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

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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 below 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.

We highly recommend testing the gDNA extraction efficiency and PCR conditions on mock samples prior to processing gDNAs from your screen (see **Protocol: Isolation of genomic DNA with Nucleospin Blood Kits 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.

Illumina PCR primer sets

| | P7_KERMIT | P7_BEAKER | P7_GONZO | P7_MISS PIGGY |
|--------------|--|--|----------|---------------|
| P5_NEON | pLKO.1 pLKO.5 | | pLX_317 | |
| P5_ARGON | pXPR_003 pXPR_049 pXPR_050 pRDA_052 | pXPR_023 pXPR_034 pXPR_048 pXPR_051 pXPR_206 pRDA_336 pRDA_429 pRDA_478 pRDA_479 | | pRDA_550 |
| P5_MAGNESIUM | | pXPR_207 | | |

**A more complete list of vectors is at the end of this document

| Primer name | Sequence |
|---------------|--|
| P5_NEON | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT[s]TCTTGTGGAAGG*A*C*G*A |
| P5_ARGON | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT[s]TTGTGGAAGGACGAAAC*A*C*G |
| P5_MAGNESIUM | Refer to sequence file |
| P7_KERMIT | CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTCTACTATTCTTTCCCTGCA*C*T*G*T |
| P7_BEAKER | CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCAATTCCACTCCTTTCAAG*A*C*C*T |
| P7_GONZO | CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTAAAGCAGCGTATCCACATA*G*C*G*T |
| P7_MISS PIGGY | CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTACCGACTCGGTGCCACTTTT*T*C*A*A |

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

Primer specifications

➤ P5_NEON and P5_Argon primers

Order 8 primers in individual tubes then make an equimolar mix
100uM
IDTE pH 8.0 Standard desalting

➤ P5_MAGNESIUM

Order as ultramers in 96-well plate
100uM
IDTE pH 8.0 Standard desalting

➤ P7 primers

Order 96 primers in a 96-well plate 100uM
IDTE pH 8.0 Standard desalting
Dilute to 5uM in DNase and RNase free H₂O

Materials for PCR

- 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

PCR set-up and protocol

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:

- 10 µL 10x Titanium *Taq* PCR Buffer
- 8 µL dNTPs
- 5 µL DMSO
- 0.5 µL P5 primer mix, 100 µM
- 10 µg or less of gDNA, but no more than 50 µL by volume

- 10 μL of P7 primer 5 μM
 - up to 98.5 μL with water
 - finally, 1.5 μL Titanium *Taq* polymerase, 100 μL total volume
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:

1. 95°C 5 minutes
2. 95°C 30 seconds (denaturation)
3. 53°C 30 seconds (annealing)
4. 72°C 20 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)

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 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 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 ~ 2 minutes.
12. Remove the eluted product and store in an eppendorf. The sample is now ready to be sequenced.

II. Gel extraction

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 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:
 - 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. Re-suspend pellet in 25 μ L TE. The sample is now ready to be sequenced.

Illumina PCR primer sets continued

| Perturbation | Vector name | Vector Type | P5 Primer | P7 Primer |
|--------------|-------------|---------------------|-----------|-----------|
| CRISPR | pXPR_001 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_003 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_004 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_005 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_006 | CRISPRko-All-In-One | ARGON | KERMIT |

| | | | | |
|--------|--------------|---------------------|-------|------------|
| CRISPR | pXPR_016 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_023 | CRISPRko-All-In-One | ARGON | BEAKER |
| CRISPR | pXPR_024 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_025 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_027 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_028 | CRISPRko-All-In-One | ARGON | KERMIT |
| CRISPR | pXPR_034 | CRISPRalt | ARGON | BEAKER |
| CRISPR | pXPR_036 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_037 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_043 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_044 | CRISPRko-All-In-One | ARGON | BEAKER |
| CRISPR | pXPR_045 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_048 | CRISPRko-All-In-One | ARGON | BEAKER |
| CRISPR | pXPR_049 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_050 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_051 | CRISPRko-All-In-One | ARGON | BEAKER |
| CRISPR | pXPR_052 | CRISPRko-GuideOnly | ARGON | |
| CRISPR | pXPR_053 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_055 | CRISPRko-GuideOnly | ARGON | KERMIT |
| CRISPR | pXPR_066 | CRISPRi-All-In-One | ARGON | BEAKER |
| CRISPR | pXPR_206 | CRISPRalt | ARGON | BEAKER |
| CRISPR | pXPR_212 | CRISPRalt | ARGON | BEAKER |
| CRISPR | pXPR_501 | CRISPRa | ARGON | KERMIT |
| CRISPR | pXPR_502 | CRISPRa | ARGON | KERMIT |
| CRISPR | pRDA_026_027 | CRISPRalt | ARGON | BEAKER |
| CRISPR | pRDA_052 | CRISPRalt | ARGON | KERMIT |
| CRISPR | pRDA_550 | CRISPRalt | ARGON | MISS PIGGY |
| CRISPR | pRDA_118 | CRISPR_GUIDE_ONLY | ARGON | KERMIT |
| CRISPR | pRDA_186 | CRISPR_GUIDE_ONLY | ARGON | KERMIT |
| CRISPR | pRDA_199 | CRISPR_ALL_IN_ONE | ARGON | BEAKER |
| CRISPR | pRDA_207 | CRISPR_GUIDE_ONLY | ARGON | KERMIT |
| CRISPR | pRDA_336 | Base Editing | ARGON | BEAKER |
| CRISPR | pRDA_429 | Base Editing | ARGON | BEAKER |
| CRISPR | pRDA_478 | Base Editing | ARGON | BEAKER |
| CRISPR | pRDA_479 | Base Editing | ARGON | BEAKER |
| ORF | pLX_317 | ORF-Constitutive | NEON | GONZO |
| shRNA | pLKO.1 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC005 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC006 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC008 | shRNA-constitutive | NEON | KERMIT |

| | | | | |
|-------|-------------|--------------------|------|--------|
| shRNA | pLKO_TRC009 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC016 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC017 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC018 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC019 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC020 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC021 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC022 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC023 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC024 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC039 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC040 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC044 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC046 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC047 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLKO_TRC060 | shRNA-constitutive | NEON | KERMIT |
| shRNA | pLI_TRC912 | shRNA-inducible | NEON | KERMIT |
| shRNA | pLI_TRC913 | shRNA-inducible | NEON | KERMIT |
| shRNA | pLI_TRC914 | shRNA-inducible | NEON | KERMIT |
| shRNA | pLI_TRC931 | shRNA-inducible | NEON | KERMIT |
| shRNA | pLI_TRC950 | shRNA-inducible | | KERMIT |

Revisions from previous protocol 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*

**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.*