gRNA Amplification and Next Generation Sequencing (NGS)

Note: If doing a positive selection screen, regular Sanger sequencing can be performed instead of NGS.

The representation of each gRNA is detected by next generation sequencing (NGS). The gRNA sequence integrated into the targeted cells genome can be amplified using common sequences flanking the hairpin. Indices are then added to each sample using the indexed primers (Appendix 1) and the pooled PCR product from all samples may be analyzed in parallel using NGS analysis. Index primers can be ordered from Eurofins or Integrated DNA Technologies.

Individual gRNA are amplified from experimental genomic DNA with two rounds of PCR. The primary PCR amplifies the gRNA and the flanking region. The secondary PCR uses nested primers to enrich for the primary PCR amplicons using modified primers adapted for NGS on an Illumina® sequencer.

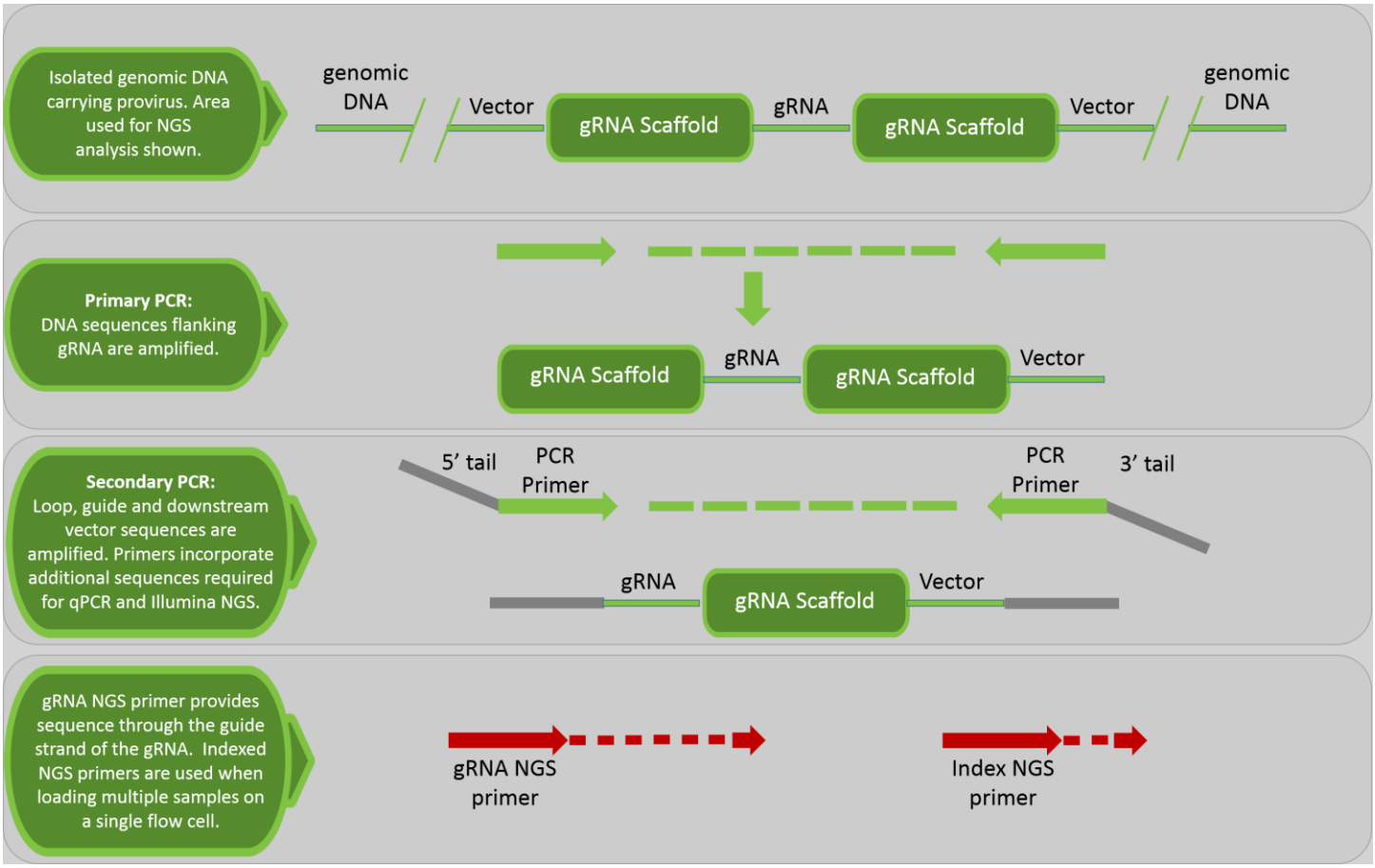

Figure 1. Schematic of PCR primers and sequencing primers as aligned with viral insert.

Primary PCR

Primary PCR reactions should be carried out with no more than ~850 ng of genomic DNA per 50 µl reaction; therefore, multiple PCR reactions will be required per sample. The combined PCR reactions for each sample should include all extracted genomic DNA to maintain representative of gRNA coverage used during the screen of the pool (500-1000 times the number of gRNA in the pool). See the example below for calculations. Primer sequences are listed in Appendix 1.

For example:
A pool containing 1500 gRNA used for screening with coverage of 1000 viral integrants per gRNA would require 9.9 µg of gDNA (genomic DNA) per sample be used for amplification.

- 1500(gRNA) x 1000(coverage) x 6.6^{-12} (g/diploid genome)= 9.9^{-6} grams gDNA (or 9.9µg)

Each 50 µl PCR reaction will contain 850 ng of gDNA.

- 9.9 µg (or 9900 ng) / 850 ng DNA = 11.6 reactions

Twelve PCR reactions per sample will be required for gDNA amplification from this pool.

Required materials

- KOD Hot Start Polymerase (Millipore Sigma Cat# 71086-4)
- 96 well PCR plates
- Agarose gel
- Qubit® dsDNA BR Assay or Quant-iT™ PicoGreen ® dsDNA Kits (recommended for their specificity to quantitate dsDNA in solution)
- PCR purification column (*QIAquick PCR Purification Kit, Qiagen Cat# 28104*)
- DNA gel extraction kit (*QIAquick Gel Extraction, Qiagen Cat# 28104)*
- PCR primers (Eurofins or Integrated DNA Technologies custom order, see Appendix 1)

Required equipment

- Qubit® fluorometer or standard spectrofluorometer with fluorescein excitation and emission wavelengths
- NGS platform

Primary PCR Protocol

Note: KOD Hot Start Polymerase, Millipore Sigma Cat# 71086-4 is recommended for PCR.

1. Based on the genomic DNA quantitation determine the total number of reactions required to amplify genomic DNA using no more than 850 ng of genomic DNA per 50 µl PCR reaction.
2. Set up PCR reactions using components and volumes outlined below for each sample. A 96-well PCR plate is recommended.

A master mix of all components (excluding genomic DNA) should be made and then added to wells containing the same volume and concentration of genomic DNA.

Components	µl per reaction
10X KOD Buffer	5
dNTP Mix (2 mM each)	5
$MgSO_4$ (25 mM)	4
Betaine (5 M)	5
Primary PCR Forward (10 µM)	1.7
Primary PCR Reverse (10 µM)	1.7
KOD Hot Start Polymerase	1.5
Genomic DNA (850 ng)	--
Nuclease free water	--
Total Volume	50

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
25	95	30 sec
	57	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, briefly centrifuge the 96 well plate containing PCR reactions.
6. Select at least 4 PCR reactions per sample and run 10 µl from each on an agarose gel to verify the **346 bp** amplicon.
7. Pool PCR reactions from each sample and purify following manufacturer's protocol ensuring that column capacity is not exceeded (it may be necessary to use multiple columns per sample for purification). *QIAquick PCR Purification Kit, Qiagen Cat# 28104 is recommended.*
8. Quantitate purified PCR reactions using a fluorometric assay specific for double stranded DNA (dsDNA).

Secondary PCR

Note: Each sample should be amplified using a uniquely indexed (6 base pair index) Secondary PCR Reverse primer if multiple samples are run in parallel on the sequencer. Primer sequences are listed in Appendix 1.

Protocol

1. 2.0 µg of primary PCR product should be amplified across 4 reactions (500 ng DNA per 50 µl PCR reaction) for each sample.
2. Set up PCR reactions using components and volumes outlined below for each sample. A 96 well plate is recommended.

   A master mix of all components should be made for each Secondary PCR Reverse- Indexed primer used (excluding DNA) which can then be added to wells containing the same volume and concentration of DNA.

Component	µl per reaction
10X KOD Buffer	5
dNTP Mix	5
$MgSO_4$ (25mM)	4
Betaine (5M)	2
Secondary PCR Forward (10 µM)	7.5
Secondary PCR Reverse(10 µM)- Indexed	7.5
KOD Hot Start Polymerase	1.5
Pooled Primary PCR DNA (500 ng)	--
Nuclease-free water	--
Total Volume	50

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
15	94	30 sec
	52	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, centrifuge the 96 well plate containing PCR reactions.
6. Pool PCR reactions from each sample.
7. Gel purify the **452 bp** product following manufacturer's recommendations from the kit used. Elute in 30 µl EB (or molecular-grade water).

   We recommend using QIAquick Gel Extraction, Qiagen Cat# 28104.

Preparing NGS adapted Amplicons for sequencing

After secondary PCR and purification, it is necessary to accurately quantitate each sample prior to NGS analysis. Quantitate purified PCR reactions using a fluorometric assay specific for double stranded DNA (dsDNA). Consult your sequencing facility for dilution and denaturing of libraries for NGS on a Illumina platform. It is recommended that you achieve 500-1000X reads relative to the number of constructs in your pool.

Sequencing NGS adapted Amplicons

An NGS core facility is recommended if assistance is needed for NGS sequencing and analysis. The instructions below describe changes (to standard Illumina NGS protocols) that are required to prepare and sequence gRNA amplicon libraries.

A 50 base pair, single read sequencing run is sufficient to sequence through the unique 20 bases of the gRNA and identify gRNA. Follow manufacturer protocols for preparing libraries (including denaturing, diluting and loading) and sequencer runs on an Illumina® sequencer.

Note the following when using Illumina kits:

- The custom gRNA Read 1 Primer is provided as 100 µM, dilute accordingly (typically 1:200 in HT1 buffer).
- Illumina® provided Read 1 and Read 2 Primer Mixes should not be used/selected for sequencing gRNA amplicon libraries.
- PhiX control should not be run with gRNA amplicon libraries as the custom gRNA Read 1 primer is not compatible with PhiX control.
- If samples were amplified using Secondary Indexed Reverse Primers, Illumina provided Index Primer Mix should be used/selected for index reads (indexes correspond to Illumina® TruSeq indices 2, 4, 5, 6, 7, 12)

Data Analysis

The custom gRNA Read 1 Primer binds to the scaffold of the gRNA (as shown in **Figure 1**) therefore, the first base read is the 5' end of the gRNA sequence. The 20 bases of the gRNA sequence can be used to identify the gRNA. Multiplexed samples should be separated and analyzed according to their index reads.

Analysis of NGS data from a gRNA screen is complex and can be difficult to evaluate without the proper experience or guidance. There are many web-based tools that can be used for alignment and analysis of NGS data; however, for optimal analysis with viable hit identification, it is recommended to have screen data analyzed by an expert in the field of NGS and bioinformatics. Once data has been analyzed and individual gRNA representation has been identified as statistically significant between control and experimental samples, secondary validation of these hits by gRNA should be performed for confirmation of hits. Individual gRNA constructs can be used for hit validation. In many cases, further validation can be performed from over expression as well.

Appendix 1: PCR and NGS sequencing primer sequences pCLIP-ALL and pCLIP-gRNA vectors

Primary NGS Forward (5'->3')
GTACCGAGGGCCTATTTCCCATG

Primary NGS Reverse (5'->3')
GACTCGGTGCCACTTTTTCAAGTTG

Crispr Secondary PCR Forward primer (5'->3')
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGCCTATTTCCCATGATTCC

CRSP-primary-F	CRSP-primary-F	gtaccgagggcctatttcccatg
CRSP-primary-R	CRSP-primary-R	gactcggtgccacttttcaagttg
CRISPR-Secondary-F	CRSPR-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGCCTATTTCCCATGATTCC
CRISPR-Secondary-R-Index 2	CRSPR-R Index #2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 4	CRSPR-R Index #4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 5	CRSPR-R Index #5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 6	CRSPR-R Index #6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 7	CRSPR-R Index #7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 12	CRSPR-R Index #12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
Custom Read 1 sequencing primer	CRSPR_NGS Read 1	CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

If low level multiplexing is needed (6 samples or less), please refer to *Multiplexing Sample preparation Guide (Illumina® Part# 1005361)* for recommendations on which sets of the indexes above can be used together.