Library Cloning Protocol – CROPseq-iT7-Brunello

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July 2024

Materials:

Plasmids/DNA: CROPseq-iT7 (Addgene Plasmid #211699)
Addgene Brunello pooled library #73178

Oligonucleotides:

cropseq_fwd	TATCTTGTGGAAAGGACGAAACA
cropseq_rev	GCCTTATTTTAACTTGCTATTTCTAG
MiSeq_gRNA_fwd_S0	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCACCATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCAACATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_fwd_S8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGACCCATCTTGTGGAAAGGACGAAACAC
MiSeq_gRNA_rev	TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Barcoding primer fwd	AATGATACGGCGACCACCGAGATCTACAC X_{10} ACACTCTTTCCCTACACGACGCT
Barcoding primer rev	CAAGCAGAAGACGGCATACGAGAT X_{10} GTGACTGGAGTTCAGACGTGTGCT

Enzymes/Buffers: FastDigest Esp3I (ThermoFisher, Cat.No. FD0454)
FastDigest ClaI (ThermoFisher, Cat.No. FD0143)
10x FastDigest Buffer (ThermoFisher, Cat.No. B64)
FastAP (ThermoFisher, Cat.No. EF0651)
DTT
NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs, Cat.No. M0541L)
2x Gibson Assembly MasterMix (New England Biolabs, Cat.No. E2611L)

Bacteria/Media: Endura™ Competent Cells DUOs (BiosearchTechnologies Cat.No. 60242-1)
Endura Recovery Medium
LB medium
LB agar plates
Ampicillin (final concentration 100 µg/mL)

Materials/Kits: QIAprep 2.0 Spin Miniprep Columns (Cat.No. 27115)
Qiagen buffer PE (Cat. No. 19065)
Qiagen buffer PB (Cat. No. 19066)
Zymo DNA Clean & Concentrator® -25 (Cat.No. D4005)
BioRad cuvette 0.1 cm (Cat.No. #1652089)
PureLink™ HiPure Plasmid Filter Maxiprep Kit (ThermoFisher, Cat.No. 210017)

Protocol:

Library cloning

- Digest vector plasmid CROPseq-iT7 with Esp3I and ClaI
  - Prepare a master mix for three reactions containing:
    - 9 µL 10X FastDigest buffer
    - 3 µg backbone plasmid
    - 4.5 µL Esp3I FastDigest
    - 4.5 µL ClaI FastDigest
    - 4.5 µL Fast AP
    - 0.9 µL 100 mM DTT
    - Ad 90 µL with H_2O
  - Prepare 30 µL reactions in PCR stripes and put in thermocycler at 37°C for 2.5 hours
  - Take 2 µL aliquots for running on an agarose gel, pool reactions, purify using a Qiagen PCR cleanup column (blue column), elute in 30 µL H_2O, and quantify using a NanoDrop
  - store at 4°C
- PCR Brunello sgRNA library
  - Prepare a master mix for 16 reactions containing:
    - 150 µL 2x NEBNext HighFidelity PCR MasterMix
    - 12 µL Lenti-guide Puro Brunello sgRNA plasmid library (10 ng/µL)
    - 30 µL mix of primers cropseq_fwd and cropseq_rev, 5 µM each
    - 108 µL H_2O
  - Prepare 25 µL reactions in PCR stripes and run the following program in a thermocycler:
    - 98°C 30 sec
    - 98°C 10 sec |
    - 55°C 10 sec | x20
    - 72°C 15 sec |
    - 72°C 2 min
    - 12°C hold
  - Take 2 µL aliquots for running on an agarose gel, pool reactions after incubation, purify using a QIAprep 2.0 Spin Miniprep Column, pool eluate (3x 25 µL), run 2 µL of purified DNA on an agarose gel, and quantify pooled DNA using a NanoDrop
  - Store at 4°C

- Gibson Assembly
  - Set up a master mix for 10 reactions:
    - 19 µL digested backbone
    - 10 µL amplified sgRNA insert
    - 100 µL 2X Gibson Assembly master mix
    - 71 µL H_2O
  - Prepare 20 µL reactions and incubate at 50°C for 1h
  - Pool 10 reactions and concentrate using the Zymo DNA Clean & Concentrator kit
  - Elute in 25 µL H_2O, measure concentration using a NanoDrop

Amplification of library - Electroporation of Endura competent cells

- Prepare eight 0.1 cm cuvettes (BioRad) and place the cuvettes on ice to cool down
- Set the electroporator to exponential mode with 1800 V,10 µF, 600 Ohms
- Thaw four vials (50 µL each) of Endura competent cells (BioSearch Technologies) on ice (~10-20 minutes)
- Per cuvette, prepare a 1.5 mL Eppendorf tube with 975 µL Endura recovery medium at room temperature
- Once the cells are thawed, prepare 25 µL aliquots in a PCR stripe on ice and add 2 µL (60.2 ng) library, mix by stirring in tube with the pipet tip – do not pipet up and down, since this may warm up the cells
- Transfer the mix to the gap of the pre-cooled cuvettes, tap cuvette on the bench a few times to ensure distribution over the whole gap, dry metal contacts, and quickly electroporate before water is condensing on the contacts again
- If electroporation was successful, transfer bacteria to one of the prepared tubes with recovery medium
- Allow recovery period of all electroporated tubes for 1 hour at 37°C, 400 rpm
- In the meantime, pre-warm 6 LB-Agar plates and prepare a 1L flask with 500 mL LB-Amp
- After recovery period, pool library samples in separate tubes and mix by inverting
- Prepare dilutions for plating, library A-D
  - A: 10 µL plus 990 µL LB (final: 1:100)
  - B: from (A) 10 µL plus 90 µL LB (final: 1:1,000)
  - C: from (A) 10 µL plus 990 µL LB (final: 1:10,000)
  - D: from (B) 10 µL plus 990 µL LB (final: 1:100,000)
  - plate 80 µL of A-D on LB Agar-Amp plates and incubate at 37°C o/n
- Pool remaining library dilution (A) with pooled culture, transfer to 1L flask with medium, and incubate in big shaker at 37°C, 230 rpm o/n

Calculation of library diversity:

- Count the colonies on the agar plates with less than 500 colonies, e.g.:
  Plate B (1:1,000) – 220 colonies
  Plate C (1:10,000) – 21 colonies
  Plate D (1:100,000) – 2 colonies
- Multiply with the count of each plate with the dilution factor from the corresponding plate:
  - B: 220 colonies x 1,000 = 220,000
  - C: 21 colonies x 10,000 = 210,000
  - D: 2 colonies x 100,000 = 200,000
- Multiply with the relative amount of dilution plated from the start culture (80 µL of 8 mL →100x) to estimate the total number of clones:
  - B: 100 x 220,000 = 22,000,000 total clones
  - C: 100 x 210,000 = 21,000,000 total clones
  - D: 100 x 200,000 = 20,000,000 total clones

→ Estimated library complexity after electroporation between 20 and 22 Mio → mean of all three plates calculated ~21 Mio.
→ For a library with 80.000 sgRNAs each guide is present ~263 times

- Purify the DNA from maxiprep culture with PureLink™ HiPure Plasmid Filter Maxiprep Kit according to manufacturer's instructions and dissolve the DNA pellet in 300-1000 µL H_2O

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Validation via Sequencing

- Prepare a 10 ng/µL dilution of the library maxiprep
- Prepare a primer mix consisting of the eight staggered forward (fwd) primers MiSeq_sgRNA_S0-S8 and the reverse (rev) primer MiSeq_sgRNA_rev
    - Each fwd primer should have a final concentration of 0.625 µM, the rev primer should have a final concentration of 5 µM
    - Mix 2 µL of each fwd primer with 16 µL of the rev primer
    - Label as "5 µM Brunello staggered primer mix"
- Prepare a master mix for PCR1 for 4 replicates, each at a total volume of 20 µL:
    - 10 µL 2x NEBNext HighFidelity PCR MasterMix
    - 2 µL 5 µM Brunello staggered primer mix
    - 1 µL DNA
    - 7 µL H_2O
- Prepare reactions in PCR stripes and run the following program in a thermocycler:
    - 98°C 3 min
    - 98°C 20 sec |
    - 65°C 20 sec | x20
    - 72°C 30 sec |
    - 72°C 3 min
    - 4°C hold
- Prepare a master mix for PCR2, each at a total volume of 20 µL:
    - 10 µL 2x NEBNext HighFidelity PCR MasterMix
    - 2 µL unique fwd / rev barcoding primer mix (5 µM each)
    - 2 µL PCR1
    - 6 µL H_2O
- Run thermocycler using the same program as for PCR1
- Pool reactions after incubation, purify using a QIAprep 2.0 Spin Miniprep Column, pool eluate, run 2 µL of purified DNA on an agarose gel, and quantify pooled DNA using a NanoDrop
- Run a quality control of the sequencing library with ~10,000 reads on an Illumina MiSeq
- If QC looks good, run the library with at least 100,000,000 reads on an Illumina NextSeq 2000

Analyse Library Coverage:

- In web browser (Firefox or Google Chrome) open https://www.jsb-lab.bio/LibCounter.htm
- In the blank field "Library" paste the reference library copied from a text file consisting of two columns separated by tab:
    - name of the targeted gene
    - sequence of the sgRNA
- Load the sequencing FASTQ files below at "choose files"
- Press "Start" to run the analysis and retrieve a count table of sgRNAs present in your library
- Sort the list of sgRNAs by reads per sgRNA from lowest to highest read count
- Calculate the fold change between the 10th and 90th percentile as a measure of even coverage
- A fold change below 15 is considered sufficient to perform genome-scale screens
- If the library quality is sufficient, proceed with production of lentivirus

Lentivirus production

Day 0: Seed HEK cells
Seed 15x10^6 HEK293T cells in DMEM 10% FCS + Ciprofloxacin per 15 cm dish

Day 1: transfection

- Prepare Lipofectamine 2000 in OptiMEM, per 15 cm dish mix:
    - 120 µL Lipofectamine 2000 + 1.66 mL OptiMEM
    - Incubate 5 minutes at room temperature
- Prepare a DNA mix in OptiMEM, per 15 cm dish mix:
    - 7,040 ng pMD2.G
    - 10,560 ng psPAX2
    - 14,080 ng CROPseq-iT7-Brunello plasmid library
    - Fill up with OptiMEM to 1.78 mL
- Combine both mixes and incubate for 20 minutes at room temperature
- Add the mix dropwise to the HEK cells (go in a spiral pattern from the outside of the dish to the center)
- After 4-6 hours, change the medium in each dish to 30 mL fresh DMEM supplemented with 10% FCS and 10 µg/mL Ciprofloxacin

Day 3: harvest

- After 48 hours incubation, harvest the lentivirus
- Pool supernatant from all dishes in 50 mL falcon tubes
- Clear lentiviral supernatant by filtering through a 0.45 µm filter using a syringe
- Aliquot in Sarstedt screw cap 1.5 mL tubes and flash freeze in liquid N_2
- Store in labeled box at -80°C