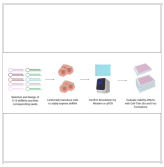

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shRNA Selection and Quality Control for Cancer Target Gene Validation

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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

Proper design of shRNA sequences can be challenging, requiring empirical testing of up to 10-20 shRNA sequences. To address these issues, we leverage data from RNAi loss of function screens aggregated by the Cancer Dependency Map project (Depmap.org). We identify and prioritize shRNAs more likely to have both strong “on-target” effects and lower off-target effects ("shRNA Seed effects"). Throughout this protocol, we detail how to identify top shRNAs from these large-scale screens, and provide guidance on the design of seed-matched controls. We then describe how to evaluate the selected shRNAs to identify the shRNAs with the maximal differential between on-target and off target effects. It is important to test multiple shRNAs and their corresponding seed control sequences to ensure they do not retain partial on target activity or have broad off-target toxicity. shRNAs and their seed controls can have different viability effects depending on the design, and the cell line being used, so it is crucial to rigorously test multiple shRNAs in multiple cell lines. If used properly, shRNAs can be a great genetic perturbation reagent for target validation, particularly for inducible *in vivo* experiments.

PROTOCOL CITATION

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1. shRNA Selection and Evaluation: Genes with high-quality shRNAs tested in RNAi Data

1 For genes that were profiled in the RNAi datasets, we can use the shRNA quality estimates calculated using DEMETER2 to identify high-quality shRNAs (5).

1.1 Choose hairpin sequences
(a)Select hairpins that that have high “G_eff” and a low “S_eff” and “unpred_offset_mean” to maximize on target effects and minimize off target effects.

2 Access the excel sheets on shRNA quality metrics and shRNA sequence to gene mapping from the DEMETER2 found on Figshare.

2.1 For the shRNA sequence to gene mapping, download "**shRNA-mapping.csv**"

2.2 For the shRNA Quality Control Excel metrics, download **"D2_combined_hp_data.csv"**

3 Map the gene(s) of interest to the shRNA sequences for that gene

3.1 Look up the gene of interest in '**shRNA-mapping.csv"** in the "Gene Symbol" column and extract the matching shRNA target sequences in the "Barcode Sequence" column

4 Find the shRNA sequences in the "**D2_combined_hp_data.csv file"**

5 Choose hairpin sequences

5.1 Select hairpins that that have high "G_eff" and a low "S_eff" and "unpred_offset_mean" to maximize on target effects and minimize off target effects.

6 A description of the table columns is provided below:

6.1 Table of model parameters estimated for each shRNA (shRNAs are indexed by their targeting sequence) includes the following columns:

1. Geff: Estimated gene knockdown efficacy of each shRNA (alpha_i).
2. Seff: Estimated off-target efficacy of each shRNA (beta_i)
3. unpred_offset_mean: Posterior mean of 'unpredicted' across-cell-line average off-target effect per shRNA (c_i)
4. unpred_offset_sd: Posterior std dev of 'unpredicted' across-cell-line average off-target effect per shRNA (c_i)
5. hairpin_offset_mean: Posterior mean of additive offset per shRNA and batch (theta_ik), averaged across batches k.
6. hairpin_offset_sd: Posterior std dev of additive offset per shRNA and batch (theta_ik), averaged across batches (as sqrt(<sigma_k^2>))

6.2 NOTE: Not all genes have quality shRNA sequences from the shRNA libraries and additional shRNA sequences may need to be designed and tested empirically. It is our experience that some genes may not have suitable shRNA sequences unless 10-20 sequences are tested. In those cases RNAi may be less tractable than alternative experimental systems to inactivate a gene or protein product, such as CRISPR or degron tags.

1. shRNA Selection and Evaluation: Genes without shRNAs quality control from RNAi Data

7 It is recommended that you design multiple on-target sequences for each gene. Some helpful tools are below:

7.1 Whitehead siRNA Design Tool or Horizon Discovery siRNA Design Tool (note both are for siRNA design so alter sequences accordingly)

7.2 Various protocols recommend 30-50% GC content

1. shRNA Selection and Evaluation: Cloning shRNAs into Tet Inducible Cellecta vector

8 To clone shRNA oligonucleotides we prefer the Cellecta shRNA Tet Inducible Expression Vector. The general design for this vector is as follows:

8.1 5'-accgG-(21sense)-(Stem-Loop-Stem)-(21antisense)-TTTT-3'

Sense oligo:

5'-accgGNNNNNNNNNNNNNNNNNNNNNGTTAATATTCATAGCNNNNNNNNNNNNNNNNNNNNNTTTT-3'

Antisense oligo:

5'cgaaAAAANNNNNNNNNNNNNNNNNNNNNGCTATGAATATTAACNNNNNNNNNNNNNNNNNNNNNC-3'

8.2 The sense strand is the shRNA target sequence from the Achilles/DRIVE library found in the "D2_combined_hp_data.csv" file

9 To design a seed control, nucleotides in the 9-11 position in the oligos should be altered to the complement nucleotides. This should disrupt on-target binding, but maintains knockdown of off-target genes.

9.1 Example- 2-8 Seed Sequence, C9-11 Alteration

On-target 21nt Sense Oligo: 5'- CGAGAAGCTGAAGGATTATTT

Seed C9-11 21nt Sense Oligo: 5'- CGAGAAGCTGTTCGATTATTT

9.2 Notice in the above example that the seed (nucleotides 2-8) remains constant between the on-target shRNA and the seed, hence these sequences being called seed-matched controls.

9.3 In certain cases, C9-11 seed controls retain partial on-target knockdown. In those cases it is possible to design seed controls with additional base pair alterations to further reduce on-target gene affinity. (For example, controls with positions 9-13 nt can be mutated that retain the seed sequence).

9.4 Link to C9-11 design tool

10 Once you have designed 3-5 targeting sequences and their corresponding seed controls, clone sequences into the Cellecta tet-inducible vector and lentivirally transduce cells to create cell lines stably expressing inducible shRNA vector.

2. Evaluation of KD by qRT-PCR or western blot

11 We evaluate on-target knockdown performance of each shRNA and seed control. We use standard lab techniques for both qPCR and western blotting to determine the effect of knockdown in the cell lines of interest.

4. Assessing cell viability effects with 10-14 day Foci Formation and 7 day Cell Titer Glo assays: CV Assay

12 To determine the short-term and long-term viability effects of the on-target and seed shRNAs *in vitro*, we use 7-Day CTGs and Crystal Violet Assays. The 7-Day CTG provides a quantitative short-term readout, while the crystal violet assay provides long-term qualitative data. Both assays are important when assessing on-target viability effects and seed toxicity, as the potency and viability timeline of shRNAs can vary widely between cell lines; seed effects that appear minimal at seven days may have a larger viability effect at the 14-day time point or vice versa. Additionally, seed effects and on-target viability that appear strong in one cell line may not have the same phenotype in another. Therefore, vigorous testing of each cell line is essential in determining its use for future *in vivo* experiments.

13 Trypsinize and count the on-target shRNA and associated seed control cell lines

14 Dilute the cells to $*0.18*10^5$/ $\mathbf{.06*10^5}$cells/mL in 14 mL media
Italicized - Slow growing cell Line
Bold- Fast Growing Cell Line

15 Plate cell suspension at 1 mL/well according to the plate map below

shX	**shX**	**shX**	**shX**	**shX**	**shX**
seedX	**seedX**	**seedX**	**seedX**	**seedX**	**seedX**

16 Add 4 mL of media to cell suspension for a final concentration $*0.12*10^5$/ $\mathbf{.04*10^5}$cells/mL in 12 mL

17 Plate cell suspension in a new 24 well plate at 1mL/well according to the plate map above

18 Add 6 mL of media to the original cell suspension for a final concentration of $*0.06*10^5$/ $\mathbf{0.02*10^5}$cells/mL at 12mL

19 Plate cell suspension in a new 24 well plate at 1mL/well according to the 24 well plate map above

20 Add doxycycline at a final concentration of 0.5mg/mL, to half the wells according to the plate map below

shX -dox	**shX -dox**	**shX - dox**	**shX +dox**	**shX +dox**	**shX +dox**
seedX -dox	**seedX -dox**	**seedX -dox**	**seedX +dox**	**seedX +dox**	**seedX +dox**

4. Assessing cell viability effects with 10-14 day Foci Formation and 7 day Cell Titer Glo assays: CTG

21 Plate cells in a 96 well for a 7-day CTG according to the plate map below at 2000 cells/well at 200 uL/well. (Note: Seeding density can be optimized for each cell line)

PBS	PBS	PBS	PBS	PBS	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	shX -dox	shX +dox	Seed - dox	Seed +dox	PBS						
PBS	PBS	PBS	PBS	PBS	PBS						

4. CTG readout 1w

22 Monitor the – dox wells to ensure the wells are confluent by day 7 1w

23 Add CTG reagent and readout by the Envision

5. Crystal Violet Fixation 2w

24 *Continuously monitor the 24 well plates to determine when the -dox wells become 100% confluent. Once the 24 well plates become confluent, fix and stain the plate according to the below protocol.* 2w

25 Gently aspirate the media and add 250 uL of 1X PBS to wash the wells. Add PBS to the sides of the wells to not dislodge any cells

26 Aspirate the PBS and add 250 uL of 4% Paraformeldahyde in 1X PBS. Leave the plates on the shaker for 15 minutes to completely fix the cells

27 Remove the 4% paraformeldahyde and wash wells with 250 uL of water.

28 Remove the water and add 250 uL Crystal Violet to each well. Leave plates on shaker for 20 minutes to stain cells

29 Remove the crystal violet stain and wash wells multiple times with water until the residual crystal violet is completely removed.

30 Let the plates dry for 24 hours and then image these plates on a scanner (600 dpi, increased brightness).

31 To quantify the crystal violet stain, add 250 uL of 10% acetic acid to each of the wells. Place plates on the shaker for 20 minutes to remove crystal violet stain from cells

32 In a 96-well plate, take 25 uL from each well and dilute it in 75 uL water according to the plate map below. Measure the OD at 600 nm.

shX-1 -dox	shX-1 -dox	shX-1 -dox	shX-1 -dox	seedX-1 -dox	seedX-1 -dox	seedX-1 -dox	seedX-1 -dox				
shX-1 +dox	shX-1 +dox	shX-1 +dox	shX-1 +dox	seedX-1 +dox	seedX-1 +dox	seedX-1 +dox	seedX-1 +dox				
shX-2 -dox	shX-2 -dox	shX-2 -dox	shX-2 -dox	seedX-2 -dox	seedX-2 -dox	seedX-2 -dox	seedX-2 -dox				
shX-2 +dox	shX-2 +dox	shX-2 +dox	shX-2 +dox	seedX-2 +dox	seedX-2 +dox	seedX-2 +dox	seedX-2 +dox				
shX-3 -dox	shX-3 -dox	shX-3 -dox	shX-3 -dox	seedX-3 -dox	seedX-3 -dox	seedX-3 -dox	seedX-3 -dox				
shX-3 +dox	shX-3 +dox	shX-3 +dox	shX-3 +dox	seedX-3 +dox	seedX-3 +dox	seedX-3 +dox	seedX-3 +dox				