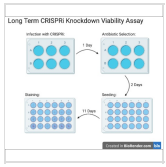

Jun 17, 2020

Single-gene long-term CRISPRi knockdown viability assay

Nolan Bick[1], Benjamin Gaeta[2], Tsukasa Shibue[1], Francisca Vazquez[2]

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

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

As an alternative to '**Single-gene short-term CRISPR ko viability assay** (https://www.protocols.io/edit/single-gene-short-term-crispr-ko-viability-assay-bc6jizcn/description)', we have also developed a method for assessing the **long-term viability effect** upon suppression of a gene of interest. Using CRISPRko (CRISPR/Cas9-mediated gene knockout) is not compatible with evaluating long-term viability effects due to incomplete penetrance of gene inactivation. Instead, we use **CRISPRi** with catalytically dead Cas9 (dCas9) fused to Krüppel associated box (KRAB) domain to suppress gene expression with higher penetrance. Following perturbation with CRISPRi, cells are seeded at a clonogenic density and their growth is monitored for up to 2 weeks.

We provide sequences of CRISPRi negative and positive sgRNAs controls, which are critical for evaluating the strength of the dependency, as well as a series of templates to record and normalize the data produced throughout this assay. In addition to permitting evaluation of long-term viability effects, this CRISPRi-based assay might also enable the assessment of the effects of partial suppression of gene expression if appropriate gRNAs are available.

ATTACHMENTS

Excel template for CV quantification.xlsx

PROTOCOL CITATION

Nolan Bick, Benjamin Gaeta, Tsukasa Shibue, Francisca Vazquez 2020. Single-gene long-term CRISPRi knockdown viability assay. **protocols.io** https://protocols.io/view/single-gene-long-term-crispri-knockdown-viability-bdm6i49e

KEYWORDS

Colony Formation, cell viability, CRISPRi, target validation, genetic dependency, CRISPR Interference

CREATED

Mar 13, 2020

LAST MODIFIED

Jun 17, 2020

PROTOCOL INTEGER ID

34206

GUIDELINES

While the Single-gene short-term CRISPR ko viability assay described here provides a good starting point for evaluating genetic dependency in selected cell line models, this assay cannot assess vability effect that appears long (> 1 week) after gene depletion. The **Single-gene long-term CRISPRi knockdown viability assay**

described in this section is useful for evaluating such slow viability effects.

MATERIALS

NAME	CATALOG #	VENDOR
Paraformaldehyde fixative: 4% paraformaldehyde in phosphate buffered saline (PBS)		
Crystal violet	CB0331.SIZE.100g	Bio Basic Inc.
EZ-LINE Multiwell TC Plates, 24-well TC plate, 50/case, Treated	SP41135.SIZE.1CS	Bio Basic Inc.
Acetic acid	320099-6X2.5L	Sigma – Aldrich
15-Dimethyl-15-diazaundecamethylene polymethobromide Polybrene	H9268	Millipore Sigma
DNA Ligation Kit Mighty Mix	6023	Takarabio

SAFETY WARNINGS

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

BEFORE STARTING

- Obtain and propagate 3+ models (i.e., cell lines) that are putatively sensitive on the genetic perturbation to be tested as well as 3+ 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)

Reagent Preparation/Set-Up

1 **Select cell lines** that are putatively dependent (dep) or nondependent (nondep) on a gene of interest using depmap.org. Usually, 3 or more cell lines from both dep and nondep classes are selected.

2 ✱

OPTIONAL: If the original medium for the cell line is not RPMI, consider adapting the cells with RPMI + 10% fetal bovine serum (FBS) so that all the cell lines are grown in the same media.

3 **Plasmids/Cloning**

We created the pXPR_BRD023-D lentivector to express dCas9 + KRAB alongside sgRNAs targeting our gene of interest in addition to positive and negative controls (sequences included in materials).

Step 3.1: describes our method to create the pXPR_BRD023-D vector
Steps 3.2 to 3.7: describes insertion of specific sgRNA sequences for the gene of interest. and controls.

3.1 The Cas9 sequence was removed from all-in-one CRISPR KO plasmid, pXPR_BRD023, and replaced with the dCas9-KRAB sequence from pXPR_BRD121. Restriction enzymes NheI (BRD023), XbaI(BRD121), and BamHI(both) were used for digestion. The dCas9-KRAB insert and BRD023 backbone were subsequently ligated, transformed, and plasmid DNA extracted.

3.2 Order oligos for sgRNA sequences:

3 sgRNAs targeting the gene to be tested

2 positive controls and 1 negative control:

Negative control (intergenic sequence) Chr2-2	GGTGTGCGTATGAAGCAGTG

Positive control 1 POLR2D	CGGCGGGTGGCAGCGATCCG
Positive control 2 SF3B1	AAGATCGCCAAGACTCACGA

CRISPRi sgRNA sequences of negative and positive controls

3.3 Anneal oligo sgciRNA oligo pairs.

For each pair, add the following in a PCR tube.

·1.5 µL of forward oligo (100uM)
·1.5 µL of reverse oligo (100uM)
·5 µL of 10x NEB buffer 3.1
·42 µL ddH20

50ul total

·Incubate for 4 min at 95°C, 10 minutes at 70C and then let cool to room temperature.

3.4 Digest the generic pXPR_BRD023-D vector with NEB enzymes, EcoRI and BsmBI (visit NEB website for digestion procedures consistent with enzyme parameters). Purify backbone and insert via agarose gel.

3.5

·Ligate digested BRD023-D backbone to each pair of annealed sgciRNA oligos.

·Immediately transform ligation mix to Stbl3 competent cells from ThermoFisher (Cat: C737303).

·Leave on ice for 10 minutes, heat shock at 42C for 30 seconds, recover by adding 200ul of SOC media per transformation, and incubate at 37C for 1 hour.

·Plate 50ul of each transformation mixture on LB agar + Carbenicillin plates and incubate at 37C overnight (16 hours).

3.6 Pick 6 bacterial colonies per plate and amplify each colony in 4ml of LB + 50ug/ml Carbenicillin media for 16 hours at 37C in a bacterial shaker.

3.7 Perform miniprep (per manufacturer's instructions) on all samples to extract plasmid DNA.

4 **Prepare lentivirus** for each CRISPRi all-in-one construct using 15 cm-plates (protocol for generic virus prodocution is included in attached files).

4.1 Determine the number of 15 cm plates for virus production per type of virus based on the need and resource available (we typically need 5 or more 15 cm plates per virus type per target).

4.2 Filter media containing virus via 0.45 um filter.

4.3 Concentrate the virus to approximately 2 mL per 15 cm plate, using Amicon Ultra-15 Centrifugal FIlter Unit (30K NMWL, MIlipore SIgma, C7715).

4.4 To concentrate virus, spin ~15mLs of virus-containing media at 2500g's for 30 minutes at 4°C. We use 30kDa or 100kDa 15-mL filter centrifuge tubes. This approach typically yields ~ 1mL of concentrated virus.

Virus Titration 30m

5 Viral Titration

30m

5.1 Mix following in a 12-well plate,

cells	500,000 cells
virus	(see below; with differing amount of virus)
8 mg/mL polybrene	1 uL
10% FBS, RPMI	up to 2 mL

Plate map (volume of virus)

0 uL (0 uL)	0 uL (0 uL)		
25 uL (200 uL)	50 uL (400 uL)		
100 uL (800 uL)	200 uL (1600 uL)		

volume of concentrated virus (use volume in the parenthesis if the virus is not concentrated)

Centrifuge at 900xg, 1.5h, 37ºC

Virus titration/infection: Day 0 2h

5.2 24 hours after seeding/infecting change medium

Puro	no Puro		
Puro	Puro		
Puro	Puro		

Puro: 10% FBS RPMI + 2 ug/mL puromycin, 1 mL
no Puro: 10% FBS RPMI, 1 mL

5.3 48 hours after starting Puro selection, pick the well with
- No apparent death by Puromycin
- No apparent virus toxicity

Virus Titration 30m

5.4 A no infection well with antibiotic selection is helpful to check on the selection progress of cells

5.5 Titrate the virus (see titration protocol above STEP5.1-5.3) to determine the amount of virus giving you maximal infection and minimal toxicity. Archiving >90% infection efficiency (post-antibiotic selection) is optimal.

If titrating the virus is not possible, select 3 concentrations to use during the experiment. We found that for most cell lines we can use 100 uL of concentrated virus or 800 uL on non-concentrated virus

6 Add optimal virus to cells and spin plate at 900g (RCF) @ 37°C for 90 minutes. 1h 30m
Aspirate media and wash with PBS and add selection antibiotic

Seeding: Day 3 55m

7 Check to see cells are selecting out from selection (Ideally >10% dead cells). 5m

When testing 3 different MOIs, pick the one with minimal viral toxicity and maximal survival against selection.

8 Trypsinize cells and count cells **(SELECTION-FREE MEDIA)** 5m

9 Seed cells into colony formation plate, seeding density varies based on cell doubling time (make dilutions if needed) 45m

9.1 24-well: Total volume to 500ul

Fast growing (cell doubling time 24-72 hours): 500 cells per well at lowest density
Medium growing (cell doubling time 72-120 hours): 1000 cells per well at lowest density
Slow growing (cell doubling time >120+ hours): 2500 cells per well at lowest density

9.2 Plate layout (24-well):

(seeding density)	Ch2-2 (neg control)	POLR2D (pos control)	SF3B1 (pos control)	Target- sgRNA-1	Target- gRNA-2	Target-sgRNA-3
1x						
2x						
4x						
8x						

9.3 DO NOT add antibiotics at this point (antibiotic treatment will be restarted on Day 6)

9.4 Plate rest of negative control and target cells for Western blot if desired

Culturing: Day 6 5m

10 Working quickly, aspirate media (collect and spin if suspension cells), and re-add media with ***antibiotic selection*** 5m

10.1
1. Do not wash with PBS
2. Avoid drying in center of wells
3. Don't touch bottom of well
4. Carefully add media to wells (avoid detachment of cells)

Culturing: Day 9 5m

11 Working quickly, aspirate media (collect and spin if suspension cells), and re-add media with ***antibiotic selection*** 5m

Culturing: Day 12 5m

12 Working quickly, aspirate media (collect and spin if suspension cells), and re-add media with ***antibiotic selection*** 5m

Staining with CV: Day 14 1h 5m

13 Staining with Crystal Violet (Adherent Cells) 1h

13.1 Aspirate Media and gently wash with PBS

13.2 Add 4% PFA or 10% Neutral-buffered formalin (under fume hood) to wells and put on shaker for 15 minutes

For 24-well, 250ul

13.3 Remove PFA or Formalin (under fume hood) and dispose of in appropriate hazardous waste container

13.4 Wash wells with DI water

For 24-well, 250ul

13.5 Remove water and dispose of in PFA/Formalin waste container

13.6 Add crystal violet to each well (under fume hood) and put on shaker for 20 minutes

For 24-well, 250ul

13.7 Remove crystal violet (under fume hood) and dispose of in appropriate hazardous container

13.8 Wash wells with DI water

For 24-well, 250ul

13.9 Remove water and dispose of in crystal violet waste container

13.10 Fill ice bucket with DI water and gently dunk plates to rinse, repeat for a total of 2 dunks

13.11 Let plates air dry (overnight is usually best)

14 Take images of plates once dry using scanner 5m

Place plates face up (bottom of well is closer to scanner)
Resolution: 600 dpi

Quantification of CV: After day 14 10m

15 After scanning pictures of plate(s), add 10% acetic acid to each well and put on shaker for at least 20 minutes 30m

For 24-well, 325ul

16 Mix well, add 100ul in triplicates into clear bottom 96-well plate 5m

16.1 Add 10% acetic acid as account for background to empty well(s)

17 Read absorbance at 595 nm 5m

17.1 Use an Excel template attached here to analyze the result

18 Dispose of acetic acid + crystal violet in appropriate hazardous waste container 5m

Quantification with CTG: Day 14 45m

19 Quantifying with Cell Titer-Glo (Suspension Cells) 45m

19.1 Without removing media, add 150 uL/well of CTG reagent (24-well plates) (undiluted)

19.2 Shake the plate on the shaker, 30 min @ Room Temp.

19.3 Aliquot 100 uL x 3 wells into opaque (white/black) 96-well plate

19.4 Read luminescence
Use the same Excel template as above (see STEP 17.1) for the analysis of the result.

The intensity of crystal violet staining (or luminescence) of experimental samples (with knockdown) relative to those of negative and positive control samples will indicate how strong the viability effect is.