BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

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Protocol: PCR of sgRNAs, shRNAs, and ORFs from genomic DNA for Illumina sequencing

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At the conclusion of a pooled screen, genomic DNA (gDNA) is extracted from cell pellets and the integrated construct containing a barcode sequence is amplified by PCR. Subsequent sequencing determines the abundance of each construct in the sample. This protocol describes the PCR step prior to sequencing.

Each PCR well can accommodate up to 10 µg of gDNA in a final reaction volume of 100 µL. There is no particular minimum amount of gDNA required for PCR, although <100 ng gDNA will benefit from additional cycles of PCR, up to 32 cycles. If amplifying from plasmid DNA, use 50 – 200 ng of DNA as input.

Material needed

-Ex Taq packets (10X ExTaq buffer, dNTPs, Taq polymerase, Cat# RR001A)

-PCR plates

-P7 primer (listed at the end)

-P5 primer (listed at the end), pick one depending on your construct

-gDNA

-Molecular biology grade water

-DNase Away (Thermo Fisher Cat# 7010)

-70% EtOH

PCR set-up

Prepare mix inside a PCR hood, clean the surface with DNase Away and 70% EtOH.

Final contents of each reaction:

- 10 µL 10x reaction buffer
- 8 µL dNTP
- 0.5 µL P5 primer mix, 100 µM
- 1.5 µL Takara ExTaq polymerase
- 10 µg or less of gDNA, but no more than 50 µL by volume
- 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 gDNA to each well, reserving at least one well as no-template control by adding water instead.
3. Finally, 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

Purify PCR product with one of the methods described below

I. AMPure XP- PCR purification (recommended)

Material needed
AMPure purification system (Beckman Coulter, Cat # A63880)
96-well round bottom plate (Costar Cat# 07-200-103)
Magnet (Example: Alpaqua Cat# A0011322)
70% EtOH
TE buffer

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 by shaking the bottle, add 100 µL of beads to each well
4. Mix thoroughly 5 times, try not to make bubbles, incubate at room temperature for 5 minutes. This step binds PCR products 100bp and larger to the magnetic beads. Pipette mixing is recommended as it tends to be more reproducible. 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. If beads are drawn out, leave a few microliters of supernatant behind.
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.

II. Gel extraction

Material needed

QIAquick Gel Extraction kit (Qiagen Cat# 28704)
GlycoBlue (Life Technologies Cat# AM9515)
Isopropanol
5M NaCl
TE buffer

1. Run samples on a 2% agarose gel and extract band of size ~360 nts. Purify using QIAquick Gel Extraction kit, incubating in Buffer QG at 40˚C instead of 50˚C. After elution, isopropanol precipitate sample:
   - 50 µL eluate
   - 4 µL 5M NaCl
   - 1 µL GlycoBlue
   - 55 µL isopropanol
2. Incubate at room temperature for 30 minutes. Centrifuge for 30 minutes. Remove isopropanol and wash 2x with 70% ice-cold ethanol. Re-suspend pellet in 25 µL TE. The sample is now ready to be sequenced.

Illumina PCR primer sets

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.

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

sgRNA inserts in pXPR_003: size 354 nts

P5 XPR/LKO1 primer mix:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[s]TTGTGGAAAGGACGAAACACCG

P7 index 2:
CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT

sgRNA inserts in pXPR_023: size 285 nts

P5 XPR/LKO1 primer mix:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[s]TTGTGGAAAGGACGAAACACCG

P7 XPR023:
CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAATTCCCACTCCTTTCAAGACCT

shRNA inserts in pLKO.1 or pLKO.5: size 298 nts

P5 ORF primer mix:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[s]TCTTGTGGAAAGGACGA

P7 index 2:
CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT

ORF inserts in pLX_317: size 271 nts

P5 ORF primer mix:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[s]TCTTGTGGAAAGGACGA

P7 index ORF:
CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAAGCAGCGTATCCACATAGCGT

P5 ORF primer mix	
Name	Sequence
P5_ORF Stag 0a	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTTCTTGTGGAAAGGACGA
P5_ORF Stag 1 A	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTATCTTGTGGAAAGGACGA
P5_ORF Stag 2 GA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTGATCTTGTGGAAAGGACGA
P5_ORF Stag 3 CGA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCGATCTTGTGGAAAGGACGA
P5_ORF Stag 4 ACGA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTACGATCTTGTGGAAAGGACGA
P5_ORF Stag 6 CTAGAA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCTAGAATCTTGTGGAAAGGACGA
P5_ORF Stag 7 GACGACA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTGACGACATCTTGTGGAAAGGACGA
P5_ORF Stag 8 TGGACACA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTTGGACACATCTTGTGGAAAGGACGA
P5_ORF Stag 0b	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTTCTTGTGGAAAGGACGA
P5_ORF Stag 1 C	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCTCTTGTGGAAAGGACGA
P5_ORF Stag 2 AG	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTAGTCTTGTGGAAAGGACGA
P5_ORF Stag 3 GCC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTGCCTCTTGTGGAAAGGACGA
P5_ORF Stag 4 AAGC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTAAGCTCTTGTGGAAAGGACGA
P5_ORF Stag 6 GAAACA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTGAAACATCTTGTGGAAAGGACGA
P5_ORF Stag 7 CGAGAAA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCGAGAAATCTTGTGGAAAGGACGA
P5_ORF Stag 8 TTGAAGCA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTTTGAAGCATCTTGTGGAAAGGACGA

P5 XPR/LKO1 primer mix	
Name	Sequence
P5_XPR/LKO1_1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTCTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTGCTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTAGCTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTCAACTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTTGCACCTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTACGCAACTTGTGGAAAGGACGAAACACCG
P5_XPR/LKO1_8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTGAAGACCCTTGTGGAAAGGACGAAACACCG

P7 barcode (index) sequences for all designs						
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