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Protocol: PCR of sgRNAs/shRNAs/ORFs from gDNA for Illumina Sequencing

Brief Description.....	1
Materials, Reagents & Instrumentation.....	2
Primer specifications.....	3
PCR Set-Up.....	3
Final contents of each reaction.....	3
Thermal cycler parameters.....	4
Purify PCR product.....	4
PCR primer sets (in alphabetical order).....	5

Brief Description

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 100 pg of plasmid DNA in each of 4 wells.

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. Use the table at the end of the document listing common vectors to determine which primer pair to use. A comprehensive table can be found at the end of this document. The amplicon size should range 250-550bp. If your vector is not listed, please email gpp-gDNAsubmission@broadinstitute.org.

We highly recommend testing the gDNA extraction efficiency and PCR conditions on mock samples prior to processing gDNAs from your screen (see [Protocol: Genomic DNA Isolation and PCR Pre-Check](#)). For the test PCR, run a few wells with 10 µg of gDNA and a couple of

No Template Control (NTC) wells. After the PCR, run the product(s) on a gel to confirm the size of the band and spot any contamination.

Materials, Reagents & Instrumentation

- Titanium *Taq* DNA Polymerase and PCR buffer (Clontech Takara Cat# 639242)
- dNTPs (Clontech Takara Cat# 4030)
- DMSO (Sigma Aldrich Cat# D9170-5VL)
- 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

Illumina PCR primer sets

Primer name	Sequence
P5_NEON	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT[s]TCTTGTTGGAAGG*A*C*G*A
P5_ARGON	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT[s]TTGTGGAAGGACGAAAC*A*C*G
P5_HELIUM	AATGATACGGCGACCACCGAGATCTACACAGGTGCTTCTCGATCT*G*C*A*T
P7_KERMIT	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTCTACTATTCTTTCCCTGCA*C*T*G*T
P7_BEAKER	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCAATTCCCACTCCTTTCAAG*A*C*C*T
P7_GONZO	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTAAAGCAGCGTATCCACATA*G*C*G*T
P7_MISS PIGGY	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTACCGACTCGGTGCCACTTTT*T*C*A*A
P7_SCOOTER	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTGAAGACCCTGAGACCACTTTT*C*C*C*G
P7_FOZZIE*	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTG*T*G *C*T

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

*Fozzie's vector primer binding sequence is the same as the Illumina sequencing primer sequence.

Primer specifications

➤ P5_Neon and P5_Argon primers (staggered)

Order 8 (Argon) or 16 (Neon) primers in individual tubes then make an equimolar mix

100uM

IDTE pH 8.0 Standard desalting

[P5 Argon](#)

[P5 Neon](#)

➤ P7 primers (index barcoded)

Order 96 primers in a 96-well plate 100uM

IDTE pH 8.0 Standard desalting

Dilute to 5uM in DNase and RNase free H₂O

[P7 Kermit](#)

[P7 Beaker](#)

[P7 MissPiggy](#)

[P7 Gonzo](#)

[P7 Scooter](#)

[P7 Fozzie](#)

PCR Set-Up

Prepare mix inside a PCR hood if available, clean the surface with DNase Away and 70% EtOH. Extreme care should be taken to avoid contamination from / to other DNA preparations.

Final contents of each reaction

Reagent	Vol. per PCR well
Nuclease-free Water	X µL
10x Titanium Taq PCR Buffer	10 µL
dNTP Mix	8 µL
DMSO	5 µL
P5 Primer (100 µM)	0.5 µL
Titanium Taq Polymerase	1.5 µL

gDNA (up to 10 µg)	up to 50 µL
P7 Primer (5 µM)	10 µL
Total	100 µL

1. Make a master mix of water, reaction buffer, dNTP, P5 primer mix, and finally Titanium *Taq* polymerase. 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

Step 1	95°C	5 minutes
Step 2	95°C (denaturation)	30 seconds
Step 3	53°C (annealing)	30 seconds
Step 4	72°C (extension)	20 seconds
Back to Step 2 x 27 (<i>see note</i>)		
Step 5	72°C	10 minutes
Step 6	4°C	infinitely

Note: For low input gDNA samples, where in the final plating the concentration will be ≤ 1 ug/well, you may want to test both 28 and 32 PCR cycle numbers. We do not recommend going over 32 cycles in order to avoid PCR bias.

Purify PCR product

Choose one of the methods described below:

AMPure XP-PCR purification (recommended)

Materials 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 μ L 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 μ L 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 homogeneous after mixing.
5. Place the reaction plate onto a magnet for 5 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 100 μ L 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 μ L of TE buffer to elute the PCR product (elution is rapid—approximately 30 seconds). Smaller elution volumes (down to 15 μ L) can be used to increase library concentration.
11. Place the plate back onto the magnet for $\sim$ 2 minutes.
12. Remove the eluted product and store in an eppendorf. The sample is now ready to be sequenced.

Gel extraction (if desired)

Materials 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 interest. Purify using QIAquick Gel Extraction kit, incubating in Buffer QG at 40 $^{\circ}$ C instead of 50 $^{\circ}$ C. After elution, isopropanol precipitate sample:
 - i. 50 μ L eluate
 - ii. 4 μ L 5M NaCl
 - iii. 1 μ L GlycoBlue
 - iv. 55 μ L isopropanol
 2. Incubate at room temperature for 30 minutes. Centrifuge for 30 minutes. Remove isopropanol and wash 2x with 70% ice-cold ethanol. Resuspend pellet in 25 μ L TE. The sample is now ready to be sequenced.

PCR primer sets (in alphabetical order)

Vector Name	P5 Primer	P7 Primer
pLKO_TRC005	NEON	KERMIT
pLKO.1	NEON	KERMIT
pLX_317	NEON	GONZO
pMV_AA013	ARGON	MISS PIGGY
pMV_AA050	ARGON	MISS PIGGY
pMV_AA089	ARGON	BEAKER
pMV_AA091	ARGON	MISS PIGGY
pMV_AA147	ARGON	MISS PIGGY
pMV_AA148	ARGON	MISS PIGGY
pMV_AA150	ARGON	MISS PIGGY
pRDA_026_027	ARGON	BEAKER
pRDA_052	ARGON	KERMIT
pRDA_118	ARGON	KERMIT
pRDA_120	ARGON	KERMIT
pRDA_122	ARGON	KERMIT
pRDA_124	ARGON	KERMIT
pRDA_170	ARGON	KERMIT
pRDA_199	ARGON	BEAKER
pRDA_206	ARGON	KERMIT
pRDA_207	ARGON	KERMIT
pRDA_208	ARGON	BEAKER
pRDA_336	ARGON	BEAKER
pRDA_343	ARGON	MISS PIGGY
pRDA_429	ARGON	BEAKER
pRDA_478	ARGON	BEAKER
pRDA_479	ARGON	BEAKER
pRDA_526	ARGON	KERMIT

pRDA_550	ARGON	MISS PIGGY
pRDA_734	ARGON	MISS PIGGY
pRDA_769	HELIUM	FOZZIE
pRDA_771	HELIUM	FOZZIE
pRDA_887	ARGON	BEAKER
pRDB_054	ARGON	MISS PIGGY
pRDB_055	ARGON	MISS PIGGY
pRDB_099	ARGON	MISS PIGGY
pRDB_345	ARGON	KERMIT
pRDB_347	ARGON	KERMIT
pXPR_003	ARGON	KERMIT
pXPR_016	ARGON	KERMIT
pXPR_023	ARGON	BEAKER
pXPR_024	ARGON	KERMIT
pXPR_028	ARGON	KERMIT
pXPR_034	ARGON	BEAKER
pXPR_036	ARGON	KERMIT
pXPR_043	ARGON	KERMIT
pXPR_044	ARGON	BEAKER
pXPR_045	ARGON	KERMIT
pXPR_048	ARGON	BEAKER
pXPR_049	ARGON	KERMIT
pXPR_050	ARGON	KERMIT
pXPR_051	ARGON	BEAKER
pXPR_052	ARGON	MISS PIGGY
pXPR_053	ARGON	KERMIT
pXPR_055	ARGON	KERMIT
pXPR_056	ARGON	KERMIT
pXPR_066	ARGON	BEAKER

pXPR_204	ARGON	MISS PIGGY
pXPR_206	ARGON	BEAKER
pXPR_212	ARGON	BEAKER
pXPR_502	ARGON	KERMIT

Revisions from previous versions:

- Addition of base editing vectors to list
- Addition of P7_MISS PIGGY primer
- Switched to Titanium *Taq* Polymerase from Ex *Taq**
- Addition of 5% DMSO to the PCR reaction*
 - o *Note: These changes to the protocol have resulted in higher PCR success rates with both low and high input samples, in addition to Titanium Taq Polymerase being a cheaper enzyme.*
- Vector list and Primer pairs updated to include current relevant vectors
- Addition of links to full primer sequences (including staggers and index barcodes)