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Protocol: PCR of sgRNAs/shRNAs/ORFs from gDNA 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. 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

	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]TCTTGTGGAAAGG*A*C*G*A
P5_ARGON	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT[s]TTGTGGAAAGGACGAAAC*A*C*C*G
P5_MAGNESIUM	Refer to sequence file
P7_KERMIT	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCA*C*T*G*T
P7_BEAKER	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCAATTCCCACTCCTTTCAAG*A*C*C*T
P7_GONZO	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTTAAAGCAGCGTATCCACATA*G*C*G*T
P7_MISS PIGGY	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTACCGACTCGGTGCCACTTTT*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 H_2O

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

- 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℃ 5 minutes
2. 95℃ 30 seconds (denaturation)
3. 53℃ 30 seconds (annealing)
4. 72℃ 20 seconds (extension)

Back to step 2, total of 28 cycles

5. 72℃ 10 minutes
6. 4℃ forever

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 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 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). Smaller elution volumes (down to 15 μL) 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.

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℃ instead of 50℃. After elution, isopropanol precipitate sample:
    i. 50 μL eluate
    ii. 4 μL 5M NaCl
    iii. 1 μL GlycoBlue
    iv. 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, 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 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*

➢ *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.*