Supplementary protocol for: *Design of highly functional genome editors by modeling the universe of CRISPR-Cas sequences*

HEK293T transfection protocol

Preparation of plasmid DNA for transfection:

1. **Add spacer of interest immediately upstream of the sgRNA scaffold.**
    a. Flanking regions for the spacer sequence should be:
        i. Upstream: TGTGGAAAGGACGAAACACC
        ii. Downstream (OpenCRISPR-1 sgRNA, 16 nt stem): GTTTTAGAGCTGTGTTGAAA
        iii. Downstream (OpenCRISPR-1 sgRNA, 12 nt stem): GTTTTAGAGCTGGAAACAGC
        iv. Downstream (SpCas9 sgRNA, 12nt stem): GTTTTAGAGCTAGAAATAGC
    b. KLD Enzyme Mix (NEB) is an efficient cloning approach for spacer insertion, but NEBuilder® HiFi DNA Assembly Master Mix (NEB) will also work with the appropriate input fragments.
    c. Platinum™ SuperFi II PCR Master Mix (Thermo Fisher) is a high fidelity polymerase able to generate very long amplicons, which is very useful for the whole plasmid amplifications needed for the KLD cloning method.
        i. Reverse primer for KLD assembly: GGTGTTTCGTCCTTTCCACAAG
        ii. Forward primer for KLD assembly (OpenCRISPR-1 sgRNA, 16 nt stem): [spacer]GTTTTAGAGCTGTGTTGAAAAACAC
        iii. To generate PCR products for KLD cloning use 10 ng of template plasmid in a 25 µL PCR reaction. Only 1 µL of this unpurified PCR product will be needed for the KLD reaction.
    d. NEB® Turbo Competent *E. coli* (High Efficiency) work well for cloning and plasmid propagation, but many other types of *E. coli* competent cells can also be used.

2. **Plasmid DNA purification.**
    a. From a fresh colony or glycerol stock of cells transformed with the nuclease and guide plasmids inoculate 500 µL of terrific broth (Difco™, BD Life Sciences) in a 2 mL deep well plate (Greiner Bio-One, 780271).
    b. Incubate for 12 - 24 hours at 37°C, shaking at 1000 rpm.
    c. Purify plasmid DNA using an endotoxin-free plasmid purification kit.
        i. Mag-Bind® Ultra-Pure Plasmid DNA 96 Kit (Omega Bio-tek) is an option that scales well to large numbers of plasmids.
    d. Measure the concentration of the eluted plasmid DNA.

i. QuantiFluor® dsDNA System (Promega) is an option that scales well to large numbers of plasmids.

e. Normalize the DNA concentrations to 25 ng/µL by diluting in nuclease-free water.
   i. Diluted DNA solutions should be stored at -20°C before use in transfection.

Transfection of HEK293T cells:

3. Thaw and expand HEK293T cells (ATCC, CRL-3216).

a. Standard media conditions are high glucose DMEM with 4 mM L-glutamine, 1 mM sodium pyruvate and phenol red pH indicator (Gibco), supplemented to a final concentration of 10% fetal bovine serum (Gibco) and 1X penicillin-streptomycin (Gibco).
b. Standard growth conditions are 37°C and 5% (v/v) CO_2 in a humidified incubator.
c. Cells should be passaged once after freezing before use in a transfection.

4. Deliver nuclease and guide plasmids by transfection.

a. 24 hours prior to transfection seed cells at a density of $1x10^3$ cells/well in 200 µL of supplemented DMEM in 96-well tissue culture-treated plates (Nunc™ Edge™, Thermo Fisher).
b. Premix nuclease and guide plasmids.
   i. Each transfection well should receive 100 ng of plasmid DNA: 50 ng of the nuclease plasmid and 50 ng of the guide plasmid.
   ii. Premix 4 µL each of the 25 ng/µL stocks of nuclease and guide plasmid. (This prepares a double volume stock of the plasmid mix to allow for dead volume during the transfection.)
c. Prepare and deliver transfection mixes.
   i. Mix 4 µL of the nuclease and guide plasmid solution with 5 µL of Opti-MEM (Gibco).
   ii. Mix 0.2 µL of TransIT®-2020 transfection reagent (Mirus Bio) with 4 µL of Opti-MEM (Gibco).
   iii. Combine the DNA and TransIT®-2020 mixes.
   iv. Incubate at room temperature for 15 - 30 minutes.
   v. Add the entire mixture dropwise to the cells seeded 24 hours previously.
   vi. Gently rock the plate to distribute the liposome complexes.
   vii. Incubate the plate for 72 hours prior to harvesting for sequencing analysis.
      1. If the edited cells will be used for other downstream assays that require cell propagation, the cells should be passaged into larger culture vessels between 24 - 36 hours post-transfection.

Sample preparation for NGS sequencing analysis:

5. **Harvest cell lysates.**
    a. Prepare lysis buffer: 100 mM Tris-HCl, pH 7.5 (VWR); 0.05% SDS (VWR); 25 µg/mL Proteinase K (NEB).
    b. Carefully aspirate the culture media from each transfection well to be harvested. Then wash each well with 100 µL 1X PBS (Gibco), taking care to not disturb the cell layer.
    c. Add 25 µL of lysis buffer per well.
    d. Incubate at 37°C for 1 hour.
    e. Add 25 µL of nuclease-free water (Qiagen) per well.
    f. Harvest lysates into a 96-well PCR plate (VWR).
    g. Collect lysates to the bottom of the well by centrifugation (1000 xg, 2 minutes).
    h. Boil lysates at 98°C for 15 minutes in a thermocycler.

6. **Prepare and pool NGS amplicons.**
    a. Use 5 µL of cell lysate (approximately $1x10^3$ harvested cells) as template for the target specific PCR reaction (25 µL total volume, 25 amplification cycles, 60°C primer annealing) (Invitrogen™ Platinum™ SuperFi II PCR Master Mix, Thermo Fisher), which will amplify the region of interest from the genome and append adapter sequences to allow for sample barcoding.
    b. Dilute the PCR products 1:100 in nuclease-free water (Qiagen).
    c. Use 8 µL of the diluted PCR product as template for the barcoding PCR (25 µL total volume, 12 amplification cycles, 60°C primer annealing) (Q5® High-Fidelity DNA Polymerase, NEB) which will append barcode sequences and Illumina-compatible adapters.
        i. Barcoding primers: xGen UDI 10nt Primer Plates 1-16 (IDT).
    d. Pool 10 µL of each barcoded PCR product and mix thoroughly.
    e. Submit pooled library for NGS analysis.