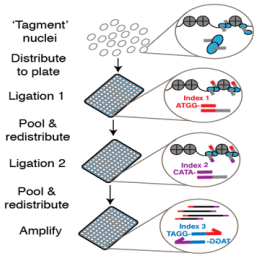
Nov 01, 2020

sci-ATAC-seq3

DOI
dx.doi.org/10.17504/protocols.io.be8mjhu6



Silvia Domcke[1], Andrew J. Hill[1], Riza M. Daza[1], Cole Trapnell[1,2], Darren A. Cusanovich[1,3,4], Jay Shendure[1,2,5,6]

[1]Department of Genome Sciences, University of Washington School of Medicine, Seattle, WA, USA;
[2]Brotman Baty Institute for Precision Medicine, Seattle, WA, USA;
[3]Department of Cellular and Molecular Medicine, University of Arizona, Tucson, AZ, USA;
[4]Asthma and Airway Disease Research Center, University of Arizona, Tucson, AZ, USA;
[5]Allen Discovery Center for Cell Lineage Tracing, Seattle, WA, USA;
[6]Howard Hughes Medical Institute, Seattle, WA, USA

Human Cell Atlas Metho...

Silvia Domcke

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.be8mjhu6

Protocol Citation: Silvia Domcke, Andrew J. Hill, Riza M. Daza, Cole Trapnell, Darren A. Cusanovich, Jay Shendure 2020. sci-ATAC-seq3. **protocols.io https://dx.doi.org/10.17504/protocols.io.be8mjhu6**

Protocol status: Working

Created: April 16, 2020

Last Modified: November 01, 2020

Protocol Integer ID: 35821

Keywords: single cell ATAC-seq, single cell, chromatin accessibility, sci-ATAC-seq, sci-ATAC-seq3, combinatorial indexing, sci, ATAC,

Abstract

We developed an improved assay for single cell profiling of chromatin accessibility that both uses three levels of combinatorial indexing and, in contrast with previous iterations of sci-ATAC-seq and related methods, does not rely on molecularly barcoded Tn5 complexes (sci-ATAC-seq3). Rather, the first two rounds of indexing are achieved by ligation to either end of the conventional, uniformly loaded Tn5 transposase complex (standard Nextera™), while the final round of indexing remains through PCR. Relative to two-level sci-ATAC-seq but similar to sci-RNA-seq3, sci-ATAC-seq3 reduces the per-cell cost of library preparation as well as the rate of collisions, opening the door to experiments on the scale of 10^6 cells. This protocol no longer requires cell sorting, and we also optimized ligase and polymerase choice, kinase concentration, and oligo designs and concentrations, to maximize the number of fragments recovered from each cell. Of note, while maintaining an enrichment in accessible regions, we made the explicit choice to maximize complexity at the expense of specificity for accessible sites. In particular, we found that the fixation conditions could be tuned to adjust the sensitivity (i.e. complexity) vs. specificity (i.e. enrichment in accessible sites) of the assay.

Guidelines

The protocol workflow is as follows:

1. Reagent preparation
2. Nuclei isolation and fixation from cell lines
3. Tissue pulverization, nuclei isolation and fixation from frozen tissues (steps 3-4)
4. sci-ATAC-seq3 library construction, QC and sequencing (steps 5-13)

Oligo and primer sequences:

- Order the barcoded oligos (see Table S7 from Domcke, Hill, Daza et al. 2020) (96-well plate format, 25 nmole DNA Oligo, standard desalting, resuspended at 100 uM in nuclease-free water, full yield) and store at -20°C until further use. Dilute oligos to 50 uM in nuclease-free water and store at -20°C.

- Order the sequencing oligos from Table S7 from Domcke, Hill, Daza et al. 2020 (standard desalted), resuspend at 100 uM in nuclease-free water and store at -20°C.

Required equipment:

Tissue culture hood
Tissue culture incubator
Freezer (-20°C, -80°C) and refrigerator (4°C)
Eppendorf Mastercycler (thermal cycler)
FACSAria III cell sorter (BD)
Microscope
Bright-Line™ Hemacytometer (Sigma)
Centrifuge (cooled to 4°C) (Eppendorf, 5810R)
Agilent 4200 TapeStation System
Gel imager
NextSeq 500 platform (Illumina)
Liquid nitrogen tank for sample storage
DynaMag™-96 Side Skirted Magnet (Thermo Fisher Scientific, 12027)
Gel box
Ice bucket
Multi-channel pipettes (10ul, 200ul) (Rainin Instrument)
Rainin Liquidator 96 Manual Pipetting System
Pipettors

Materials

0.5 M EDTA (Thermo Fisher Scientific, AM9260G)
100 bp ladder (New England Biolabs (NEB), N3231L)
1000X Sybr (Invitrogen (Gibco/BRL Life Tech), S7563)
10mM ATP (New England Biolabs (NEB), PO756S)
10X HBSS (Gibco/BRL Life Tech, 14065-056)
10X PNK Buffer (New England Biolabs (NEB), M0201L)
1M MgCl2 (Thermo Fisher Scientific, AM9530G)
1X DPBS (Thermo Fisher Scientific, 14190-144)
5% Digitonin (Thermo Fisher Scientific, BN2006)
5M NaCl (Thermo Fisher Scientific, AM9759)
6% TBE PAGE (Invitrogen (Gibco/BRL Life Tech), EC6265BOX)
6x Orange dye (New England Biolabs (NEB), B7022S)
AMPure Beads (Beckman Coulter, A63882)
BSA, Molecular Biology Grade (New England Biolabs (NEB), B9000S)
DNA LoBind Tube 1.5 ml, PCR clean (Eppendorf North America, 22431021)
DL-Dithiothreitol, 1M 10 × 0.5ML (Sigma Aldrich, 64563-10x.5ML)
EB Buffer (Qiagen, 19086)
Falcon Tubes, 15 ml (VWR Scientific, 21008-936)
Falcon Tubes, 50 ml (VWR Scientific, 21008-940)
Falcon® 5mL Round Bottom w/ Cell Strainer (Fisher Scientific, 352235)
Green pack LTS 200ul filter tips (GP-L200F) (Rainin Instrument, 17002428)
Green pack LTS 20ul filter tips (GP-L20F) (Rainin Instrument, 17002429)
Glycerol (Sigma Aldrich, G5516-500ML)
Glycine (Sigma Aldrich, 50046-250G)
IGEPAL CA-630 (Sigma Aldrich, I8896-50ML)
Liquidator tips - 10 ul (Rainin Instrument, 17011117)
Liquidator tips - 200 ul (Rainin Instrument, 17010646)
LoBind clear, 96-well PCR Plate (Eppendorf North America, 30129512)
Low-Profile 0.2 ml 8-tube white tube w/o cap (Bio-rad Laboratories, TLS0851)
Magnesium acetate tetrahydrate (Sigma Aldrich, M5661-50G)
Microseal ‘B’ Adhesive seal (Bio-Rad Laboratories, MSB1001)
Nalgene MF 75 Sterilization Filter Unit, 0.2 um – 250 ml (VWR, 28199-112)
Nalgene MF 75 Sterilization Filter Unit, 0.2 um – 500 ml (VWR, 28198-505)
NEBNext Hi-Fidelity master mix (2x) (New England Biolabs (NEB), M0541L)
NextSeq 500 High Output kit (150 cycle) (Illumina Inc., FC-404-2002)
Non-woven gauze (Dukal, 6114)
Nuclease-free water (Thermo Fisher Scientific, AM9937)
Optical flat 8-cap strips (Bio-Rad Laboratories, TCS-0803)
Protease Inhibitors (Sigma Aldrich, P8340-1 ml)
RT-L250WS wide-orifice LTS 250 ul (Rainin Instrument, 30389249)
Reagent reservoirs (Fisher Scientific, 07-200-127)

Spermidine (Sigma Aldrich, S2626-1G)
Sybr gold (Invitrogen (Gibco/BRL Life Tech), S-11494)
Steriflip, Disposable Vacuum Filter Unit, 0.22 um pore (Fisher Scientific, SCGP00525)
T4 PNK (New England Biolabs (NEB), M0201L)
T7 Ligase (New England Biolabs (NEB), M0318L)
T7 Ligase Buffer (New England Biolabs (NEB), M0318L)
Tapestation (D5000 reagent) (Agilent Technologies, 5067-5589)
Tapestation (screentape) (Agilent Technologies, 5067-5588)
TD Buffer (2x) (Illumina Inc., FC-121-1031)
TDE1 (Tn5) (Illumina Inc., FC-121-1031)
Tris-HCl pH 7.5 (1M) (Thermo Fisher Scientific, 15567027)
Tween-20 (Thermo Fisher Scientific, BP337-500)
UltraPure Distilled Water (DNAse, RNAse, Free) (Thermo Fisher Scientific, 10977023)
DNA Clean and Concentrate (DCC-5) (Zymo Research, D4014)

Reagent preparation (30-45 mins)

1

- ATAC-RSB buffer (Corces et al. Nature Methods 2017 PMCID: PMC5623106)

In a 50 ml falcon tube, combine 500 ul 1M Tris-HCl pH 7.4 (10 mM Tris-HCl final), 100 ul 5M NaCl (10 mM NaCl final), 300 ul 0.5M MgCl2 (3 mM MgCl2 final) and 49.1 ml nuclease free water. Filter sterilize by using Millipore "Steriflip" Sterile Disposable Vacuum Filter Unit, PES membrane; Pore size: 0.22 um (SCGP00525). Store buffer at 4°C for up to 6 months.

- Make 10% Tween-20 in nuclease free water and filter sterilize by using Millipore "Steriflip" Sterile Disposable Vacuum Filter Unit, PES membrane; Pore size: 0.22 um (SCGP00525). Store buffer at 4°C for up to 6 months.

- Make 10% IGEPAL CA-630 in nuclease free water and filter sterilize by using Millipore "Steriflip" Sterile Disposable Vacuum Filter Unit, PES membrane; Pore size: 0.22 um (SCGP00525). Store buffer at 4°C for up to 6 months.

- Dilute 5% digitonin to 1% with nuclease-free water, store at 4°C for up to 6 months

- Freezing buffer (FB) (Saunders et al. Genes Dev 2013 PMCID: PMC3672648)

In a 50 ml falcon tube, combine 50 mM Tris at pH 8.0, 25% glycerol, 5 mM Mg(OAc)2, 0.1 mM EDTA, and nuclease free water. Filter sterilize by using Millipore "Steriflip" Sterile Disposable Vacuum Filter Unit, PES membrane; Pore size: 0.22 um (SCGP00525). Store buffer at 4°C for up to 6 months. On the day of nuclei isolation, mix 975 ul of FB, 5 ul 5 mM DTT (Sigma-Aldrich cat. no. 646563-10X0.5ml) and 20 ul 50× protease inhibitor cocktail (Sigma-Aldrich cat. No. P8340).

- 2.5 M glycine

Make 2.5 M glycine, combine 46.92 g of glycine in 250 ml of water, then filter sterilize (Nalgene filtration system, 0.2 um cellulose nitrate membrane (VWR, 28199-112). Store reagent at room temperature for up to 6 months.

- 40 mM EDTA

Make 40 mM EDTA from 0.5 M EDTA stock (Invitrogen, AM9262) with water, then filter sterilize (VWR, 28198-505). Store reagent at room temperature for up to 6 months.

On the day of experiment prepare the following fresh and keep on ice:

- Omni-ATAC lysis buffer (ATAC-RSB, 0.1% NP40, 0.1% Tween-20 and 0.01% Digitonin)

- ATAC-RSB with 0.1% Tween-20

Nuclei isolation and fixation of cell lines (~2 hours for 4 cell lines)

2 Before starting, prepare Omni-ATAC lysis buffer and ATAC-RSB with 0.1% Tween-20.

Note

GM12878 cells were cultured and maintained in RPMI 1640 medium (Thermo Fisher Scientific cat. no. 11875-093) with 15% FBS (Thermo Fisher cat. no. SH30071.03) and 1% Pen-strep (Penicillin and Streptomycin, Thermo Fisher cat. no 15140122). They were counted and split at 300,000 cells/ml three times a week.

CH12-LX murine cell line was gifted by Michael Snyder's lab (Stanford). The cells were cultured in RPMI 1640 medium with 10% FBS, 1% Pen-strep and 10 uM beta-mercaptoethanol. They were counted and maintained at a density of 100,000 cells/ml, splitting three times a week to maintain cell concentration.

Both cell lines were incubated at 37°C with 5% CO2.

- Pellet ~10-100 million cells by centifugation at 500 x g for 5 min at room temperature.
- Aspirate supernatant, resuspend pellet in 1 ml Omni-ATAC lysis buffer and incubate on ice for 3 min.
- Add 5 ml of ATAC-RSB with 0.1% Tween-20 and pellet nuclei for 5 min at 500 x g at 4°C.
- Aspirate supernatant and resuspend nuclei in 5 ml 1X DPBS (Thermo Fisher cat. no. 14190144).
- To crosslink the nuclei, add 140 ul of 37% formaldehyde (VWR cat. no. MK501602) in one shot for a final concentration of 1%.
- Incubate the fixation mixture at room temperature for 10 minute inverting every 1-2 min.
- To quench the crosslinking reaction, add 250 ul of 2.5 M glycine and incubate at room temperature for 5 minutes and then on ice for 15 minutes to stop cross-linking completely.
- Take 20 ul of the quenched crosslinked mixture and combine with 20 ul of trypan blue for counting on a hemocytometer.
- Spin crosslinked nuclei at 500 x g for 5 minutes at 4°C and aspirate the supernatant.
- Resuspend fixed nuclei in appropriate amount of freezing buffer to obtain 2 million nuclei per 1 ml aliquots, snap freeze in liquid nitrogen and store at -80°C.

Note

This is a good stopping point. All samples can be stored at -80°C until further processing.

Tissue pulverization, nuclei isolation and fixation of frozen tissues (~3 hours for 6 tissues)

3 Before starting, label 2 tubes per tissue sample. The number of tubes may vary from tissue to tissue depending on size and weight of tissue.

Note

Tissues were rinsed in 1X HBSS pH 7.4 (with Ca2+ and Mg2+, no phenol red (Gibco BRL (500 ml) 14065-056) then blotted dry on semi-damp non-woven gauze (Dukal # 6114). Dried tissues were placed on heavy duty foil (Fisher Scientific, NC19180132) or in cryotube. Tissues were snap-frozen in liquid nitrogen and stored at -80°C.

Note: cryotubes can create “frost” of water crystals inside the tube due to trapped air/moisture during the snap-freeze process.

- On the day of pulverization, cool pre-labeled tubes and hammer on dry ice beforehand with a cloth towel between the dry ice and metal.
- Create tin foil “padding” by taking an 18” x 18” heavy duty foil, fold in half twice creating a rectangle. Fold twice more to create a square.
- Place frozen tissue inside the foil “padding” then place padded tissue inside a pre-chilled 4 mm plastic bag to prevent tissue from falling out onto the dry ice in case the foil should rupture.
- Chill this tissue packet between 2 slabs of dry ice.
- Using the pre-chilled hammer, manually pulverize the tissue inside the packet; 3 to 5 impacts avoiding a grinding motion before taking a break to avoid heating the sample.
- Chill the hammer and repeat pulverization as needed until the tissue is uniform.
- Aliquot pulverized tissue into pre-labeled and pre-chilled 1.5 ml LoBind and nuclease-free snap cap 1.5 ml tubes (Eppendorf cat. no. 022431021).
- Aliquots of powdered tissues can be stored at -80°C.

Note

This is a good stopping point. All samples can be stored at -80°C until further processing.

4 Before starting, prepare Omni-ATAC lysis buffer and ATAC-RSB with 0.1% Tween-20.

Note

On day of nuclei isolation, add lysis buffer directly to tube or pour out the frozen aliquot into a 60 mm dish with cell lysis buffer and further mince with a blade. As long as the aliquot has not thawed at some point during storage, the powdered tissue aliquot should easily slide out of the storage tube without sample loss.

We estimate ~20,000 cells per mg of original tissue weight and performance may vary from tissue to tissue.

- Resuspend pulverized tissue in 1 ml Omni-ATAC lysis buffer, then transfer to a 15 ml falcon tube.
- Incubate these nuclei for 3 minutes on ice then add 5 ml ATAC-RSB + 0.1% Tween-20
- Centrifuge the nuclei at 500 x g for 5 minutes at 4°C.
- Aspirate supernatant and resuspend in 5 ml 1X DPBS.
- Pass nuclei in 1X DPBS through a 100 um cell strainer (VWR cat. no. 10199-658) to remove tissue clumps.
- In the fume hood, crosslink the nuclei by adding 140 uL of 37% formaldehyde (VWR, MK501602) for 1% final concentration. Mix quickly but gently by inverting the tube several times.
- Incubate at room temperature for exactly 10 minutes with gentle inversion of the tube every 1-2 min.
- Add 250 uL of 2.5 M glycine to quench the cross-linking reaction, mix well by inverting the tube several times.
- Incubate for 5 minutes at room temperature, then on ice for 15 minutes to stop the cross-linking completely.
- Count nuclei using a hemocytometer to know the final volume of freezing buffer to add. The goal is to freeze ~1-2 million nuclei in 1 ml of freezing buffer per tube.
- Centrifuge the cross-linked nuclei at 500 x g for 5 minutes at 4°C, aspirate the supernatant and resuspend pellet in the appropriate amount of freezing buffer supplemented with 1x protease inhibitors and 5 mM DTT.
- Snap-freeze nuclei in liquid nitrogen and store nuclei at -80°C.

Note

This is a good stopping point. All samples can be stored at -80°C until further processing.

Library construction, QC and sequencing

5 **Thawing, permeabilization, counting and tagmentation (~4 hours)**

- Before starting, prepare the Omni-ATAC lysis buffer (200 ul per sample). Also prepare RSB supplemented with 0.1% Tween-20.
- Take frozen fixed nuclei out of the -80°C and place on a bed of dry ice.
- Incubate nuclei in 37°C water bath until thawed (~30 sec – 1 min) and transfer nuclei to a 15 ml tube.
- Pellet nuclei at 500 x g for 5 minutes at 4°C.
- Aspirate supernatant without disturbing the pellet and resuspend pellet in 200 ul of Omni-ATAC lysis buffer.
- Incubate on ice for 3 minutes.
- Wash out the lysis buffer with 1 ml ATAC-RSB supplemented with 0.1% Tween-20 and gently invert the tube 3 times to mix.
- Count the nuclei by combining 20 ul of nuclei and 20 ul of Trypan blue and loading 10ul on a hemocytometer. While counting and throughout the remainder of the protocol, keep nuclei on ice whenever possible.
- For 3-level indexing experiments (with 384 barcodes introduced at each level for a total of 56,623,104 unique barcode combinations), we typically start with 4.8 million nuclei
- For tagmentation, these nuclei are distributed at 50,000 nuclei per well spread across a 96-well LoBind plate. Different tissues or samples can be arranged in different wells of this plate.
- Make a master mix for tagmentation reaction (to account for volume loss when multi-channel pipetting from troughs to plates we make enough for 110 reactions):

	Tagmentation Master Mix	1 reaction (in uL)	110 reactions (in uL)
	TD buffer 2X (110 rxn)	25	2750
	1X DPBS	8.25	907.5
	1% Digitonin	0.5	55
	10% Tween-20	0.5	55
	Water	13.25	1457.5
	NexteraV2 enzyme	2.5	
	Total	50	5225

- Pellet nuclei at 500 x g for 5 min at 4°C.
- Aspirate supernatant and resuspend pellet in tagmentation reaction master mix.
- Aliquot 47.5 ul of nuclei resuspended in the tagmentation master mix using a wide bore tip (Rainin Instrument Co cat. no.30389249) across wells of a LoBind 96-well plate (Eppendorf cat. No. 30129512).

Note

Note: for the experiments described in Domcke, Hill, Daza, et al. 2020, we processed 24 samples at a time laid out as shown in the diagram below.

	S1	S1	S1	S1	S9	S9	S9	S9	S17	S17	S17	S17
	S2	S2	S2	S2	S10	S10	S10	S10	S18	S18	S18	S18
	S3	S3	S3	S3	S11	S11	S11	S11	S19	S19	S19	S19
	S4	S4	S4	S4	S12	S12	S12	S12	S20	S20	S20	S20
	S5	S5	S5	S5	S13	S13	S13	S13	S21	S21	S21	S21
	S6	S6	S6	S6	S14	S14	S14	S14	S22	S22	S22	S22
	S7	S7	S7	S7	S15	S15	S15	S15	S23	S23	S23	S23
	S8	S8	S8	S8	S16	S16	S16	S16	S24	S24	S24	S24

S = sample

- Add 2.5 ul of Nextera v2 enzyme (Illumina Inc cat. no. FC-121-1031) per well, seal plate with adhesive tape and spin at 500 x g for 30 sec.
- Incubate the tagmentation plate at 55°C for 30 minutes.
- During the incubation, make a 2X stop reaction master mix by combining 25 ml of 40 mM EDTA and 3.9 ul of 6.4 M Spermidine (final concentration will be 20 mM EDTA and 1 mM Spermidine)
- After incubation, add 50 ul of stop reaction mixture to each well and re-seal the plate.
- Incubate at 37°C for 15 min.

6 **Pooling, PNK reaction and N5 ligation (2.5 hours)**

- Using wide-bore tips, pool tagmented nuclei into an Eppendorf tube. Note: if more than one sample was tagmented, each sample should be pooled separately.
- Pellet the nuclei at 500 x g for 5 minutes at 4°C.
- Aspirate supernatant and resuspend the pellet in 500 ul of ATAC-RSB with 0.1% Tween-20.
- Pellet the nuclei at 500 x g for 5 minutes at 4°C and aspirate the supernatant.

Note

Note: for the experiments described in Domcke, Hill, Daza, et al. 2020, we processed 24 samples at a time.

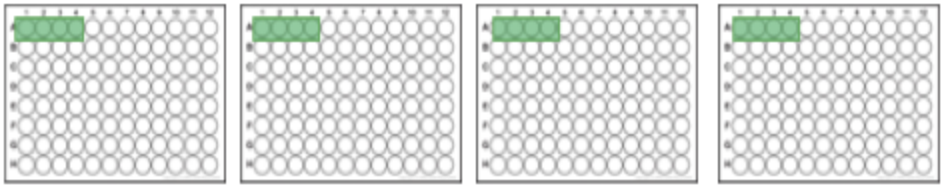
- Resuspend the pellet in 18 ul ATAC-RSB with 0.1% Tween-20 per sample.
- Create a PNK reaction master mix

	PNK master mix	1 reaction (in uL)	440 reactions (in uL)
	10x PNK buffer	0.5	220
	rATP 10 mM	0.5	220
	water	1	440
	T4 PNK	2	880
	Total		1760

- Add 72 ul of PNK master mix to each sample
- Aliquot 5 ul of PNK reaction mix across 16 wells of four 96-well plates

Note

Note: Alternatively, aliquot 22.5 ul across 4 wells per sample of a 96-well plate. Use a multichannel to stamp out 5 ul of PNK reaction with nuclei across an additional three 96-well plates.



- Seal with adhesive tape and spin at 500 x g for 5 minutes at 4°C.
- Incubate PNK reactions at 37°C for 30 minutes.
- Create a N5 ligation master mix for 440 reactions

	N5 Ligation Master Mix	1 reaction (in uL)	440 reactions (in uL)	
	2X T7 ligase buffer	10	4400	
	1000 uM_N5_splint	0.18	79.2	
	water	1.12	492.8	
	T7 DNA ligase	2.5	1100	
	50 uM_N5_oligo	1.2		Add separately
	Total		6072	

- Using a multichannel, directly add 13.8 ul of ligation master mix to each PNK reaction.
- Using a multi-channel or a 96 head dispenser (Liquidator, cat. No. 17010335), add 1.2 ul of 50 uM N5_oligo (IDT) to each well across four 96-well plates.
- Seal with adhesive tape and spin at 500 x g for 30 seconds then incubate at 25°C for 1 hour.
- After the first round of ligation, add 20 ul of EDTA and Spermidine mix (20 mM EDTA and 1mM Spermidine) from a trough to stop ligation reaction and incubate at 37°C for 15 minutes.
- Using wide bore tips, pool each well in a trough and transfer into a 50 ml falcon tube.
- Pellet nuclei at 500 x g for 5 minutes at 4°C, aspirate supernatant and resuspend nuclei in 1 ml of ATAC-RSB with 0.1% Tween-20 to wash any residual ligation reaction mix.
- Pellet nuclei at 500 x g for 5 minutes at 4°C and aspirate supernatant without disturbing the pellet.

7 N7 ligation (2 hours)

- Create N7 ligation master mix, making enough for 440 reactions and resuspend the nuclei with the ligation master mix.

	N7 Ligation Master Mix	1 reaction (in uL)	440 reactions (in uL)	
	2X T7 ligase buffer	10	4400	
	1000 uM_N7_splint	0.18	79.2	
	water	6.12	2692.8	
	T7 DNA ligase	2.5	1100	
	50 uM_N7_oligo	1.2		Add separately
	Total		8272	

- Transfer nuclei suspended in master mix into a trough and using wide bore tips, aliquot 18.8 ul of ligation master mix into four 96-well LoBind plates then add 1.2 ul of 50 uM N7_oligo (IDT) to each well across four 96-well plates.
- Seal plates with adhesive tape and spin at 500 x g for 30 seconds then incubate at 25°C for 1 hour
- Stop ligation by adding 20 ul of EDTA and Spermidine mix (20 mM EDTA and 1 mM Spermidine) and incubate at 37°C for 15 minutes.

8 Pooling, counting and dilution (1.5 hours)

- Pool wells in a trough using wide bore tips and then transfer into a 50 ml falcon tube.
- Pellet nuclei at 500 x g for 5 minutes at 4°C, aspirate supernatant and resuspend nuclei in 2 ml of Qiagen EB buffer (Qiagen cat. no. 19086).

- Filter nuclei using FACS tube with 40 um filtered cap (Fisher Scientific cat. no # 352235)
- Take 20 ul of resuspended and filtered nuclei and 20 ul of trypan blue to count nuclei with hemocytometer.
- Dilute nuclei to 100 – 300 nuclei per ul and aliquot 10 ul per well into four 96-well LoBind plates.
- In another 96-well LoBind plate, aliquot 10 ul per well into 8 wells, this will be used as a test to determine the optimal cycle number.

Note

This is a good stopping point. All 96-well plates including test PCR plate can be stored at -20°C until further processing. Otherwise, proceed to uncrosslinking step.

9 **Reverse crosslink nuclei (overnight incubation)**

- To reverse crosslink the nuclei, make a reverse crosslink master mix for 440 reactions consisting of EB buffer, Proteinase K (Qiagen, cat. no. 19133) and 1% SDS; 1ul/0.5 ul/0.5 ul respectively per well) and add 2 ul to each well of nuclei.
- Seal with adhesive tape, spin at 500 x g for 30 seconds and incubate at 65°C for 16 hours.

10 **Test PCR and gel QC (~2 hours)**

- Briefly spin down uncrosslinked plates before starting.
- Make a PCR master mix for 6 reactions

	Test PCR Master Mix	1 reaction (in uL)	6 reactions (in uL)
	P7_10uM_row____	1.25	Add separately
	P5_10uM_column____	1.25	Add separately
	NEBNext Hi-Fidelity 2x Master Mix	25	150
	100X BSA	1.0	6
	100X SYBR Green	0.25	1.5
	Water	9.25	55.5
	Total		213

- Aliquot 35.5 ul of PCR master mix into an 8-strip tube (white without cap) (Bio-Rad Laboratories, TLS0851).
- Add 1.25 ul each of 10 uM P7 and P5 primers.
- Add 12 ul of uncrosslinked nuclei to the PCR and primer mix.

- Cap reaction tubes with optical flat 8-cap strips (Bio-Rad Laboratories, TCS-0803).
- Place test PCR reactions in the qPCR machine and monitor amplification to determine optimal cycle number.

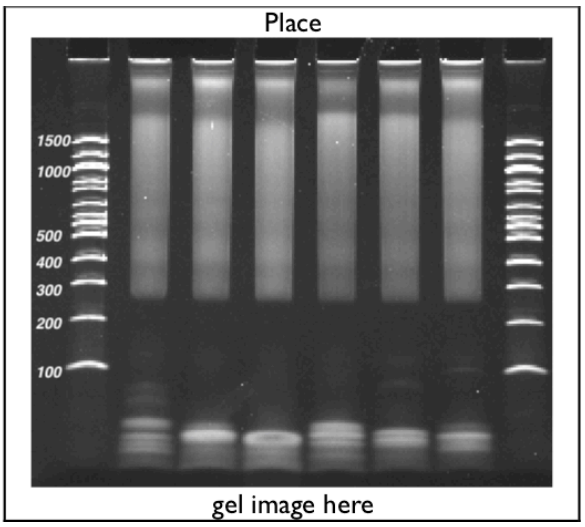
  72ºC 5 min
  98ºC 30 sec
  10-20 Cycles:
  98ºC 10 sec
  63ºC 30 sec
  72ºC 1 min
  Hold at 10ºC

Note: Based on the test wells, choose cycle number such that test wells were all clearly amplifying but before the fluorescence intensity in any of the wells has saturated

- Take 1 ul of PCR product for QC.

Samples = 1 ul + 9 ul nuclease free water + 2 ul 6x loading dye
100 bp ladder (1:10) = 1 ul + 9 ul nuclease free water + 2 ul 6x loading dye

- Run 6% TBE polyacrylamide gel, 180 volts for 35 mins.
- Stain with 5 ul SYBR Gold in 50 ml 0.5X TBE buffer, incubate at room temperature for 5 min.



Test PCR QC - image above is unpurified PCR product run with 100 bp ladder from NEB. Nucleosome banding is evident betwen 280-300 bp (subnucleosomal), 400-500 bp (mononucleosomal) and above 500 bp for dinucleosomal etc.

11 **PCR plate set-up for the rest of the plates (1 hour)**

- Briefly spin down the plate. Set aside on ice until the test PCR result becomes available.
- Make a PCR master mix

	PCR Master Mix	1 reaction (in uL)	440 reactions (in uL)
	P7_10uM_row____	1.25	Add separately
	P5_10uM_column____	1.25	Add separately
	NEBNext Hi-Fidelity 2x Master Mix	25	11000
	100X BSA	1.0	440
	Water	9.5	4180
	Total		15620

- Using a multi-channel pipette, aliquot 35.5 ul of PCR master mix into each well containing uncrosslinked nuclei (make sure to change tips at every addition).
- Add 1.25 ul each of 10 uM P7 and P5 primers.

Note row and column primer combination used during amplification.

	Experiment_plate_ID	3lv2_PCR_P7	3lv2_PCR_P5
	Ex. Exp1_P1	Row A	Column 1
	Ex. Exp1_P2	Row B	Column 2
	Ex. Exp1_P3	Row C	Column 3
	Ex. Exp1_P4	Row D	Column 4

Example for combination of row and column indices.

- Seal with an adhesive tape then spin at 500 x g for 30 sec.
- Run PCR plate with the optimal cycle number from test PCR result:
  72ºC 5 min
  98ºC 30 sec
  10-20 Cycles:
  98ºC 10 sec
  63ºC 30 sec
  72ºC 1 min
  Hold at 10ºC

12 **PCR Amplification Clean-up and QC (~1.5 hour)**

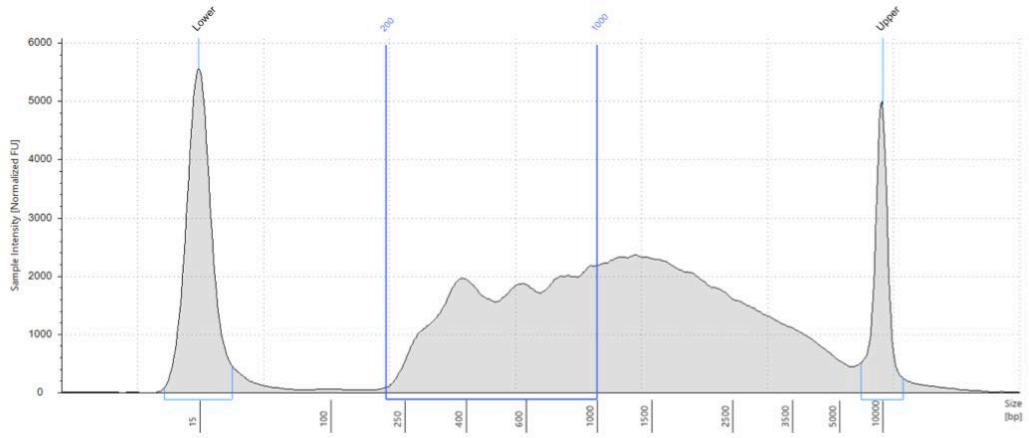
Clean PCR products with Zymo Clean&Concentrator-5

- Combine 25 ul of each PCR reaction into a trough (2.4 ml).
- Add 2 volumes binding buffer (4.8 ml).
- Split across 4 C&C columns (600 ul spun 3 times in each column).
- Add 200 ul Zymo wash buffer and spin (2 washes total).
- Use an extra spin to dry columns for 1 min after last wash.

- Elute in 25 ul Qiagen elution buffer (let buffer stand on column 1 min, then spin 1 min at max speed).
- Combine all 4 eluates and clean a second time with 1X AMPure beads: Add 100 ul AMPure beads.
- Place on MPC (magnetic particle collector) until supernatant is clear.
- Aspirate supernatant.
- Wash beads with 200 ul 80% ethanol twice.
- Dry beads for 30 sec – 1 min until beads are dull in color without over drying the beads.
- Elute beads in 25 ul Qiagen EB buffer, place back in MPC and transfer supernatant to a fresh tube.

For library QC using Tapestation, follow manufacturer's specification using D5000 ScreenTape Assay.

- For fragment analysis with the Tapestation software, create a region table from 200 – 1000 bp in which a region molarity is calculated. Use that nM concentration to dilute library to 2 nM with buffer EB (Qiagen) and 0.1% Tween-20.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total	Region Comment	Color
200	1000	580	17.8	54.8	42.81		

	Sample_ID	Avg length	nM	Library	0.1% Tween/EB
	Example. Exp1_P1	430	17.6	5 ul	39

- If pooling multiple libraries, normalize each library to 2 nM and create an equimolar pool for sequencing.
- Purified libraries and normalized 2 nM dilutions can be stored at -20°C for long term storage.

13 **Sequencing - Illumina 150 cycle kit, high output (~1.5 hours)**

Libraries can be assessed for quality with a NextSeq run before deep sequencing on a NovaSeq.

Library denaturation

- Dilute 2N NaOH to 0.2N NaOH (10 ul 1N to 90 ul nuclease-free water).
- In a new 1.5 Lo-Bind tube, transfer 10 ul 0.1N NaOH and add 10 ul 2nM pooled libraries.
- Incubate at room temperature for 5 minutes.
- Add 980 ul HT1 to dilute denatured libraries to 20 pM.
- Dilute denatured library to 1.8 pM loading concentration (135 ul 20 pM + 1365 ul HT1).
- Dilute custom primers to 0.6 uM (stock primers are in 100 uM)

	Position		Final conc. 0.6 uM (ul)	HTI (ul)	Volume (ml)
	7	Read 1	9	1491	1.5
	8	Read 2	9	1491	1.5
	9	Index 1 + Index 2	9 + 9	1491 + 1491	3.0
	10	sample	1.8 pM	1500	1.5

NextSeq Sequencing recipe name: 3LV2_sciATAC_high

NOTE: remember to check boxes for custom primers 7, 8 and 9

R1 - 50 bases for gDNA

R2 - 50 bases for gDNA

Index 1 - 20 bases (10 bases for N7 oligo, 15 dark cycle, 10 bases PCR barcode)

Index 2 - 20 bases (10 bases for N5 oligo, 15 dark cycle, 10 bases PCR barcode)

	Sequencing Primers	
	3Lv2_R1_seq	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
	3Lv2_R2_seq	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
	3Lv2_IDX1	CTCCGAGCCCACGAGACGACAAGTC
	3Lv2_IDX2	ACACATCTGACGCTGCCGACGACTGATTAC