgCH2-2/-sgAAVS1)
- 3 positive controls (BRD003-sgPOLR2D/sgSF3B1/-sgKIF11)
- 3 target-specific guides (BRD-003-sgXXX-A/-sgXXX-B/-sgXXX-C)

Prepare virus in 1x15cm dish; this will yield approx 17 mL of virus. Aliquot the virus into 1.5 mLx11 vials and assemble as below:

sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ	sgLacZ
sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2	sgCH2-2
sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1	sgAAVS1
sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D	sgPOLR2D
sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1	sgSF3B1
sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11	sgKIF11
sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A	sgTarget-A
sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B	sgTarget-B
sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C	sgTarget-C
sgLacZ	sgCH2-2	sgAAVS1	sgPOLR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	---

last row: 1 vial each **FOR TITRATION**

sgRNA sequence for all controls:

sgLacZ (non-targeting control)	AACGGCGGATTGACCGTAAT
sgCh2-2 (intergenic control)	GGTGTGCGTATGAAGCAGTG
sgAAVS1 (intergenic control)	GGGCCACTAGGGACAGGAT
sgPOLR2D (positive control)	AGAGACTGCTGAGGAGTCCA
sgSF3B1 (positive control)	AAAAGGATCAAGACGCTCTG
sgKIF11 (positive control)	TCTTGTGTAGGAGTATACGG

sgRNA sequences for positive and negative controls

16 Pick 3 cell lines representing HIGH/MEDIUM/LOW infection sensitivity based on the 'Assay Development' result

e.g. SNU503(**LOW**), **S**W837 (**MEDIUM**), HCC202(**HIGH**)

17 (Day 0) **Seeding**: Prepare 15ml of cell suspension with:

*[# of cells to seed per well] x 150 cells in R10, containing 10ul f 8 uG/uL polybrene

17.1 Seed 100 uL/well into two 96-well plates per cell line:

----	no infection	sgLacZ	sgCh2-2	sgAAVS1	sgPOLR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	----
----	100	100	100	100	100	100	100	100	100	100	ul
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	----	----	----	----	----	----	----	----	----	----	----

Plate A : Puro (-)

----	no infection	sgLacZ	sgCh2-2	sgAAVS1	sgPOLR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	----
----	100	100	100	100	100	100	100	100	100	100	ul
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	100	100	100	100	100	100	100	100	100	100	----
----	----	----	----	----	----	----	----	----	----	----	----

Plate B : Puro (+)

18 Make virus 'Master Plate':

Create a master plate (enough virus for both seeded plates) by serial diluting pure virus in R10 with a multichannel pipette:

----	----	----	----	----	----	----	----	----	----	----	----
----	Med	150	150	150	150	150	150	150	150	150	ul
----	Med	150	150	150	150	150	150	150	150	150	----
----	Med	150	150	150	150	150	150	150	150	150	----
----	Med	150	150	150	150	150	150	150	150	150	----
----	Med	150	150	150	150	150	150	150	150	150	----
----	Med	300	300	300	300	300	300	300	300	300	----
----	----	----	----	----	----	----	----	----	----	----	----

Example for a single cell line (Medium Infectability)

1. Fill row G (columns 3-11) with indicated amount of virus.
2. Fill rows B-F (columns 3-11) with indicated amount of medium (eg. R10).
3. Fill rows B-F (columns 3-11) with indicated amount of medium (eg. R10).
4. Transfer half the volume from row G to row F and mix well.
5. transfer half the volume from row F to row E and mix well.
6. Transfer half the volume from row E to row D and mix well.
7. Transfer half the volume from row D to row C and mix well.
8. Transfer half the volume from row C to row B and mix well.

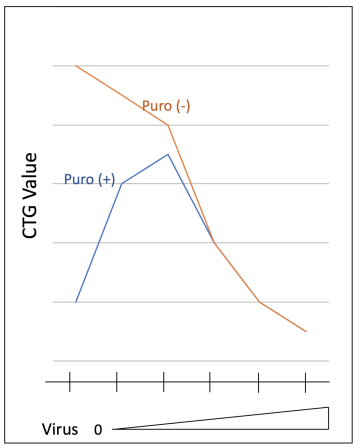
19 (Day 0) **Infection**: Transfer 40 uL of virus to cell-seeded plates form the corresponding well in Master Plate. Centrifuge at 900 x g, 37-degree for 1.5h.

20 (Day 1) Change medium (Plate A: just medium; Plate B: medium with 2 uG/uL Puro)

21 (Day 4) **Read CTG**

22 Determine appropriate amount of virus to add per cell line

22.1 Plot the titration result (CTG value; see below)



22.2 Determine the amount of virus (X) that fulfills both:

A) CTG $_{virus=x, Puro(-)}$ / CTG $_{virus=0, Puro(-)}$ ≥0.8,
B) CTG $_{virus=x, Puro (+)}$ / CTG $_{virus=0, Puro(-)}$ ≥0.8

22.3 When multiple virus amounts fulfill the above criteria, pick the amount of virus that gave the largest CTG $_{Puro(+)}$ value. This will usually match the peak of the Puro (+) CTG curve.

22.4 Make a table (see below) and use this as a reference for the amount of virus to be used for cell lines with HIGH/MEDIUM/LOW infection sensitivity:

	LacZ	Ch2-2	AAVS1	...	Target B	Target C
Low	5 uL	2.5uL	10		5	5
Medium	10	5	20		10	10
High	20	10	40		20	20

7-Day Viability Assay

23 Before starting the assy, you should know: **Seeding Density** and **Infection Sensitivity** (for each cell line tested) and Viral Titer (for each virus tested).

(Day 0) Create a copy of the "CTG normalization template" and fill out the information (there are 6 lines per template; if testing > 6 cell lines, create multiple sheets).

24 Cell seeding; seed # of cells in accordance with the result from the seeding density measurement; One 96-well plate (rows B-G; columns 1-11).

25 (Day 1) Infection

25.1 Dilute Virus with 10% FBS RPMI; use the "Virus Calculation for CTG Assay" template to calculate how much virus/medium to be mixed.

25.2 Aliquot 325 uL of diluted virus to single row (columns 1-11)/cell line of the Master Plate(s).

Cell Line												
a	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
b	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
c	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
d	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
e	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
f	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	-	
-	-	-	-	-	-	-	-	-	-	-	-	-

Master Plate

25.3 Replace the medium of cell-seeded plates with 50uL of 10% FBS RPMI containing 8ug/mL polybrene.

25.4 Add 50 uL of virus from Master Plate to corresponding wells (in rows B-G) of cell-seeded plate.

25.5 Spin plate at 900 x g for 1.5 hours at 37C.

25.6 (for sensitive cells) replace medium with 100uL of fresh cell-specific medium right after spinning.

26 (Day 2) **Selection**: Replace medium with 100 uL of fresh medium [for antibiotics (-)] or antibiotics-containing medium [for antibiotics (+)] .

-												
(-)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
(-)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
(-)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
(+)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
(+)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
(+)	-	no infection	sgLacZ	sgAAVS1	sgCh2-2	sgPolR2D	sgSF3B1	sgKIF11	sgTarget-A	sgTarget-B	sgTarget-C	
-	-	-	-	-	-	-	-	-	-	-	-	-

Master Plate

27 (Day 5) **Medium change**: Replace medium with 100 uL of fresh medium [for antibiotics (-)] or antibiotics-containing medium [for antibiotics (+)]

28 (Day 7) **CTG Assay**

28.1 Replace medium with 100 uL/well of warm 10% FBS RPMI

28.2 Add 25 uL/well of CellTiter-Glo Reagent (Promega, G7573)

28.3 Shake on a plate shaker for 30 min RT

28.4 Read luminescence using a plate reader (eg., EnVision, PerkinElmer, #2105-0010)

29 Final CTG values from a plate reader can be copied and pasted directly into "CTG Normalization Template. The results for the final assay will be normalized such that 0 is equal to an average of our negative (cutting) controls and -1 is equal to the average of our positive controls.