

Illumina Sequencing Sample Preparation for use with CRISPRi/a-v2 Libraries **Version 4, 2019/02/26 (originally created by Max Horlbeck and Chong Park, updated by Christina Liem and Marco Jost)**

Overview

This protocol describes the three 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. PCR of sgRNA-containing fragments to amplify and append Illumina sequencing adapters
3. PCR clean-up and submitting for sequencing

Note: Previous versions of this protocol included an additional gel extraction step between steps 1 and 2. With newer PCR products capable of handling large amounts of genomic DNA inputs, this step can now be omitted, at the cost of setting up ~100 PCRs per sample. A version of the protocol including gel extractions is available at:

https://weissman.wi.mit.edu/resources/IlluminaSequencingSamplePrep_old.pdf

Reagents, kits, and resources

- Macherey Nagel Blood
 - Mini, L, or XL depending on number of cells; if XL, 1 column per 100 million cells
- NEBNext Ultra II Q5 MasterMix (NEB)
 - expect to use ~1 kit of 250 reactions per 100 million cells, depending on yields from preceding steps
- Custom PCR and sequencing primers (sequences in protocol)
- SPRIselect beads (BeckmanCoulter; expensive but pre-made) or Seramag speed beads (Thermo scientific; much cheaper but requires some preparation steps)
- Magnetic stand
- 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 @ 400 x g, 5 min

or

2c. Freshly Harvesting Cells

1. Spin @ 400 x g, 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 @ 400 x g, 5 min

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

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 but ideally leave them to cool at room temperature for ~60 min.
- 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

Step 2: 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 (V5)

PCR amplification of insert:



Submit for Illumina sequencing (SE50) with the sample-specific sequencing primer:



Notes: This protocol is optimized for sequencing on the Illumina HiSeq4000. If you are using other sequencers, consult your core facility or test the requirements for sequence diversity.

Previous versions of this protocol included two different primer sets for the PCR, set A and set B, which generated two different products that were sequenced in opposite orientations. This strategy had been implemented to increase sequence diversity during the sequencing run. However, **with recent updates to Illumina flow cell chemistry and software, the HiSeq4000 is more robust to low-diversity regions and thus it is feasible to sequence all samples in a single orientation with a 5-10% phiX spike-in. We now recommend sequencing all samples in a single orientation using a 10% phiX spike-in.**

Illumina Hiseq4000 compatible Primers (for PCR and sequencing)

5' Primers with TruSeq indices:

Sample id	TruSeq ID	5' PCR Primer (orange indicates sample index for demultiplexing after sequencing)
1	12	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacCTTGTAgca caaaggaaactcacct
2	6	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacGCCAATgca caaaggaaactcacct
3	14	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacAGTTCCgca caaaggaaactcacct
4	10	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacTAGCTTgca caaaggaaactcacct
5	3	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacTTAGGCgca caaaggaaactcacct
6	1	aatgatacggcgaccaccgagatctacacgatcgggaagagcacacgtctgaactccagtcacATCACGgca caaaggaaactcacct

Common 3' PCR Primer CAAGCAGAAGACGGCATACGAGATCGACTCGGTGCCACTTTTTTC

Submit pooled samples for sequencing along with the following sequencing primer:

GTGTGTTTTGAGACTATAAGTATCCCTTGGAGAACCACCTTGTG

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

PCR

Expect to set up between 50 and 200 PCRs per 100M cell sample, depending on the yield of the genomic DNA extraction. The recommended input of 10 ug gDNA per 100 uL reaction can be adjusted, although higher inputs can lead to inhibition of the PCR or uneven amplification. If you find you will be setting up a very high number of PCRs based on the 10 ug input, it is recommended that you process an unimportant stock of sgRNA library-containing cells to optimize this step.

PCR parameters

Reagent	uL per 100 uL reaction
Common primer (100 uM)	1
Index primer (100 uM)	1
NEBNext Ultra II Q5 MasterMix	50
gDNA	10 ug
Water	bring to final volume of 100 uL

Protocol

- 1) Measure gDNA concentration and determine # of reactions to set up (10 ug per PCR)
- 2) Transfer gDNA into a 5 mL Eppendorf or a 15 mL Falcon tube (should have ~1.2-1.6 mL from one gDNA extraction column).
- 3) Add corresponding common and indexing primers (1 uL per reaction).
- 4) Bring to half the final volume with water.
- 5) Add corresponding volume of 2x PCR MasterMix.
- 6) Transfer into a clean, segmented multichannel reservoir and then transfer into a 96-well PCR plate using a multichannel pipette.
- 7) Run PCR using cycling conditions below.

PCR conditions

- 98 °C, 30 s
- 22-24 cycles (see note below)
 - 98 °C, 10 s
 - 65 °C, 75 s
- 65 °C, 5 min
- 4 °C hold

Note on number of cycles: empirically, 22 cycles produce sufficient amount of product. To be absolutely certain that enough PCR product will be obtained, cycle number can be increased to 23 or 24, with the caveat that each additional cycle may induce non-linearities.

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 be represented at much greater abundance than, 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.

Step 3a: 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 in Appendix 2.

Note: with this protocol there will be a large amount of primers in the PCR. If you see a substantial amount of primer in the BioAnalyzer, consider running a second round of purification to remove the excess primer.

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

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.

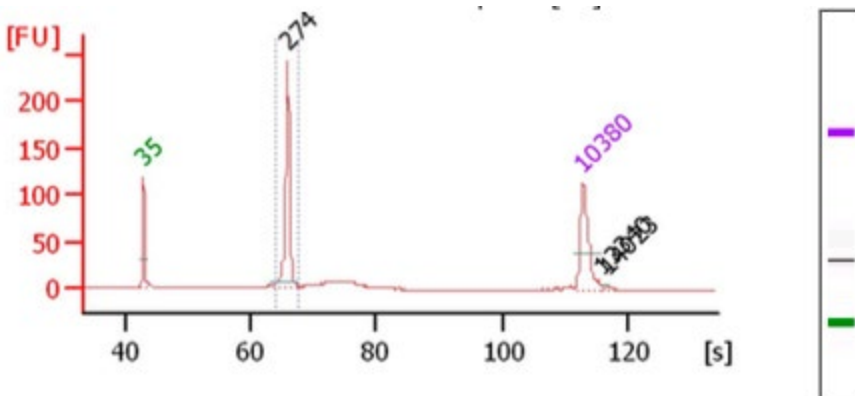
- Air Dry 5-15 min. The beads will look glossy after you remove the EtOH. The texture will turn from glossy (wet) to matte (dry).
- Once dry, elute in 20 uL of EB.

STEP 3b: 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 custom sequencing primer 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 primer; Read 2, standard Illumina index primer**
- **PhiX Spike-in: Request 5-10% phiX (we recommend 10%) to ensure robust read calling.**

Appendix 1. Detailed genomic DNA extraction protocol

Materials per sample (up to 100 M cells)

- 1 column
- 500 uL proteinase K
- 10 mL BQ1
- 10 mL ethanol (+ extra to make BQ2)
- 15 mL BQ2
- 1.6 mL EB

Preparation:

- Preheat water baths to 56C and 70C
- Resuspend proteinase K in proteinase buffer PB (for NucleoSpin Blood XL: 5.75 mL per tube)
- add ethanol to buffer BQ2 concentrate
- heat buffer EB to 70C
- label 1 falcon tube, 1 column tube, 1 collection tube for each sample

Step-by-step protocol:

1. Thaw cells: RT (or 37C)
2. Transfer cells to 50 mL falcon tube with 25 mL PBS.
3. Pellet cells. Spin @ 1300 rpm (~400 x g), 5 min.
4. Resuspend each pellet in 10 mL PBS.
5. Add 500 uL proteinase K to each tube.
6. Add 10 mL BQ1 to each tube. Mix by vortexing vigorously (or by inverting 15 times).
7. Incubate at 56C for 15 min.
8. Cool to RT for at least 30 min. **THIS IS CRITICAL**
9. Add 10 mL 100% EtOH to each tube, mix by vortexing vigorously (or by inverting 15 times).
10. For each lysate, load 15 mL onto one column.
11. Spin 3 min at 4000 x g, discard flow-through. (or keep in tube)
12. Load remaining lysate onto column.
13. Spin 3 min at 4000 x g, discard flow-through.
14. Add 7.5 mL BQ2 to each column.
15. Spin 2 min at 4000 x g.
16. Add 7.5 mL BQ2 to each column.
17. Spin 10 min at 4000 x g.
18. Move column to a new collection tube.
19. Add 800 uL pre-heated buffer BE to each column.
20. Incubate at RT 5 min, then spin 4 min at 4000 x g.

21. Repeat steps 19+20.

22. Measure DNA concentrations (transfer 2 uL to a fresh tube, measure on NanoDrop).

Appendix 2. 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 uL 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 uL of eluate (insert is here)
 - b. 1.5 uL-2uL of glycoblu (this will help you visualize your pellet)
 - c. 25 uL 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 uL water or Qiagen EB