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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 1ng of plasmid DNA (50ul @ 20pg/ul) in 4 wells.

We highly recommend testing the gDNA extraction efficiency and PCR conditions on mock samples prior to processing gDNAs from your screen.

Material needed

-Ex Taq packets (10X ExTaq buffer, dNTPs, Taq polymerase, Clontech Takara 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 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 reaction buffer
- 8 µL dNTP
- 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 Takara ExTaq polymerase, 100 µL total volume

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

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

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)

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 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.

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.

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 will range 250-550bp.

	P7_KERMIT	**P7_BEAKER**	**P7_GONZO**
P5_NEON	pLKO.1 pLKO.5		pLX_317
P5_ARGON	pXPR_003 pXPR_049 pXPR_050	pXPR_023 pXPR_034 pXPR_048 pXPR_051 pXPR_206	
P5_MAGNESIUM		pXPR_207	

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

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

Vector primer binding sequence where * indicates a phosphorothioate bond to prevent degradation by nuclease.

Primers specifications

P5_NEON and P5_Argon primers

Order 8 primers in individual tubes then make a 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

Perturbation	Vector name	Vector Type	P5 Primer	P7 Indexed 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_053	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_054	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_206	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_208	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_209	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_210	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_212	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_501	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_502	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_503	CRISPRi	ARGON	KERMIT
CRISPR	pXPR_505	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_506	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_508	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_509	CRISPRi	ARGON	KERMIT
CRISPR	pXPR_510	CRISPRi	ARGON	KERMIT
CRISPR	pXPR_511	CRISPRi	ARGON	KERMIT
CRISPR	pXPR_512	CRISPRi	ARGON	KERMIT
CRISPR	pXPR_513	CRISPRa	ARGON	KERMIT
ORF	pLX_317	ORF-Constitutive	NEON	GONZO
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	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	pLKO.1	shRNA-constitutive	NEON	KERMIT