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 x $10^{6,}$, midi: 2 x 10^{7}; maxi: 1 x 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.

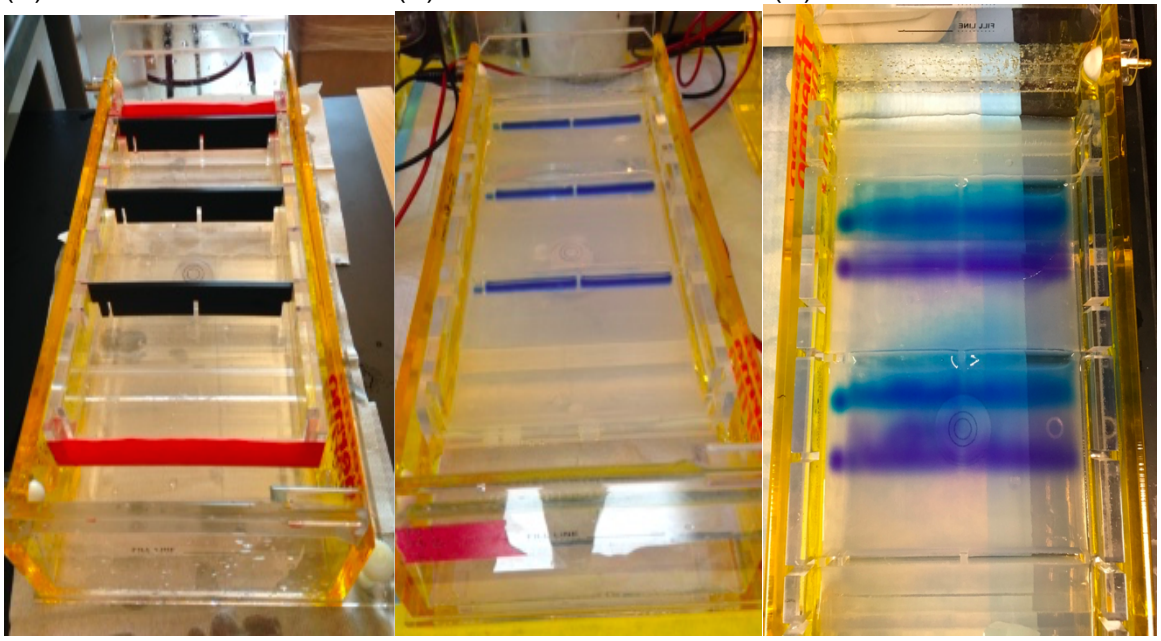
4. Load samples. [Final] of loading dye is 1X.

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.

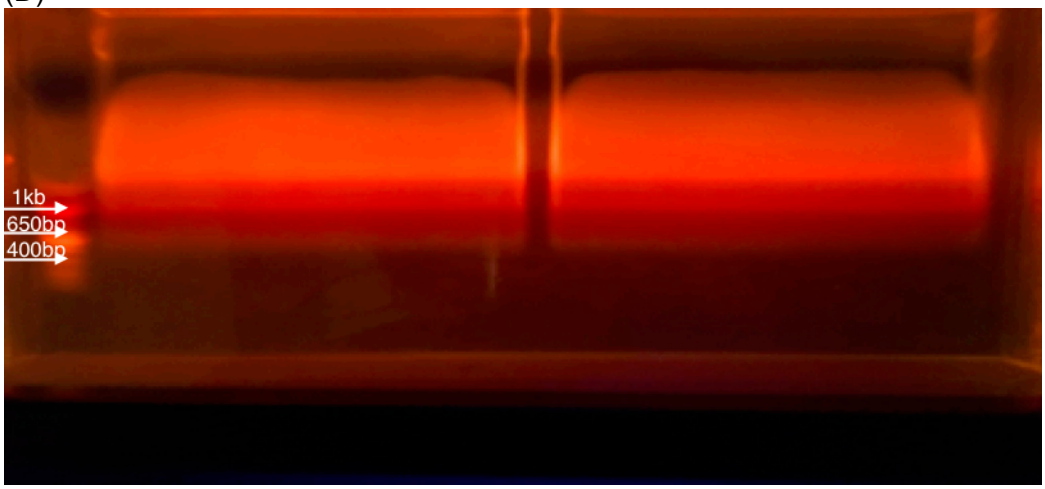
6. Make size selection. Be sure to change blades between samples. I loaded 35 uL of NEB 100 bp ladder at 0.25 ug/uL, and my gel was stained with Sybrsafe.
For CRISPRi/a-v2 cut above and below 500 bp. I give myself a generous window and cut from 700 bp to 350 bp. See image (D). There is a line that runs 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).

(A) (B) (C)



(D)



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 uL 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.

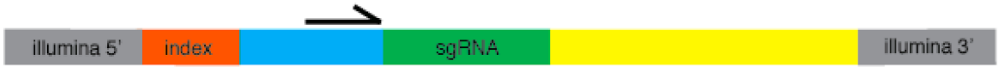
Preparation of sgRNA-containing inserts for Illumina sequencing (V4)

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