**The amplification of the sgRNA library**

After receiving the library, for long-term usage, amplify it as backup for use in the future. We typically use *E. coli* DH10B chemically competent cells as host. For 100 μL chemically competent cells, 50 μg library plasmid can be used for the transformation according to the standard protocol. Typically, we can obtain ~ 5×104 colonies. Hence, due to ~ 6×104 sgRNAs in our library, 30 or more transformation experiments (100 μL competent cells each) are needed to maintain an acceptable coverage of the library. We suggest testing the efficiency of DH10B or other competent cells prior to large-scale experiment to determine the needed batches of experiments.

After transformation of the plasmid library into the competent cells and 1 hour recovery in LB medium (LB: competent cell, 4:1, v:v) at 37 °C, streak on the 150 mm LB agar plate with 100 mg/L ampicillin (5 plates per 100 μL competent cell prior to transformation). After overnight cultivation at 37 °C, evaluate the overall number of colonies, assuring the proper coverage towards the library. All colonies on plates were rinsed down using sterile LB medium supplemented with ampicillin, collected through centrifugation (4 °C; 8,000 × g for 10 min), then resuspended and thoroughly mixed to OD600 of 6 using fresh sterile LB medium containing ampicillin. You can split the culture into two fractions, while one part for plasmid extraction and another fraction mixed with 50% glycerol (v:v) by 1:1 (v:v) for long-term storage at −80 °C.

Via a different approach, we found that it is possible to avoid using a big amount of agar plates, which is very tedious and labor intensive. Here, after recovery in LB medium, directly add all the culture into flasks containing fresh LB broth with 100 mg/L ampicillin (10 mL fresh LB broth per 100 μL competent cells prior to transformation). Note that **around 8 hours of cultivation** (**important**) at 37 °C results in OD600 ~ 1.0. Harvest the cells by centrifugation and the following protocoling is all the same as the agar plate method.

Generally speaking, we find that agar plate approach can maintain the fidelity and coverage of the library better. Using LB broth directly partially impairs these metrics (they are still acceptable) but provides great convenience.

**CRISPRi screen**

Firstly, you need to construct *E. coli* strain expressing dCas9 protein. We provide pdCas9-J23111 in Addgene for routine usage, which strongly and constitutively expresses dCas9 and can provide > 99% gene repression for most target sites. We also deposit another pdCas9-J23109 with weaker expression (10% of J23111 promoter). Sometimes, if partial gene repression is preferred, you can try pdCas9-J23109 (80% repression in our chromosome-inserted-sfGFP reporter system). Both of them have a kanamycin marker.

After constructing *E. coli* cells containing dCas9-expression plasmid, prepare the electrocompetent cell used in sgRNA library transformation. High transformation efficiency can greatly reduce the labor in electroporation (number of samples to be transformed), so try to optimize the electrocompetent cell preparation protocol to maximize the efficiency. Similar to sgRNA library amplification, we also recommend to test the efficiency of prepared electrocompetent cells before usage. For 100 μL electrocompetent cells, 50 μg library plasmid can be used in the transformation experiment and 104 ~ 105 CFUs is expected for one batch. Note that freshly extracted sgRNA library plasmid also matters in achieving good transformation efficacy.

According to the measured transformation efficiency, determine the proper number of electroporation experiments to maintain the coverage towards the sgRNA library. Akin to the library amplification, 20-fold coverage or more should be applied. Also, agar plate or LB broth can be used to enrich for the successfully transformed cells (see library amplification section).

After obtaining the cell library with actively expressing dCas9 and sgRNA library, CRISPRi screen can be performed. For details, please check our paper (Wang, T., et al., Nat. Commun. 2018, (9)2475.). Typically, one stressed and one control condition is applied. 5 ~ 10 cell doublings are recommended, because such duration is enough to amplify the moderate fitness change due to gene knockdown, while minimizing the potential de novo mutation that cannot be detected by sequencing the sgRNA plasmid. Two biological replicates for each condition are also useful to test the reliability of the screen experiment. Note that the cell culture prior to the screen experiment should also be kept as the initial library for plasmid extraction and NGS profiling, which is necessary in the data processing.

**NGS profiling**

After plasmid extraction for all the cell culture, follow the steps below for NGS profiling.

KAPA HiFi HotStart polymerase (KAPA Biosystems) Kit

50 uL×4 reactions, 50 ng template plasmid/reaction

95 °C 3 min, (98 °C, 20 s; 67.5 °C, 15 s; 72 °C, 30 s) × 20 cycles, 72 °C for 1 min.

Primers:

TGCGCCGACATCATAACGGTTCTG

CGACTCGGTGCCACTTTTTCAAGTTG

One single band corresponding to 193 bp is expected with no obvious unspecific bands. The PCR product can be further used in NGS library construction and NGS profiling. The NGS library was prepared following the manufacturer’s protocol (TruSeq DNA Nano Library Prep Kit for Illumina). Briefly, the fragments were treated with End Prep Mix for end repairing, 5’ phosphorylation and purification using Sample Purification Beads (SPBs). Then fragments were treated with A-tailing Mix for adenylated 3’ ends, followed by ligation to adaptors indexed with a ‘T’ overhang. Subsequently, the products were purified using the SPBs and amplified by PCR for eight cycles using P5 and P7 primers, cleaned up using SPBs, validated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then libraries with different indexes were multiplexed and loaded on an Illumina HiSeq instrument according to the manufacturer’s instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2 × 150 paired-end configuration; image analysis and base calling were conducted with the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. Five million or more pair-end reads for each library is expected, given ~ 6×104 mutants in our sgRNA library.

**Data analysis**

Please visit our GitHub site with one-stop computational package developed in Python and comprehensive user manual.

<https://github.com/zhangchonglab/CRISPRi-functional-genomics-in-prokaryotes>