

Illumina Sequencing Sample Preparation for use with CRISPRi/a-v2 Libraries

Overview

This protocol describes the four steps required for generating sequencing samples from cells harvested from screens conducted with the CRISPRi/a-v2 libraries:

1. Isolation of genomic DNA from fresh or frozen cell pellets
2. Restriction digest and gel extraction for enrichment of sgRNA-containing genomic DNA fragments (may be omitted for <15 million cell inputs)
3. PCR of sgRNA-containing fragments to amplify and append Illumina sequencing adapters
4. PCR clean-up and submitting for sequencing

Reagents, kits, and resources

- Machery Nagel Blood
 - Mini, L, or XL depending on number of cells; if XL, 1 column per 100 million cells
- SbfI-HF restriction enzyme (NEB)
 - expect to use at least 30uL of 20,000U/uL per 100 million cells
- Large gel box ex. OWL A1 Large Gel System (Thermo Scientific) and combs
- Machery Nagel gel extraction kit with vacuum manifold
- Phusion (NEB) and dNTPs
 - expect to use 5-20uL Phusion per 100 million cells, depending on yields from preceding steps
- Custom PCR and sequencing primers (sequences in protocol)
- SPRIselect beads (Beckman and Coulter; expensive but pre-made) or SeraMag speed beads (Thermo scientific; much cheaper but requires some preparation steps)
- Magnetic stand (ex, DynaMag from Life tech)
- Access to Bioanalyzer (Agilent) with DNA High Sensitivity chips and reagents
- Access to an Illumina sequencing facility; HiSeq 4000 or 2500 preferred

A word of warning on the sample prep

The sgRNA content of the sample up to the PCR step is at extremely low abundance compared to plasmid or post-PCR DNA! This makes the samples very susceptible to contamination, which can be further amplified in the PCR. We do all of the genomic extractions, digests, PCR set-up in a separate room to avoid contamination. You don't have to be that extreme, but do use a separate bench, set of pipettes, etc. from where you clone sgRNA plasmids or do the post-PCR clean-up step.

Step 1: gDNA isolation

We use Nucleospin Blood kits from Macherey Nagel sold in mini, midi(L), and maxi(XL) form.

- mini: 5×10^6 , midi: 2×10^7 ; maxi: 1×10^8

1a. For MN NucleoSpin Blood XL prep preheat water bath to 56C, and 70C

2b. Frozen Cells:

1. Thaw cells: RT or 37C
2. Resuspend cells in 25 mL of PBS or transfer cells to 50 mL falcon tube with 25 mL PBS.
3. Pellet cells. Spin @ 1300 rpm, 5 min

or

2c. Freshly Harvesting Cells

1. Spin @ 1200 rpm, 5 min, and remove supernatant.
2. Add 25 mL of PBS to 50 mL falcon tube. Transfer cells to 50 mL falcon tube.
3. Pellet cells. Spin @ 1300 rpm, 5 min

3. **Macherey Nagel (MN) NucleoSpin Blood** (follow NucleoSpin Blood protocol and guide below):

Number of cells*:	< 100M	< 200M	< 300 M
Resuspend in PBS	10 ml	20 ml	30 ml
Add ProK	500 ul	1,000 ul	1,500 ul
Add BQ1	10 ml	20 ml	30 ml
Add 100% EtOH	10 ml	20 ml	30 ml
Split among how many columns:	1	2	3

* Round up for highest recovery. 150M use 2 columns; 250M, use 3 columns...

Notes:

- After incubating cells at 56C, remember to cool lysed cells to RT before adding 100% EtOH. You can place tubes on ice for 5-10min.
- MN recommends spinning @ 4000x g; if your centrifuge cannot reach this, double spin times to maximize recovery
- Prior to eluting, preheat EB at 70C. Add 0.8 mL EB per column, incubate at RT 5 min, spin 4,000 rpm 4 min. Repeat. Recovery per column is roughly 1.2-1.6 mL.
- You should recover ~ 1-1.5 mg of gDNA per column (in general, 3~4.5mg yield from 250 million cells)

Step 2a: Size Fractionation of gDNA

The purpose of Step 2 (a-c) is to enrich for the sgRNA-containing fragments of genomic DNA so that the gDNA input into the PCR in Step 3 is reduced, thus requiring far fewer PCR reactions. The SbfI digest and gel isolation can theoretically enrich for sgRNAs >600-fold, but some material may be lost. Therefore, this step is optional but highly recommended for samples where a large amount of cells (generally greater than 15 million) have been processed.

Set-up digest in a 15mL falcon tube.

Entire volume of gDNA (mL)	Buffer 10X (uL)	Enzyme (Sbf1 HF)
1.6-4.8 mL (3~5mg)	f.c. 1X	~400U/mg

Digest overnight at 37C.

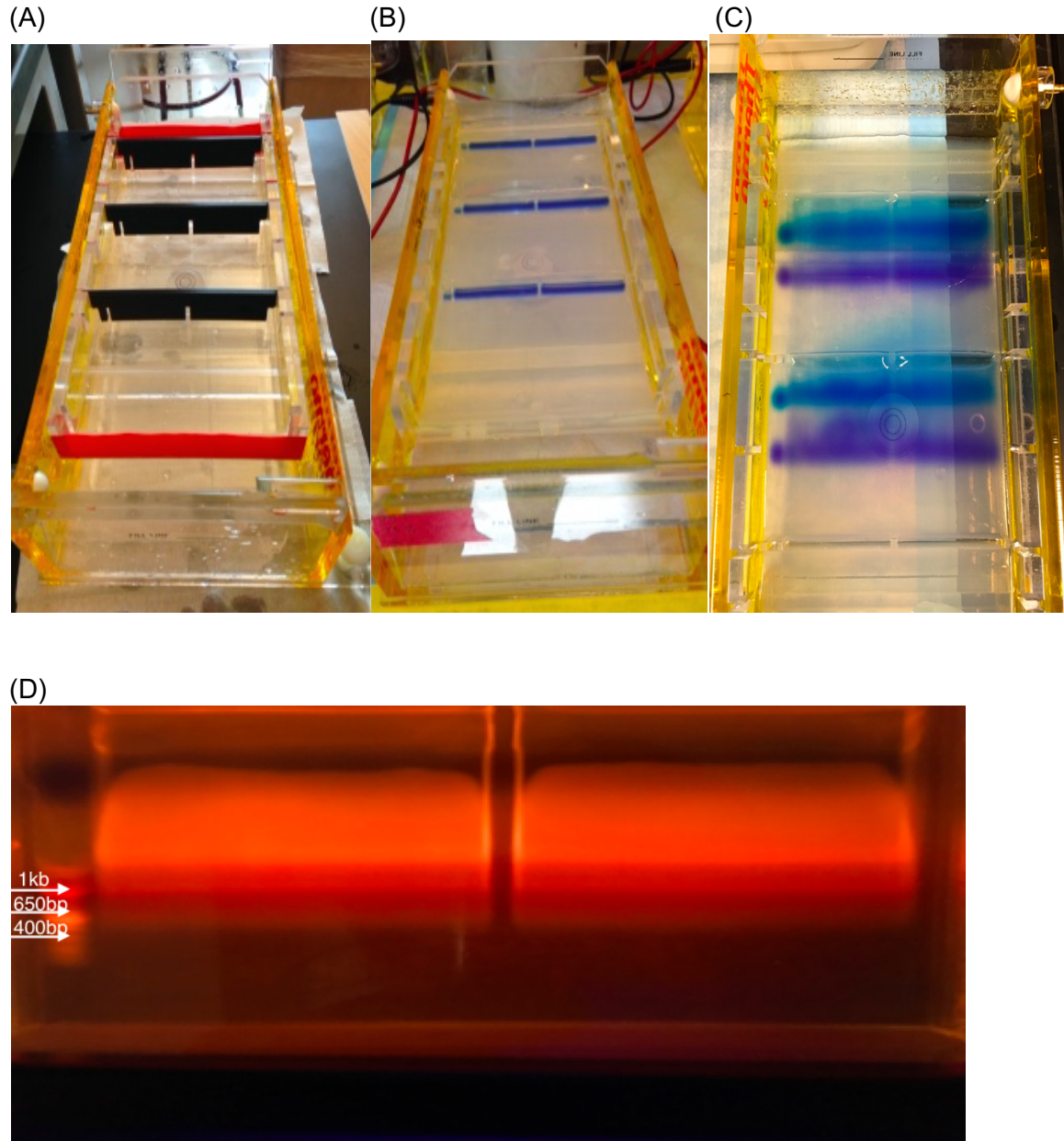
Step 2b: LARGE TAE gel

- **OWL A1 Large Gel System (Thermo Scientific), 37.5 x 21.5 x 11 cm, LxWxH (exterior)**
- **We 3D print our gel combs in Acrylonitrile-Butadiene-Styrene (ABS) plastic, and STL files for printing combs are included with this protocol**

The gel will take ~30 min to solidify in the cold room. Alternatively, you can pour the gel the night before and leave your gel in the cold room covered with foil.

Each large gel has six wells. You can load 1.2-1.5 mL of digest per well (I like loading 1.2 mL). At 1.5 mL you may have a little spill over between neighboring wells. If you're ok with spill over you can load replicates next to one another. At 300M cells, and with a ~5mL digest, you can load 1-2 samples per gel (one sample will need 3 wells). See image (B) below.

1. Clean large gel box and large combs (rinse REALLY well with water). Dry gel tray, and tape ends. See image (A) below.
2. Pour 400mL, 0.8%, 1X TAE agarose gel. Use large wells.
3. Fill chambers with 1X TAE. Keep wells DRY (Do not cover the entire gel with running buffer). Before loading samples, gently aspirate any TAE that may have entered the gel.
4. Load samples. [Final] of loading dye is 1X.
5. If some of your sample leaked into the ladder well remove it, and rinse once with running buffer. Add 25 uL of running buffer to well, then load 25 to 35 uL of 1KB plus (invitrogen) or 100 bp (NEB). Run sample at 150V, for ~1h-1h30m. This should run the purple dye front half way down the gel. See image (C) below.
6. Make size selection. Be sure to change blades between samples. I loaded 35 uL of NEB 100 bp ladder at 0.25 ug/uL, and my gel was stained with Sybrsafe.
For CRISPRi/a-v2 cut above and below 500 bp. I give myself a generous window and cut from 700 bp to 350 bp. See image (D). There is a line that runs in at 1Kb, and a fuzzy line that runs at 500 bp. You can use these lines as a guide. Aim to cut above and below the arrows marking 650 bp and 400 bp, erring on the side of cutting too wide.
7. Place excised band in a 50 mL Falcon tube
8. Weigh gel, and proceed with gel purification (my gel slices generally weigh 7.5-9 g).



Step 2c: Gel Purification (MN gel purification kit)

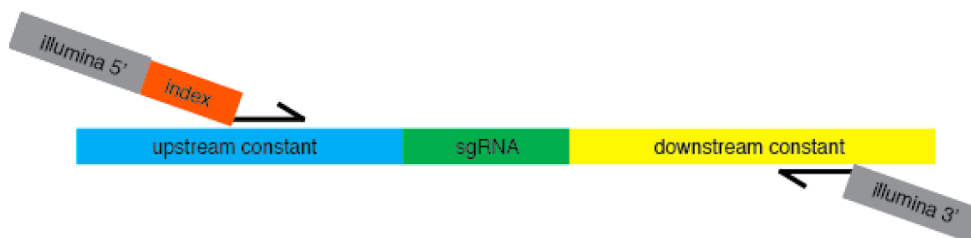
1. Add 2X NT1. Place in 56C water bath (5-10min), at RT (1h), or 4C (O/N)
2. Once gel has dissolved, add 1/100 3M NaOAc, pH 5.3
3. Load onto 1-4 columns (from initial cell count: ~100M cells per column) placed on a vacuum manifold; this may take a long time to load the full volume.
5. Follow MN protocol for washing columns
6. Heat Elution buffer at 70C. Add 20 μ L EB to column. Let sit for 5 min. Spin. Repeat.
7. Measure concentration by Nanodrop or Qubit, and place size selected and purified digest in the freezer or continue with indexing PCR

Step 3: PCR for sgRNA amplicon enrichment and Illumina adapter ligation

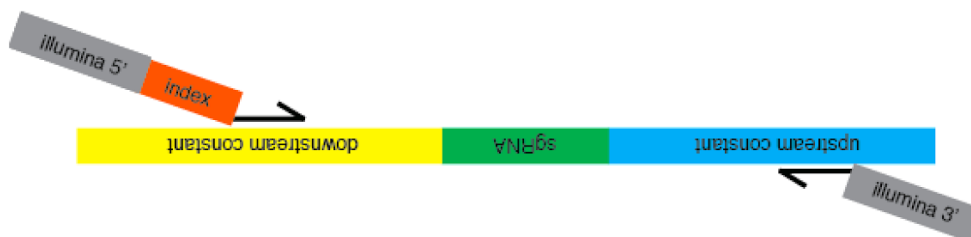
The PCR is designed to enrich (amplify) sgRNAs. During the enrichment, illumina 5' and illumina 3' adapters are added to the 3' and 5' end of the sgRNA cassette, and the index (barcode for de-multiplexing) is also added to one end. Below is a cartoon depicting the reaction, followed by sequences of our primers. We order the primers from IDT with standard desalting.

Preparation of sgRNA-containing inserts for Illumina sequencing (V4)

Primer Set A

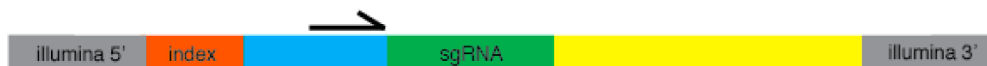


Primer Set B (switching the adapters of Set A)



Prepare roughly equal numbers of samples prepared with Set A and Set B, pool together, and submit for Illumina sequencing along with a mix of 5' and 3' sequencing primers:

Set A samples will be read by the 5' sequencing primer



Set B samples will be read by the 3' sequencing primer



This allows the sequencer to have over 25bp of diverse sequence to effectively call reads, and allows us to bioinformatically filter out any sgRNAs containing oligo synthesis errors in the sgRNA or constant region

Illumina Hiseq4000 compatible Primers (for PCR and sequencing)
Prepare roughly equal numbers of samples using Set A and Set B

Set A

Sample id	TruSeq ID	Paired End 5' Primer (orange indicates sample index for demultiplexing after sequencing)
1	12	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacCTTGTAgca caaaaggaaactcaccct
3	14	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacAGTTCCgca caaaaggaaactcaccct
5	3	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacTTAGGCgca caaaaggaaactcaccct

Common 3' Primer CAAGCAGAAGACGGCATACTGAGATCGACTCGGTGCCACTTTTTTC

Set B

Sample id	Truseq ID	Paired End 3' Primer
2	6	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacGCCAAT cgactcggtgccacttttttc
4	10	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacTAGCTT cgactcggtgccacttttttc
6	1	aatgatacggcgaccaccgagatctacacgatcggaagagcacacgtctgaactccagtcacATCACG cgactcggtgccacttttttc

Common 5' Primer CAAGCAGAAGACGGCATACTGAGATGCACAAAAGGAAACTCACCT

Submit pooled samples from both A and B for sequencing along with a mix of the following two primers:

5' Sequencing Primer GTGTGTTTTGAGACTATAAGTATCCCTTGGAGAACCACCTTGTTG

3' Sequencing Primer TGATAACGGACTAGCCTTATTTAACTTGCTATGCTGTTTCCAGCTTA

The index that is appended to sgRNA samples allows each sample to be uniquely identified, even when pooled into a single sequencing lane. Consult with your sequencing facility to find out how many reads to expect per lane, and plan for 250-500 reads per sgRNA per sample. For example, a typical genome-scale 200k library screen should have at least 50M reads per sample (e.g. T0, treated, untreated), and each should be PCR amplified such that they have a unique index. Before proceeding plan out how you want to pool/run your samples on the flow cell. If you are sharing a lane with another lab member ask them which indices they are using. If you are running fewer than 6 samples on a lane, use indices 12,6 or 12,6,14 or 12,6,14,10, etc, to ensure the indices are read correctly.

Sample id	Truseq id	Set	Index				
1	TruSeq Index 12 CTTGTA	A	CTTGTA	21	TruSeq Index 19 GTGAAA	A	GTGAAA
2	TruSeq Index 6 GCCAAT	B	GCCAAT	22	TruSeq Index 20 GTGGCC	B	GTGGCC
3	TruSeq Index 14 AGTTCC	A	AGTTCC	23	TruSeq Index 21 GTTTCG	A	GTTTCG
4	TruSeq Index 10 TAGCTT	B	TAGCTT	24	TruSeq Index 25 ACTGAT	B	ACTGAT
5	TruSeq Index 3 TTAGGC	A	TTAGGC				
6	TruSeq Index 1 ATCACG	B	ATCACG				
7	TruSeq Index 23 GAGTGG	A	GAGTGG				
8	TruSeq Index 13 AGTCAA	B	AGTCAA				
9	TruSeq Index 5 ACAGTG	A	ACAGTG				
10	TruSeq Index 4 TGACCA	B	TGACCA				
11	TruSeq Index 7 CAGATC	A	CAGATC				
12	TruSeq Index 11 GGCTAC	B	GGCTAC				
13	TruSeq Index 9 GATCAG	A	GATCAG				
14	TruSeq Index 2 CGATGT	B	CGATGT				
15	TruSeq Index 16 CCGTCC	A	CCGTCC				
16	TruSeq Index 18 GTCCGC	B	GTCCGC				
17	TruSeq Index 8 ACTTGA	A	ACTTGA				
18	TruSeq Index 22 CGTACG	B	CGTACG				
19	TruSeq Index 27 ATTCCT	A	ATTCCT				
20	TruSeq Index 15 ATGTCA	B	ATGTCA				

PCR

Expect to set up between 5-20 PCR reactions per 100M cell sample, depending on the yield of the gel extraction. The recommended input of 500ng gDNA per 100uL reaction can be adjusted, although higher inputs (>1ug) can lead to inhibition of the PCR reaction or higher background. If you find you will be setting up a very high number of PCRs based on the 500ng input, it's recommended that you process an unimportant stock of sgRNA library-containing cells for optimizing this step.

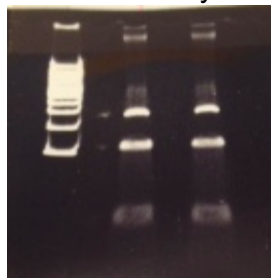
PCR Reaction	100 uL reaction (vol uL)
Water	bring final vol to 100 uL
5XBuffer	20
DMSO (100%)	3
P(Common) 100 uM	0.4
P(Index) 100 uM	0.4
dNTP (10 mM)	2
Phusion	1
gDNA	500 ng

PCR conditions

- 98°C, 30s
- 23 cycles
 - 98°C, 30s
 - 56°C, 15s
 - 72°C, 15s
- 72°C, 10min
- 7°C hold

After the PCR is completed, pool all your reactions for a given sample in a 5mL or 15 mL tube. Mix reaction well. All of the sgRNAs in the pool should now be represented at much greater abundance, so you may proceed with only a fraction of the sample for PCR purification. Remember that this material represents a significant contaminant to pre-PCR samples, so be sure to keep them separate.

You can directly test the PCR by running an aliquot on a 20% TBE gel with 100bp ladder:



Enriched PCR product @ ~274bp
Primer dimer @ ~150bp

Step 4a: PCR purification

There are several possible ways to remove the gDNA and primer dimer background from the PCR. The recommended method is listed below, but two other options are provided at the end of this protocol.

SPRI bead selection. Proceed with 100-300 uL of pooled PCR reaction

You will perform a double SPRI purification using SPRIselect Reagent (Beckman and Coulter) to select for your 270 bp sample. Double SPRI allows you to exclude anything above and below your target size. You will need: low retention/nonstick tubes, magnetic stand, 80% EtOH (make fresh), and Elution Buffer.

Some references if you are curious about how SPRI beads work:

Fisher S, *et al.*: A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol* 2011

DeAngelis MM, Wang DG, Hawkins TL: Solid-phase reversible immobilization for the isolation of PCR products. *Nucleic Acids Res* 1995

Lis JT. Size Fractionation of double stranded DNA by precipitation with polyethylene glycol. *Nucleic Acids Research* (1975)

You can also purchase Seramag speed beads (Thermo Scientific) if you want to make your own cost efficient SPRI beads: https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf

Amounts listed below are for 300uL of PCR reaction, but you can adjust volumes according to the input. Since you likely have far more sgRNA amplicon from the PCR than will be needed for sequencing, you can be conservative with the starting material for this step if you set up fewer than 6 PCRs in case an error occurs in the PCR clean-up.

1) Add 0.65X SPRI beads.

- 300 uL * 0.65= 195uL SPRIselect. At this ratio, fragments >300 will bind to the beads
- Mix well. You want a homogenous mixture. If you splash any of the mixture onto the sides of your tubes or cap spin down briefly.
- Incubate 10 min at RT
- Place tubes on magnetic stand for 5 min or until supernatant clears
 - I recommend Dyna mag 2 (Life Tech)
- Transfer the supernatant to a new tube. **KEEP supernatant.** Your sample is here.

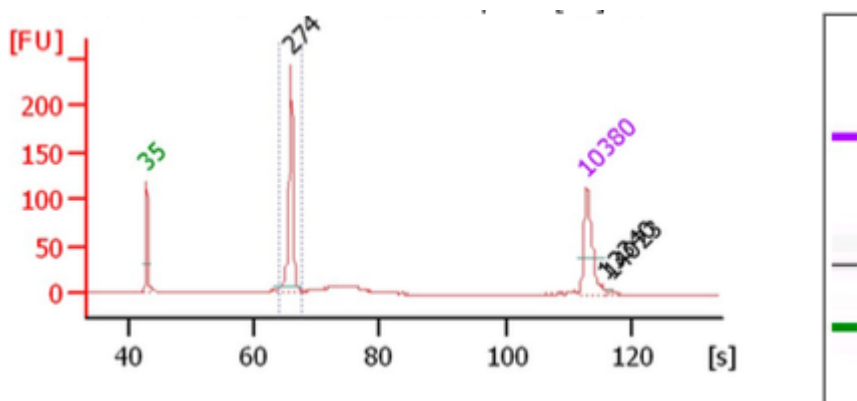
2) Add 1X SPRI beads

- 300 uL * 1= 300 uL SPRIselect. At this ratio, fragments >150 will bind to the beads.
 - Note this is based on the original PCR input volume, not the volume of the supernatant obtained from the first SPRI selection.
- Mix well and Spin tubes briefly.
- Incubate 10 min at RT
- Place tubes on magnetic stand for 5 min or until supernatant clears
- Remove supernatant. **KEEP beads.** Your sample is bound to the beads.
- Rinse beads with 1mL (make sure all you beads are covered) fresh 80% EtOH. Wait 2 min. Remove EtOH. Repeat for a total of 2 washes.
- Air Dry 5-15 min. The beads will look glossy after your remove the EtOH. The texture will turn from glossy (wet) to matte (dry).
- Once dry, elute in 20 uL of EB.

STEP 4b: Sequencing library QC and submission for sequencing

Once Your PCR Product is Purified:

- 1) Measure PCR clean-up yield by Nanodrop and/or Qubit, and dilute a small portion of your sample to 400 pg/uL based on this measurement.
- 2) Run samples on a Bioanalyzer (Agilent) with High Sensitivity DNA chip. The enriched PCR product is at 274bp, but the bioanalyzer peak will run in around 276bp-280bp. You can pool multiple samples together prior to analyzing, or analyze each sample in a separate channel to obtain a more accurate concentration of the sequencing sample prior to pooling.



Samples with higher background than shown here, in the form of a broad, low smear above or below the sgRNA amplicon, have been run without problems. Your sequencing facility may request these traces, and also request or perform additional QC in the form of qPCR.

- 3) Submit samples for sequencing, along with the mix of the two custom sequencing primers listed in the PCR section. The exact input volume and molar concentration will depend on the preferences of your sequencing facility. Our facility requests 15uL of 3-10nM (1-3ng/uL) pooled sample, with an additional 5uL for each lane.

The sequencing parameters are:

- **Run type: Single-end 50**
 - In the event you are sharing a sequencing run with other types of samples, longer sequencing formats will provide extraneous information but the sgRNA amplicons should be compatible
- **Index length: 1x6**
- **Sequencing primers: Read 1, custom primers; Read 2, standard Illumina index primer**
- **PhiX Spike-in: Depending on how evenly your samples are divided up into A and B sets, there should be sufficient diversity to request "standard 1% PhiX"; if the sequencing lane contains only A or B, there will be no diversity after the 19bp sgRNA protospacer is read, so request higher (up to 20%) spike-in**

Appendix. Other PCR Clean-up methods

Option 1: Proceed with 1mL of your pooled PCR reaction

- 1) Add: 5X vol PB and 1/100 vol 3M NaOAc, pH 5.3. Mix well.
- 2) Set up vacuum manifold. Use 1 column per 4mL of PB.
- 3) Wash 2X, PE and dry spin.
- 4) Elute 30 uL
- 5) Load 1ug-4ug (I loaded everything) on 20% TBE gel(0.5X). Run @200V, 1h
- 6) Stain gel with sybrsafe.
- 7) Prepare electroelution tubes:
 - a) label tubes (w/EtOH resistant water proof marker)
 - b) pre wet tubes, 5 min (fill tubes with 0.5X TBE)
 - c) decant
- 8) Cut out PCR band (270 bp). Use a new blade for each sample, or fill a beaker with EtOH, and clean blades between sample.
- 9) Place PCR band in prewet electroelution tubes, and fill tubes with 0.5X TBE. Make sure tubes are tightly capped. Check for air bubbles.
- 10) Place tubes in a clean gel box. There is a golden rack for midi tubes, but micro tubes will need to be taped.
- 11) Fill chamber with new 0.5X TBE, and run @160V for 90 min. Reverse current for 2 min.
- 12) Your PCR should be in the medium. Remove ALL the medium. You will not recover your entire volume as the gel absorbs some of the TBE.
- 13) Add 3.2mL PB, 40uL NaOAc. Mix well. Apply to gel extraction columns. 2, PE washes, and 1 dry spin. Elute in 35uL EB.

Option 2: Rapid Extraction

1. Follow Steps 1-6 from Option 1 (listed above)
2. While gel is running prepare tubes and tips for rapid extraction. Pierce the bottom (tip) of a 0.5 ml nonstick tube with an 18.5 gauge needle and place it inside a 1.5 ml nonstick tube. Use a clean blade or clean scissors to cut the tips off of p1000 pipette tips.
3. Excise 264 bp PCR product, and place excised gel piece inside 0.5 mL tube
4. Spin tubes at 20,000 x g for 3 min. This will force the gel through the hole, and gel will crush into tiny pieces. Check for gel pieces in small tube. Residual pieces should be transferred to large tube. If you cannot tap the residual pieces use a pipette tip.

5. Add 200 μ L of water to gel pieces, and incubate for 10 min at 70°C
6. Vortex gel slurry for 30s and use pre-cut p1000 tips to transfer gel mixture to Costar Spin-X columns.
7. Spin tubes for 3 min at 20,000 x g to recover the elution mixture free of gel debris.
8. Transfer eluate to a new 1.5 mL nonstick tube
9. Isopropanol Precipitation
 - a. 200 μ L of eluate (insert is here)
 - b. 1.5 μ L-2 μ L of glycoblue (this will help you visualize your pellet)
 - c. 25 μ L 3M NaOAc or 3M NaCl
- i. Mix Well (invert 10-15X)
10. Add 0.75 mL Isopropanol, mix well
11. -30°C, 30 min to 18h or -80°C, 30 min
12. Pellet: Spin 30 min at 20,000 x g at 4°C , remove supernatant
13. Wash pellet 2X with ice cold 80% EtOH
14. Air Dry
15. Resuspend pellet in 15 μ L water or Qiagen EB