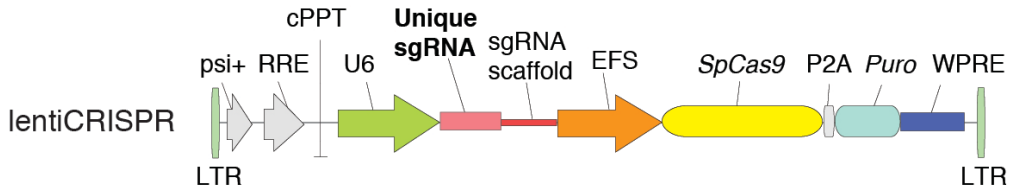
Genome-scale CRISPR Knock-Out (GeCKO) pooled 64K library

CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) is a microbial nuclease system involved in defense against invading phages and plasmids. CRISPR loci in microbial hosts contain a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage. Using lentivirus, we delivered the type II CRISPR nuclease system to facilitate genome editing in mammalian cells in a pooled library, targeting 18,080 genes with 64,751 sgRNAs in early consecutive exons (Shalem*, Sanjana*, *et al.*, Science 2014). **Here we describe how to amplify the aliquot of GeCKO library DNA plasmid to have sufficient quantity to produce lentivirus, while maintaining full library representation**.

LentiCRISPR (pXPR_001): The GeCKO library consists of 64,751 distinct sgRNAs cloned into the same lentiviral backbone (lentiCRISPR) as shown below.



Lentiviral production: Since this vector enables lentiviral delivery of both Cas9 and sgRNA for targeted gene knock out, it is important to perform these experiments in a lab with the appropriate biosafety level and controls, which can vary between different institutions. Before starting any lentiviral work, please ensure compliance with your Environmental Health and Safety office and government / organization / university. Briefly, to make lentivirus, lentiCRISPR (with sgRNA cloned) must be co-transfected into HEK293(F)T cells with the packaging plasmids pVSVg (AddGene 8454) and psPAX2 (AddGene 12260). As a positive control for viral production, we often use a CMV-EGFP lentiviral transfer plasmid (eg. AddGene 19319).

Representation: The GeCKO library is a pool of many different vectors mixed together. The library contains 64,751 unique lentiCRISPRs (each with a unique sgRNA). To ensure no loss of representation, it is important to amplify the library using the protocol given on the next page. **The GeCKO library should not be transformed using chemically competent cells or amplified in liquid cultures. Please read over the entire protocol before starting library amplification.**

Other questions? Many questions about using CRISPR for genome engineering are addressed here:
Genome-engineering using the CRISPR-Cas9 system. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013). *Nature Protocols*, 8, 2281-2308. DOI: 10.1038/nprot.2013.143
Complete plasmid sequences, protocols and a discussion forum with a large community of CRISPR and GeCKO users can be found at the Zhang Lab website: www.genome-engineering.org .

Citation: Please reference the following publication for the use of this material.

Genome-scale CRISPR-Cas9 knockout screening in human cells.
Shalem O*, Sanjana NE*, Hartenian E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F (2014). *Science*, 343, 83-7. DOI: 10.1126/science.1247005

GeCKO pooled electroporation, plating, determination of transformation efficiency and maxi prep:

1. Dilute the GeCKO library to 50 ng/uL in water or TE (if not already diluted).

2. Electroporate the library
   a. Add 2 uL of 50 ng/uL GeCKO library to 25 uL of electrocompetent cells with an efficiency of ≥10^9 cfu/ug. We have had success with many electrocompetent cells, including NEB DH5a cells, Invitrogen DH5a cells and Lucigen E. cloni 10G Elite. In our hands, the Lucigen cells (cat # 60052-1) have yielded the highest efficiency with the GeCKO library.
   b. Electroporate using the manufacturer's suggested parameters/protocol.
   c. Recover in 975 uL SOC (or media provided with cells) and transfer to a loosely capped tube with an additional 1 mL of SOC.
   d. Repeat for a total of 4 electroporations and rotate at 250 rpm for 1 hour at 37 C

3. Plate a dilution to calculate transformation efficiency
   *Note the library plasmids have **ampicillin resistance** – prepare all plates accordingly.*
   a. Pool all 8 mL of SOC. Mix well.
   b. Remove 1 uL and add to 1 mL of SOC, mix well, and plate 20uL onto a pre-warmed 10cm petri dish (ampicillin). This is a 400,000-fold dilution of the full transformation and will enable you to estimate transformation efficiency to ensure that full library representation is preserved.

4. Plate the transformations
   Follow Step a) if your lab has 24.5 cm^2 bioassay plates for large-scale bacteria culture; otherwise follow Step b), which substitutes 20 standard (10 cm round) petri dishes.
   a. Plate 4mL of transformation on each of 2 pre-warmed 24.5 cm^2 bioassay plates (ampicillin) using a spreader. Spread the liquid culture until it is largely absorbed into the agar and won't drip when turned upside down.
   b. Alternatively, spread 400 uL of transformation mix per petri onto 20 pre-warmed petri dishes (ampicillin).

5. Grow all plates inverted overnight at 37 C

6. Calculate transformation efficiency
   a. Count the number of colonies on the dilution plate.
   b. Multiple this number of colonies by 400,000 for the total number of colonies on all plates.
   c. Proceed if the total number of colonies is at least 6.5 x 10^7. This efficiency is equivalent to 1,000 colonies per lentiCRISPR construct in the GeCKO library.

7. Harvest colonies
   a. Pipette 10 mL of LB onto each 24.5 cm^2 bioassay plate (or, 500 uL per 10 cm petri dish)
   b. Scrape the colonies off with a cell spreader/scraper.
   c. Pipette off the liquid plus scraped colonies into a tube and repeat the procedure a second time on the same plate with additional 5-10 mL. *Note: you should weigh this tube prior to adding any liquid to it.*

8. Weigh the bacterial pellet to determine the proper number of maxiprep columns to use
   a. Spin down all liquid to pellet the bacteria and then discard the supernatant.
   b. Weigh the bacterial pellet and subtract the weight of the tube

9. Maxi-prep for downstream virus production and future amplification
   a. Using Qiagen's Plasmid Maxi Kit (cat # 12162), each column can handle approximately 0.45 g of bacterial pellet.
   b. Perform a sufficient number of maxi preps so as to not overload a column.

10. Proceed to transient transfection of HEK293(F)T cells with maxi-prepped GeCKO lentiCRISPR library and appropriate packaging plasmids. The lentiCRISPR backbone vector is compatible with both 2nd and 3rd generation lentiviral packaging plasmids (see www.addgene.org/lentiviral/packaging for more details.)