Propagation of Human Genome-Wide Minimized Double-gRNA library **24.2.2020**

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MATERIALS

- Library DNA (50 µl at 20 ng/µl in TE buffer)
- NEB 10-beta Electrocompetent *E. coli* (NEB, cat. no. C3020K)
- Qiagen plasmid maxi kit (Qiagen, cat. no. 12163)
- BioRad GenePulser Xcell (BioRad, cat. no. 165-2662)
- Electroporation cuvette, 0.1 cm gap (BioRad, cat. no. 165-2083)

PROCEDURE

Electroporation

1| Pre-warm 500 ml LB+amp (ampicillin, 50 µg/ml) medium in a 1000-ml flask and SOM recovery medium (Stable Outgrowth Medium supplied with the competent cells) at 37 °C for 1 h.
2| Place five electroporation cuvettes and five 1.5-ml tubes on ice.
3| Dilute the library DNA (obtained from Addgene) by mixing 5 µl of the DNA and 5 µl of sterile water to the final concentration of 10 ng/µl. Add 1 µl to each of four pre-chilled 1.5-ml tubes and keep the tubes on ice.
4| As a positive control of electroporation, dilute the control pUC19 DNA (provided with the competent cells) by 1:5 to a final concentration of 10 pg/µl using sterile water. Add 1 µl of the diluted DNA to the last pre-chilled 1.5-ml tube and keep the tube on ice.
5| Thaw two vials (or at least 125 µl) of the frozen electrocompetent cells on ice. This will take approximately 5 min. Mix the cells by flicking gently. Keep the cells on ice.
6| Set the electroporator: 2.0 kV, 200 Omega and 25 µF.
7| Bring the ice box containing the DNA tubes, the competent cells and the electroporation cuvettes, and the pre-warmed SOM recovery medium next to the electroporator.
Note: Once cells are electroporated, SOM must be added immediately. Rapid recovery of cells is critical for electroporation efficiency. Make sure that all reagents, pipettes (P200 and P1000), tips and a 50-ml tube and a 15-ml tube are by the electroporator.
8| Add 25 µl of the cells to the first tube containing the library DNA, mix gently by pipetting up and down 2-3 times and transfer them to a pre-chilled cuvette without making bubbles. Deposit cells across the bottom of the cuvette by gently hitting the bench a few times. Wipe away water and ice around the cuvette completely.
9| Electroporate.
10| Immediately add 1000 µl of the pre-warmed SOM recovery medium to the cuvette, gently mix up and down twice, then transfer the cells to a 50-ml tube.
11| Repeat Steps **8-10** for the remaining three tubes with the library DNA. Add all electroporated cells into the same 15-ml tube and place the tube in bacterial shakers at 37 °C. The total volume of the library recovery culture should be approximately 4 ml.
12| Perform the fifth electroporation for the positive control and add the cells into a 15-ml tube.
13| Shake at 37 °C for 1 h.

Electroporation efficiency measurement

14| Add 180 µl of pre-warmed SOM into 10 wells in a 96-well PCR plate (Wells A1-A5 and B1-B5).
15| Add 20 µl of the bacteria electroporated with the library into Well A1. Then add 20 µl of the bacteria electroporated with the positive control into Well B1.
16| Using multi-channel pipette, mix the bacteria in Wells A1 and B1, and then transfer 20 µl from Column 1 to 2.
17| Dilute the bacteria serially from Column 2 to 3, 3 to 4 and 4 to 5 in the same way.
18| Plate 50 µl from each well in Columns 2-5 to a LB+amp plate and incubate overnight at 37 °C.

19| In the following morning, count colonies. From pUC19 transformation, calculate the transformation efficiency in colony forming unit (cfu)/µg. This is usually higher than 1.0 x 10^{10} cfu/µg. From library DNA transformation, when *X* number of colonies are obtained on the plate derived from Column *Y* and there were *Z* µl of bacteria/SOM solution at Step 11 (*Z* is 4000 when this protocol is used), the total number of colony forming unit is given in *X* x 10^*Y* x 1/50 x *Z.* This should be >6 x 10^7 cfu for the faithful library replication.

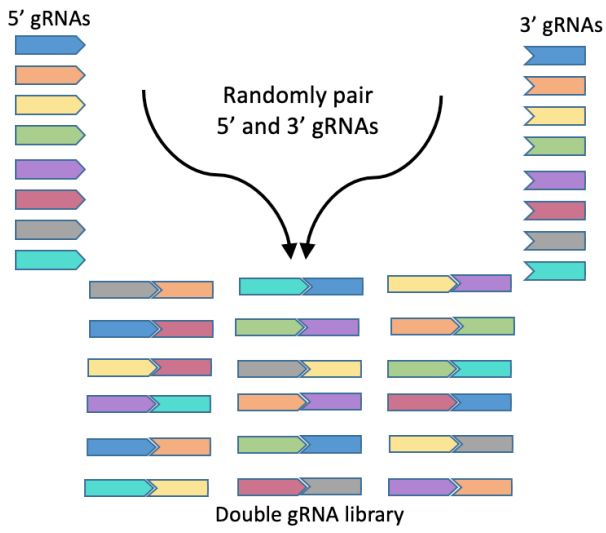
Culture bacteria transformed with the library DNA

20| Add the remaining bacteria electroporated with the library (step **15**) to the pre-warmed 500 ml LB+amp (ampicillin, 50 µg/ml) medium and incubate at 37 °C overnight with shaking.

Note. The volume of bacteria culture is very flexible. If a small amount of DNA is required, this volume can be 200 ml, which are enough for one Maxi-prep column. Or, if more plasmid is required, 1-2 L culture can also be performed although it may require a slightly longer culture time.

Plasmid purification

21| Use 2-3 columns from the Qiagen plasmid maxi kit to purify plasmid from 500 ml culture. Follow the manufacturer's instruction. Change the number of columns if the bacteria have been cultured in a different volume appropriately.



- 60,000 gRNAs picked against 19,260 genes
- Every gene is covered with 3 gRNAs
- One additional gRNA is present for 1,985 iPSC-specific genes
- For 5,546 genes with signal in Avana data, top two Avana gRNAs [by log-fold change] and top Brunello gRNA [by Rule set 2 score] are used
- For remaining genes, top two Brunello gRNAs [by Rule set 2 score] and top Avana gRNA [by log-fold change] are used
- Targeting 5' gRNAs and targeting 3' gRNAs are the same set
- Non-targeting 5' and 3' gRNAs are different
- 5' and 3' gRNAs are read out with different sets of primers

Generation of sequencing libraries from Human Genome-Wide Minimized Double-gRNA library

The double-gRNA library encodes two separate expression cassettes (indicated here as 5' and 3') which need to be separately amplified and sequenced on a SE19 platform. Amplification of both cassettes in a single amplicon, followed by paired-end sequencing, is possible in principle but not recommended. Such PCR has a very low efficiency on a genomic DNA template, leading to loss of complexity. Please contact Luca Crepaldi (lc12@sanger.ac.uk) for further details.
Sequencing libraries are prepared by two consecutive PCR reactions, the first generates an amplicon encoding part of the Illumina adapters, the second PCR (or indexing PCR) extends the adapters and adds indexes for multiplexing, according to the Illumina Unique Dual Indexing (UDI) standard.

MATERIALS

- Q5® Hot Start High-Fidelity 2X Master Mix (NEB M0494S)
- QIAGEN QIAquick PCR purification columns (28106)
- KAPA HiFi HotStart ReadyMix (2x) (KK2602)
- SPRI beads (Agencourt AMPure XP beads; Beckman, Cat.no. A63881)
- DynaMag-96 Side Magnet (Invitrogen, Cat.no. 12331D)

PROCEDURE

First PCR

1| Mix two PCR reactions, one for each of the 5' and 3' cassettes, according to the following table. Prepare enough master mix for all the samples, including a no-template negative control. Scale up reaction volumes depending on amount of template. For large scale reactions from screens, do not use more than 5µg of gDNA for every 50µl reaction aliquot. For amplification of a plasmid library, usually 0.1-1ng template are plenty.

reagent	1 rxn
Q5 2x	25
Forward primer (10µM)	1
Reverse primer (10µM)	1
DNA	x
Water	23-x
Total	50

	5' cassette	3' cassette
Forward primer (10µM)	#1	#605
Reverse primer (10µM)	#738	#737
Product size	195bp	137bp

2| Run the following program
98ºC, 30sec
98ºC, 10sec |

61ºC, 15sec | x25 cycles (less if amplifying from plasmid)
72ºC, 40sec |
72ºC, 2min

3| Run 5 µl of reaction on a ≥2% agarose gel. At least a faint amplicon should be visible. For the 5' cassette, a second band (~700bp) may appear: this is expected and not an issue, see below.
4| Purify PCR products using QIAGEN columns, elute into 30µl H2O. For large samples aliquoted in more than one reaction, prior to purification pool 5µl from each aliquot.
5| Quantify the purified PCR (Nanodrop will do) and dilute to 1 ng/µl

Indexing PCR
6| Mix PCR reactions according to the following table. Use the same primers for both 5' and 3' amplicons. Do not pool 5' and 3' products from the same sample, they are to be sequenced separately.

reagent	1 rxn
KAPA 2x	25
Forward i5 primer (10µM)	1
Reverse i7 primer (10µM)	1
1 ng/µl purified 1st PCR	1
Water	22
Total	50

	5' cassette	3' cassette
Product size	264bp	206bp

7| Run the following program
98°C, 30sec
98°C, 10sec |
66°C, 15sec | 8 to 16 cycles
72°C, 20sec |
72°C, 5min

8| Run 5 µl of reaction on a ≥2% agarose gel. At least a faint amplicon should be visible. For the 5' cassette, a second band (~700bp) may appear: this is expected and not an issue. The upper band can be removed using SPRI beads and a size selection protocol, instead of the standard purification protocol (next step).
9| Purify the indexing PCR using AMPure SPRI Beads. Follow the manufacturer's recommended protocol, using 1.2x volumes of SPRI beads (e.g 48µl beads for 40µl PCR reaction).
10| Quantify purified samples using your favourite protocol, we have good results with Life Technologies Qubit dsDNA HS and Quant_IT dsDNA HS Assay Kits.

Sequencing

11| Pooled libraries can be sequenced single-end, 19 base pairs. The first transcribed nucleotide of all gRNAs in this library is a G. Using the recommended primers, sequencing starts at the *second* transcribed nucleotide (i.e. sequenced nucleotide +1 is transcribed nucleotide +2). Complexity at 1st sequencing cycle is high enough, therefore PhiX or dark cycles are not required.

	5' cassette	3' cassette
SE19 primers	#16	#619

Note: Primers #16 and #619 do not interfere with each other's template, amplicons from 5' and 3' cassettes can be loaded and sequenced on the same lane without issues.

PRIMERS

Primer	sequence
#1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACA
#738	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCACCGACTCGGTGCCACTT
#605	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGCATGCGAGAAAAGCCTTGTTTG
#737	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGTTTCCAGCATAGCTCTTAAAC
i5 index forward	AATGATACGGCGACCACCGAGATCTACACnnnnnnnnACACTCTTTCCCTACACGACGCTCTTCCGATCT
i7 index reverse	CAAGCAGAAGACGGCATACGAGATnnnnnnnnGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
#16	TCTTCCGATCTCTTGTGGAAAGGACGAAACACCG
#619	TCTTCCGATCTATGCATGCGAGAAAAGCCTTGTTTGG