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Protocol status: In development
We are still developing and optimizing this protocol

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gRNA POOL NGS SEQUENCING LIBRARY PREPARATION

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ABSTRACT

Quantification of the frequency of each sgRNA in the library pool is necessary before proceeding to the 10x Genomic assay. We prepare genomic DNA collected after lentiviral transduction of the CRISPR library in H9 dCAS9 cell line to verify the plasmid/viral pool for the CRISPR library via Next Generation Sequencing (NGS sequencing). Genomic DNA was extracted from cells of interest, PCR amplified and the final product was sent to the sequencing facility, where the downstream cleanup and sequencing was performed.

ATTACHMENTS

gRNA POOL NGS SEQUENCING LIBRARY PREPARATION.docx

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PROTOCOL integer ID: 95912

Keywords: ASAPCRN, CRISPRi machinery, Perturb-Seq, NGS sequencing

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MATERIALS

Kits

	A	B	C
	KIT	COMPANY	CATALOG
	QIAamp DNA Blood Mini Kit	Qiagen	51104
	1x dsDNA, high sensitivity	Thermofisher Scientific	Q33231
	KAPA HiFi Hotstart PCR Kit	Roche	KK2502

Primers:

The Illumina overhang adapter sequences were added to the locus specific sequences:

- Forward overhang: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACA [locus specific sequence]
- Reverse overhang: 5'GTCTCGTGGGCTCGGAGATTGTATAAGAGACAG-[locus specific sequence]

The protospacer site within pCRISPRi_dual guide gRNA library plasmid was amplified by PCR using primers containing Illumina adapters added to the locus specific sequence.

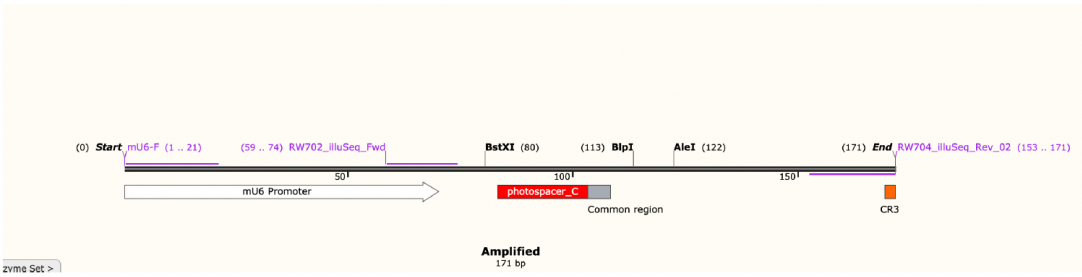

Fig:1 PCR to amplify protospacer A/C = mU6 Fwd +RW704_illuSeq_Rev, the amplicon size= 171 bp

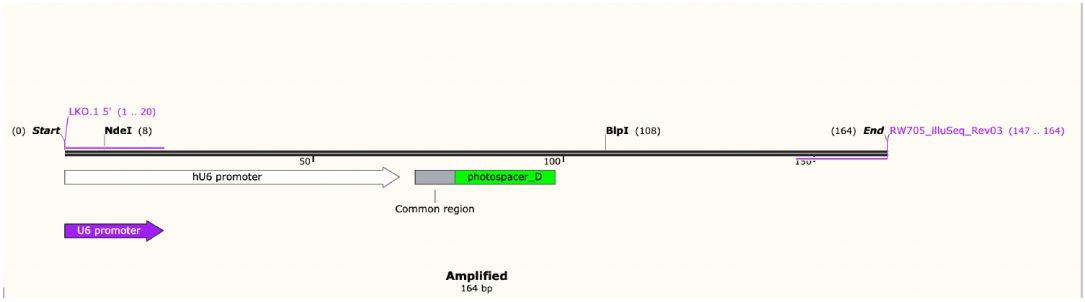

Fig:2 PCR to amplify protospacer B/D = LKO/U6 Fwd + RW705_illuSeq_Rev, the amplicon size 164 bp

	PRIMER	SEQUENCE
	mU6 -Fwd with Fwd overhang	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CA CAG CAC AAA AGG AAA CTC ACC
	RW704 illuSeq-Rev with Rev overhang	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCG GCC AAG TTG TAA ACG G
	LKO/U6 with Fwd overhang	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAC TAT CAT ATG CTT ACC GT
	RW705 illuSeq-Rev withReverse overhang	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GGC CAA GTT GAT AAC GGA

Table:1 PCR to amplify protospacer B/D = LKO/U6 Fwd + RW705_illuSeq_Rev, the amplicon size 164bp

BEFORE START INSTRUCTIONS

H9 dCAS9 CRISPRi were transduced with the pooled CRISPRi library to obtain the gDNA (genomic DNA) after lentiviral transduction.

CITATION

Renuka Ravi Gupta, Nona Farbehi, hendersa, Vikram Khurana, Gist Croft, Lorenz Studer, Joseph Powell. LENTIVIRAL TRANSDUCTION OF HUMAN PLURIPOTENT STEM CELLS. protocols.io.

LINK

https://protocols.io/view/lentiviral-transduction-of-human-pluripotent-stem-c9wtz7en

DNA Extraction

1 Collect cells after FACS sort in E8 flex media.

2 Centrifuge the cells at 300 g for 4 mins.

3 Aspirate the supernatant and freeze down the cell pellet or continue with DNA extraction using the QIAamp DNA Blood Mini Kit.

4 Measure the concentration of the DNA using nanodrop to check the quality of the DNA and using Qubit to get the precise concentration of the DNA

PCR amplification

5 PCR reactions are set up as follows:

- Template 50 ng pCRISPRi_dual guide gRNA library, 1:1 primers mU6 Fwd + RW704 illuSeq-Rev
- Template 50 ng pCRISPRi_dual guide gRNA library, 1:1 primers LKO/U6 Fwd + RW705 illuSeq-Rev

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- Inorder to ensure enough yield of DNA product we load 50 ng of DNA/ reaction for the PCR amplification for each run and set up at least 4 PCR reactions which will be pooled to ensure even distribution of the sgRNAs.
- $Volume = 50/conc$

	A	B	C
	REAGENT	1X in uL	FINAL CONCENTRATION
	2X KAPA HiFi HotStart Ready Mix	12.5	1X
	10uM Fwd Primer	0.75	0.3uM
	10uM Rev Primer	0.75	0.3uM
	Template DNA (50ng)	As required	As required
	PCR grade Water	Up to 25uL	N/A

Detailed protocol for PCR sample preparation can be found in this link: https://rochesequencingstore.com/wp-content/uploads/2023/02/KAPA-HiFi-HotStart-ReadyMix-PCR-Kit-Technical-Data-Sheet_v14-22.pdf

7 Set up the thermal cycler using the following program:
95ºC for 3 minutes
25 cycles of:
- 95ºC for 30 seconds
- 95ºC for 30 seconds
- 95ºC for 30 seconds

72ºC for 5 mins
Hold at 4ºC
Detailed protocol for PCR sample preparation can be found in this link: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

8 Pool the PCR products with similar forward and reverse primers together and send them to the sequencing platform.

9 QC for the final product was done by the sequencing platform by running the agarose gel to verify the required size of band followed by downstream sequencing.