

Genetic Screens in Human Cells Using the CRISPR/Cas9 System

The lentiviral Cas9/sgRNA-based screening system described in Wang et al enables large-scale loss-of-function genetic screening. The system consists of two components: (1) a dox-inducible Cas9 expression vector (2) pooled sgRNA expression libraries. The major advantages afforded by this system are: (1) uniform expression of Cas9 in all cells screened (2) facile production of high-titer sgRNA lentivirus. This contrasts with the screening system developed by Shalem et al where Cas9 and sgRNAs are introduced simultaneously, which has the advantage of allowing rapid screening in a large number of cell lines. To use this two vector system, one should take the following steps:

A. Derive a clonal cell line expressing FLAG-Cas9 under the control of a doxycycline responsive promoter (pCW-Cas9)

- 1) Generate Cas9 lentivirus (see: <http://www.addgene.org/tools/protocols/plko/#E>)
- 2) Transduce target cells followed by puromycin selection 24 hours after infection.
- 3) Isolate single-cell clones via limiting dilution or FACS.
- 4) Induce clones and probe for Cas9 expression via western blotting using an anti-FLAG antibody.
- 5) Select a clone with the best induction properties (ie. dox-dependent expression) for use in screens.

NOTE: The doxycycline-inducible promoter is CMV-based and will therefore silence after repeated passaging. Therefore it is important to freeze down early passages of the Cas9-expressing cells for screening. Additionally, in some cases, the minimal leakiness of the doxycycline-inducible promoter may result in sufficient Cas9 expression to induce genomic cleavage in the presence of a sgRNA. Therefore, for a screen, it is recommended that the initial population be sampled immediately after library infection.

B. Propagate sgRNA library

- 1) Transform 1 μ L of each sgRNA subpool into highly competent bacteria (XL-10 gold is recommended for chemical transformation).
- 2a) After the recovery period of the transformation, perform dilution plating to determine transformation efficiency using 1% of the total reaction.
- 2b) With the remainder of the reaction, inoculate a 100mL liquid culture.
- 3a) The transformation efficiency must be at least 20-fold greater than the pool size to ensure adequate library representation. If this efficiency is not achieved, discard the bacterial culture and re-transform.
- 3b) Harvest bacteria prior to reaching stationary phase.
- 4) Extract plasmid DNA from pelleted bacteria. It is critical that transfection-grade DNA is obtained

C. Library lentivirus production

- 1) Package lentivirus as described here: <http://www.addgene.org/tools/protocols/plko/#E> but at a larger scale. Depending on the size of the subpool library, 2 to 12 T-175 flasks should be seeded prior to transfection with the proportional amounts of DNA, transfection reagent and media used.

NOTE: Due to the small size of the vector, virus produced from these sgRNA libraries should be very high titer so it is not necessary to concentrate the virus prior to transducing targeting cells.

D. CRISPR-based screening (cell culture)

- 1) Transduce target cells according to the guidelines here: <http://www.addgene.org/tools/protocols/plko/#F>. A spin-infection may boost the effective viral titer for some cell lines. Enough cells should be used such that each sgRNA integrates into 100-1,000 cells.

- 2) 24 hours after transduction, select target cells with blasticidin for 2-3 days. Performing a kill curve on unmodified cells is highly recommended.
- 3) After selection, harvest an initial population of cells and freeze at -20oC.
- 4) If appropriate, a selection agent (ie. drug) can be applied at this time. Performing a kill curve on unmodified cells is highly recommended.
- 5) Passage cells for the duration of the experiment.
- 6) At the end of the experiment, harvest cells and freeze at -20oC.

E. CRISPR-based screening (screen deconvolution)

- 1) Harvest genomic DNA from target cells.
- 2) Amplify genomic DNA using nested PCR with the following primers:

Outer Primer 1 (*OutF*): GCCGGCTCGAGTGTACAAAA

Outer Primer 2 (*OutR*): AGCGCTAGCTAATGCCAACTT

Inner Primer 1 (*InF*): CAAGCAGAAGACGGCATAACGAGATCXXXXXXTTTCTTGGGTAGTTTGCAGTTTT
(XXXXXX denotes the sample barcode)

Inner Primer 2 (*InR*): AATGATACGGCGACCACCGAGATCTACACCGACTCGGTGCCACTTTT

- 3) Clean-up PCR reaction and quantify library

- 4) Submit for high-throughput sequencing with the following custom primers:

Illumina sequencing primer (*SeqP*):

CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Illumina indexing primer (*IndexP*):

TTTCAAGTTACGGTAAGCATATGATAGTCCATTTTAAAACATAATTTTAAAACACTGCAAACCTACCCAAGAAA

- 5) Map reads back to library sequences. The reads are the reverse complement of the sgRNA spacer sequence.

