BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

Protocol: PCR of sgRNAs for Illumina sequencing

MATERIALS

- Ex Taq DNA polymerase (Clontech, RR001A)
- PCR plates
- P5 & P7 primers (listed at the end)
- 70% EtOH
- AMPure purification system (Beckman Coulter, 63880)
- 96-well round bottom plate (Costar, 07-200-103)
- Magnet (e.g. Alpaqua, A0011322)

PROTOCOL

PCR set-up

Preferably, prepare mix inside a PCR hood, after cleaning the surface with DNase Away and 70% EtOH. If possible, we recommend setting up at least 4 parallel PCRs for a given sample.
Final contents of each reaction:

- 10 µL 10x reaction buffer
- 8 µL dNTP
- 0.5 µL P5 primer mix, 100 µM
- 1.5 µL ExTaq polymerase
- up to 10 µg of genomic DNA or 200 ng of plasmid DNA
- 10 µL of P7 primer 5 µM
- up to 100 µL with water

1. Make a master mix of reaction buffer, dNTP, P5 primer mix, taq polymerase, and water. Aliquot into a PCR plate.
2. Add template DNA.
3. Add a unique P7 primer to barcode each individual reaction.

Thermal cycler parameters

Wait for block to reach 95˚C before adding samples.

1. 95˚C, 1 minute
2. 95˚C 30 seconds (denaturation)
3. 53˚C 30 seconds (annealing)
4. 72˚C 30 seconds (extension)
   Back to step 2, total of 28 cycles
5. 72˚C 10 minutes
6. 4˚C forever

AMPure XP-PCR purification (recommended)

1. Pool PCR products into an eppendorf (15-30 µL per well is typically sufficient).
2. Distribute 100 µL of pooled products to a 96-well round bottom plate
3. Resuspend the magnetic beads included in the AMPure XP reagent, add 100 µL of beads to each well
4. Mix thoroughly by pipetting up and down 5 times, try not to make bubbles, and incubate at room temperature for 5 minutes. This step binds PCR products 100bp and larger to the magnetic beads. The color of the mixture should appear homogenous after mixing.
5. Place the reaction plate onto a magnet for 2 minutes to separate beads from the solution. Wait for the solution to clear or you see a brown ring around the perimeter of the well before proceeding to the next step.
6. Aspirate the cleared solution from the reaction plate and discard. This step must be performed while the reaction plate is situated on the magnet. Do not disturb the ring of separated magnetic beads.
7. Add 200 µL of 70% ethanol to each well and incubate for 30 seconds at room temperature; aspirate the ethanol and discard.
8. Repeat step 7 once more for a total of two ethanol washes.
9. Remove the plate from the magnet and dry plate for 1 minute and no longer than 4 minutes. A longer dry time (the bead ring appears cracked) will significantly decrease elution efficiency.
10. Add 50 µL of TE buffer to elute the PCR product (elution is rapid—approximately 30 seconds).
11. Place the plate back onto the magnet for ~ 2 minutes.
12. Remove the eluted product and store in an eppendorf. The sample is now ready to be sequenced.

Alternatively, standard gel extraction can be performed. If so, we recommend to isopropanol precipitate the sample post-extraction to remove contaminating guanidine isothiocyanate that is commonly used in gel extraction protocols, which obscures quantitation due to its absorbance at 230 nm.

PCR PRIMERS

A mix of P5 primers with stagger regions of different length is necessary to maintain sequence diversity across the flow-cell. A minimum of 8 primers is recommended. Typically, we mix all 8 primers together in one master mix of P5 primers and use that same mix in all wells.

P5/P7 flowcell attachment sequence
Illumina sequencing primer
Vector primer binding sequence
Stagger region / Barcode region

P5 primer for either vector:
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[s]TTGTGGAAAGGACGAAACACCG

P7 primer for use with lentiGuide (product size = 354 nt)
5'CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT

P7 primer for use with lentiCRISPRv2 (product size = 285 nt)
5'CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAATTCCCACTCCTTTCAAGACCT

P5 primers	
Name	Sequence
P5 0 nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
P5 1nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACACCG
P5 2 nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTTGTGGAAAGGACGAAACACCG
P5 3 nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTTGTGGAAAGGACGAAACACCG
P5 4 nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACTTGTGGAAAGGACGAAACACCG
P5 6 nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCACCTTGTGGAAAGGACGAAACACCG
P5 7nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCAACTTGTGGAAAGGACGAAACACCG
P5 8nt stagger	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGACCCTTGTGGAAAGGACGAAACACCG

P7 barcode (index) sequences for all designs (NNNNNNNN)						
Well	Sequence to include in P7 primer, 5'-3'	Index read (rev comp of sequence)		Well	Sequence to include in P7 primer, 5'-3'	Index read (rev comp of sequence)
A01	CGGTTCAA	TTGAACCG		E01	TAACTCAA	TTGAGTTA
A02	GCTGGATT	AATCCAGC		E02	CGTGAGCC	GGCTCACG
A03	TAACTCGG	CCGAGTTA		E03	ATCAGAGG	CCTCTGAT
A04	TAACAGTT	AACTGTTA		E04	TATGGAGG	CCTCCATA
A05	ATACTCAA	TTGAGTAT		E05	GCGTTCAA	TTGAACGC
A06	GCTGAGAA	TTCTCAGC		E06	CGCAAGAA	TTCTTGCG
A07	ATTGGAGG	CCTCCAAT		E07	CGACAGCC	GGCTGTCG
A08	TAGTCTAA	TTAGACTA		E08	CGACTCGG	CCGAGTCG
A09	CGGTGACC	GGTCACCG		E09	TACAAGAA	TTCTTGTA
A10	TACAGAGG	CCTCTGTA		E10	CGCAGATT	AATCTGCG
A11	ATTGTCAA	TTGACAAT		E11	ATTGCTCC	GGAGCAAT
A12	TATGTCTT	AAGACATA		E12	GCACTCGG	CCGAGTGC
B01	ATTGGATT	AATCCAAT		F01	ATGTTCTT	AAGAACAT
B02	ATACTCGG	CCGAGTAT		F02	ATGTCTCC	GGAGACAT
B03	TATGAGAA	TTCTCATA		F03	GCACTCAA	TTGAGTGC
B04	GCACAGTT	AACTGTGC		F04	TAGTAGCC	GGCTACTA
B05	CGTGGATT	AATCCACG		F05	CGTGTCAA	TTGACACG
B06	TAGTAGAA	TTCTACTA		F06	GCGTTCTT	AAGAACGC
B07	GCACGATT	AATCGTGC		F07	GCCAAGCC	GGCTTGGC
B08	CGGTAGCC	GGCTACCG		F08	GCACCTCC	GGAGGTGC
B09	TAGTTCTT	AAGAACTA		F09	GCACCTGG	CCAGGTGC
B10	TACAAGTT	AACTTGTA		F10	GCCAGACC	GGTCTGGC
B11	ATCACTGG	CCAGTGAT		F11	CGCAAGCC	GGCTTGCG
B12	CGCATCAA	TTGATGCG		F12	TACATCAA	TTGATGTA

C01	GCACGACC	GGTCGTGC	G01	GCGTAGCC	GGCTACGC
C02	TACACTCC	GGAGTGTA	G02	CGACAGAA	TTCTGTCG
C03	CGGTCTAA	TTAGACCG	G03	TAGTCTGG	CCAGACTA
C04	ATGTTCGG	CCGAACAT	G04	ATCAAGTT	AACTTGAT
C05	CGTGGACC	GGTCCACG	G05	TAGTAGTT	AACTACTA
C06	ATTGAGCC	GGCTCAAT	G06	ATACTCTT	AAGAGTAT
C07	TAGTTCGG	CCGAACTA	G07	CGGTAGTT	AACTACCG
C08	CGGTGAGG	CCTCACCG	G08	ATACGAGG	CCTCGTAT
C09	CGTGAGTT	AACTCACG	G09	CGCACTGG	CCAGTGCG
C10	ATCAGATT	AATCTGAT	G10	TACAGACC	GGTCTGTA
C11	TAGTGATT	AATCACTA	G11	GCGTGACC	GGTCACGC
C12	CGGTTCGG	CCGAACCG	G12	TATGTCGG	CCGACATA
D01	TATGGACC	GGTCCATA	H01	CGACTCTT	AAGAGTCG
D02	GCCAAGTT	AACTTGGC	H02	GCGTTCGG	CCGAACGC
D03	CGCAGACC	GGTCTGCG	H03	ATACCTAA	TTAGGTAT
D04	CGACCTCC	GGAGGTCG	H04	CGGTGATT	AATCACCG
D05	GCCACTGG	CCAGTGGC	H05	TAACGACC	GGTCGTTA
D06	GCGTAGTT	AACTACGC	H06	ATACAGCC	GGCTGTAT
D07	CGCAAGTT	AACTTGCG	H07	CGACGACC	GGTCGTCG
D08	CGACAGTT	AACTGTCG	H08	ATCACTAA	TTAGTGAT
D09	CGCATCTT	AAGATGCG	H09	CGACCTGG	CCAGGTCG
D10	ATGTTCAA	TTGAACAT	H10	TATGTCAA	TTGACATA
D11	GCGTAGAA	TTCTACGC	H11	TAACCTAA	TTAGGTTA
D12	ATGTAGCC	GGCTACAT	H12	GCCATCTT	AAGATGGC