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🌐 snm3C-seq3

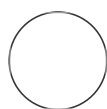
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ABSTRACT

We developed a single-nucleus multiomic sequencing technology (snm3C-seq3) to assay both the methylome and chromatin contacts from within the same cell. We further adapted this protocol to allow for mass-scale assessment, assaying thousands of cells at a time. This protocol is an expanded and updated version of snmC-seq2, adding in the chromatin contact assay and optimizing the pooling strategy of mass-scale library preps.

MATERIALS

MATERIALS-

General reagents

- FACS sorted single-nuclei into 384-well PCR plate
- HyClone™ HyPure™ Molecular Biology Grade (MB) Water (GE Life Sci. SH30538.08)
- 200-Proof (100%) Ethanol (Koptec V1001)

Nuclei Isolation

- RNaseOUT™ Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific 10777019)
- SUPERase• In™ RNase Inhibitor (ThermoFisher Scientific AM2694)
- Protease Inhibitor Cocktail (Sigma-Aldrich P8340)
- DTT (Sigma, 43816)
- Hoechst 33342 Solution (20 mM) (ThermoFisher 62249)
- anti-NeuN-488 clone A60 (Millipore MAB377)

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Protocol status: Working
We use this protocol and it's working

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**Funders
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- OptiPrepTM Density Gradient Medium (Sigma-Aldrich D1556)
- UltraPureTM BSA (50 mg/mL) (ThermoFisher AM2618)
- DPBS (1X) (ThermoFisher 14190144)
- 1% Triton X-100 (Sigma T8787-100ML)
- 1M Tris-Cl pH 8.0 (Invitrogen 15568025)
- 2M KCl (Invitrogen AM9640G)
- 1M MgCl₂ (Invitrogen AM9530G)
- 1M Sucrose (VWR VWRV0335)

3C Protocol

- Formaldehyde (Sigma F8775-25ml)
- Glycine (sigma 50046-250G)
- rCutSmart Buffer 10X (NEB B6004S)
- Arima-HiC+ Kit: Arima 3C kit 8 reactions (Arima 10113)

Collection of single nuclei by Fluorescence-activated Cell Sorting (FACS)

- M-Digestion Buffer (Zymo D5021-9)
- Proteinase K w/Storage Buffer (Zymo D3001-2-20)
- CpG Methyltransferase (M.SssI) (NEB M0226)
- Unmethylated Lambda DNA (Promega D1521)

Bisulfite Conversion and Cleanup

- CT Conversion Reagent (Zymo D5003-1)
- M-Solubilization Buffer (Zymo D5021-7)
- M-Dilution Buffer-Gold (Zymo D5006-2)
- M-Reaction Buffer (Zymo D5021-8)
- MagBead J (Zymo custom product D4100-3-100)
- M-Binding Buffer (Zymo D5021-7)
- M-Wash Buffer (Zymo D5040-4)
- M-Desulphonation Buffer (Zymo D5040-5)
- M-Elution Buffer (Zymo D5007-6)

Random primers

- Random Indexing Primer (IDT custom DNA oligo, standard desalted) - See sequences below

Sera-Mag Solid Phase Reversible Immobilization (SPRI) beads

- Sera-Mag SpeedBeads Magnetic Carboxylate Modified (GE Healthcare 5152105050250)

- Poly(ethylene glycol) PEG 8000 (Sigma cat no. 89510-250G-F)
- TE buffer pH=8.0 (Ambion AM9858)
- 5M NaCl
- 1M Tris-HCl pH=8.0
- 0.5M EDTA pH=8.0
- AMPure XP beads (Beckman Coulter cat no. A63881)
- 100 bp DNA ladder (New England Biolabs cat no. N3231L)

snmC-seq3 Library Preparation

- Blue Buffer (10x) (Enzymatics P7010-HC-L)
- Klenow Exo- (50U/μL) (Enzymatics P7010-HC-L)
- Deoxynucleotide Solution Mix (10mM each dNTP) (NEB N0447L)
- Exonuclease I (20U/μL) (Enzymatics X8010L)
- Shrimp Alkaline Phosphatase (rSAP) (1U/μL) (NEB M0371L)
- Sera-Mag SPRI beads
- M-Elution Buffer (Zymo 5007-6)
- EB buffer (Qiagen 19086)
- Accel-NGS® Adaptase™ Module (Swift Bio 330384)
- P5 Indexing Primer (IDT custom DNA oligo, standard desalted) - See sequences below
- P7 Indexing Primer (IDT custom DNA oligo, standard desalted) - See sequences below
- KAPA HiFi HS RM (Kapa KK2602)
- Qubit dsDNA BR Assay Kit (Thermo Fisher Q32850)

Equipment

- Dounce tissue grinder set (2 mL) (Sigma-Aldrich D8938)
- Dounce tissue grinder set (7 mL) (Sigma-Aldrich D9063)
- 384-Well Hardshell PCR Plate Clear, 20 Pcs/pk (Thermo Fisher 4483285)
- 96-Well Hardshell PCR Plate GPLE, 20 Pcs/pk (Thermo Fisher 4483348)
- MicroAmp™ Splash-Free 96-Well Base (Thermo Fisher 4312063)
- Plateone® Deep 384-Well 200 μL Polypropylene Plate (USA-SCI. 1884-2410)
- Reservoir Single Well 384 Bottom Low Profile, Clear (Axygen RES-SW384-LP)
- Reservoir Single Well 96 Bottom High Profile, Clear (Axygen RES-SW96-HP)
- Reservoir Single Well 96 Bottom Low Profile, Clear (Axygen RES-SW96-LP)
- 15mL Centrifuge Tubes (Olympus 28-103)
- 50mL Centrifuge Tubes (Olympus 28-106)
- 1.7mL Microtube, Clear (Olympus 24-282LR)
- Microamp Clear Adhesive Film, 100pc (Thermo Fisher 4306311)
- Speedball Deluxe Soft Rubber Brayer, 4 inches (Statesville N.C.)
- 37°C Incubator
- 384-well and 96-well Compatible Thermocycler

- DynaMagTM-96 Side Magnet (Thermo Fisher 12331D)
- DynaMagTM-2 Magnet (Thermo Fisher 12321D)
- 384-well Magnet Plate (Alpaqua A001222)
- Allegra 25R Centrifuge with S5700 2x96 Swinging Bucket Rotor (Beckman Coulter 368954)
- Axygen[®] 384-well tips, 45µL, Clear, Filtered, Sterile, Fine Point, SLAS Rack (Corning FXF-384-45FP-R-S)
- Axygen[®] 96-well tips, 200µL, Clear, Filtered, Sterile, SLAS Rack (Corning FXF-200-R-S)
- Axygen[®] 96-well tips, 30µL, Clear, Filtered, Sterile, SLAS Rack (Corning FXF-50-R-S)
- Beckman Coulter Biomek i7 dual-Multichannel with MC384 and MC96 Pipetting Heads

REAGENT SETUP-

Methylated λ DNA

Dilute supplied SAM to 1600µM with Nuclease-free Water. Set up the reaction in the following order:

A	B
Reagent	Volume
Nuclease-free H ₂ O	up to 50 µL
Methyltransferase Reaction Buffer (10x)	5 µL
Diluted SAM (1600 µM)	5 µL
DNA	1 µg (2 µL)
Methyltransferase	8 units (2 µL)

Mix by pipetting. Incubate at 37°C for 1 hr. Stop the reaction by heating at 65°C for 20 minutes. Purify product by Sera Mag bead cleanup (1.6 bead vol : sample vol ratio) or Qiagen DNA Column Cleanup.

Digestion Buffer Plates (for FACS sorting)

In a 50mL tube, combine 15mL M-Digestion Buffer (incubate for 10 minutes at 37°C to dissolve precipitate) with 14mL MB water. Resuspend Proteinase K with 1mL Proteinase K Resuspension Buffer and add 1mL to the digestion buffer mix. Add 1.5µL methylated λ DNA to aid determination of bisulfite conversion efficiency. Invert to mix. Aliquot 1µL per well to 50 x 384-well plates to use for FACS sorting of

stained nuclei (This is automated using Biomek i7). Plates can be stored for 3 months at -20°C.

Sera-Mag Solid Phase Reversible Immobilization (SPRI) Beads

1. Mix Sera-Mag SpeedBeads and transfer 1mL to a 1.5ml tube.
2. Place SpeedBeads on a magnetic stand until clears and carefully remove the supernatant. Wash the beads twice with 1ml of TE. For each wash, remove the tube from the magnet and mix by inversions. Resuspend the washed beads in 1ml of TE.
3. Add 9g of PEG 8000 to a new 50ml sterile conical tube.
4. Add 10ml of 5M NaCl to the 50ml tube.
5. Add 500µl of 1M Tris-HCl pH=8.0 and 100ul of 0.5M EDTA pH=8.0 to the 50ml tube.
6. Mix until all dissolves into solution.
7. Add 1ml of resuspended SpeedBeads to the 50ml tube and fill the volume with MB water.
8. Test against AMPure XP beads using 100bp DNA ladder.

Ethanol, 80%

To 40mL of 200-proof Ethanol, add 10 µL MB water in a 50mL tube. Keep solution sealed when not in use. Prepare ~9 tubes fresh before each 16-plate library preparation.

Random Primer solution

Sequences of Random Primers with unique barcodes are provided in the end. Transfer 1.5 µL stock primer (100 µM) into 298.5 µL MB water. Vortex to mix. If preparing for 384-well plates, aliquot into deep-well 384-well plates.

PCR Primer Mix

Sequences of indexing primers with unique dual barcodes are provided in the end. Each PCR Primer Mix contains a P5L indexing primer (600 nM) and a P7L indexing primer (1 µM).

Primer Information

Part 1: Primer structure

Random primer:

/5SpC3/TTCCCTACACGACGCTCTTCCGATCTXXXXXXXXX(H1:33340033)(H1)(H1)

(H1)(H1)(H1)(H1)(H1)(H1)

Where the XXXXXXXX is an 8-bp barcode sequence, see the barcode sequence and name below.

PCR i5 primer:

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXXACACTCTTCCCTACACGACGCTCT


Where the XXXXXXXXXX is a 10-bp barcode sequence, see the barcode sequence and name below. The i5 and i7 primers are in pairs.

PCR i7 primer:

CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTT

Where the XXXXXXXXXX is a 10-bp barcode sequence, see the barcode sequence and name below. The i5 and i7 primers are in pairs.

Primer names and sequences can be found in the attached document:

 snm3C_seq3_PrimerSequences.docx

Prepare Single Nuclei

1h 30m

1 Prepare stock solutions, stored at 4°C

10m

1. NIM: Sucrose (250 mM), KCl (25 mM), MgCl₂ (5 mM), Tris-Cl pH 8.0 (10 mM)
2. Diluent: Tris-Cl pH 8.0 (120 mM), KCl (150 mM), MgCl₂ (30 mM)

2 Prepare the following solutions fresh before each experiment

10m

1. NIMT (on ice): NIM + Triton X-100 (0.1%) + DTT (1 mM) + Proteinase Inhibitor (1:100 dilution)
2. 50% Iodixanol (Room temp) : 5 vol. Optiprep (60% Iodixanol) + 1 vol Diluent
3. 25% Iodixanol (Room temp) : 1 vol. 50% Iodixanol + 1 vol NIM
4. DPBS + 1% BSA (on ice)

3 Prepare nuclei:

45m

1. Fast cool centrifuge with swinging bucket tube rotor to 4°C.
2. Remove frozen tissue from -80°C freezer, place on ice.
3. Transfer tissue with 2.4 mL NIMT from tube into the large dounce.
4. Use loose pestle A 40 times gently without introducing bubbles.
5. Use tight pestle B 40 times gently without introducing bubbles.
6. Transfer lysed solution into a 5-mL or 15-mL tube and keep on ice.
7. If doing NeuN stain: Add 6 µL of NeuN 488 (1:500 dil). Mix and incubate 15 min on ice, keeping solution covered from light.

8. Mix the lysis suspension with 1.5 mL of 50% Iodixanol by pipetting.
9. Slowly pipette 1 mL of cell mixture onto 500 μ L 25% Iodixanol cushion, 4 x 2-mL tubes in total per sample.
10. Centrifuge in swing rotor for 20 min at 10,000 x g at 4°C.

4 Nuclei staining and count:

20m

1. Remove supernatant very carefully, avoiding debris.
2. Using ice-cold DPBS+RNase Inhibitors, resuspend and combine the pellets from each tube into a total of 1 mL nuclei solution in a new tube.
3. Add Hoechst 33342 (dil: 1:1000): Dilute the Hoechst 1:10 (5 μ L + 45 μ L DPBS), then add 5 μ L to 1 mL sample.
4. Incubate on ice for 5 min.
5. Mix 10 μ L Nuclei suspension with 10 μ L Trypan Blue and mix by pipetting.
6. Add 10 μ L stained solution to a Cell Counter Slide and read using the Bio-Rad Cell Counter TC-20.

Note: Nuclei count is Total Count minus Live Count

3C Protocol

3h

5 Crosslinking:

45m

1. Split sample into 2 tubes (1-2 million nuclei in each tube) and bring volume up to 1 mL with DPBS.
2. Add 57 μ L of 37% formaldehyde (for a final concentration of 2%) to each tube and mix well by inverting 10 times.
3. Incubate 10 min (5 min if a NeuN pre-stain was done to avoid stain degradation) at room temperature, inverting occasionally.
4. Add 91.9 μ L Stop Solution (or 2.5 M Glycine) and mix well by inverting 10 times.
5. Incubate for 5 min at room temperature, inverting occasionally.
6. Place sample on ice and incubate an additional 5 min.
7. Pellet cells by centrifuging 5 min at 2500 x g or 10 min at 1000 x g.
8. Discard supernatant, being sure to leave no residual liquid, and resuspend in 1 mL of 1x PBS.
9. Repeat steps 7-8 two more times to completely wash away residual formaldehyde/glycine.
10. Proceed directly to next section or freeze samples at -80°C up to 5 days.

6 3C Nuclei Conditioning:

30m

1. Resuspend each reaction of crosslinked nuclei in 20 μ L DPBS.
2. Add 24 μ L Conditioning Solution and mix gently by pipetting.
3. Incubate at 62°C for 10 min. If using a thermal cycler, set the lid temperature to 85°C.

4. Add 20 μL Stop Solution 2 and mix gently by pipetting.
5. Incubate at 37°C for 15 min. If using a thermal cycler, set the lid temperature to 85°C.

7 3C Enzymatic Reactions

2h

Note: Some of the next steps require addition of several reagents in the same step. These reagents should be combined into master mixes using the following tables (including 10% extra to accommodate distribution).

1. Digestion: Add 28 μL of master mix containing the following reagents:

A	B	C	D	E	F
Reagent	1 Rxn (μL)	w/10% extra		# Rxns	Final Volume
1x CutSmart	12 μL	13.2 μL	x		
10x CutSmart (or Buffer H)	7 μL	7.7 μL	x		
NLAIII/Enzyme H1	4.5 μL	4.95 μL	x		
Mbol/Enzyme H2	4.5 μL	4.95 μL	x		
Total	28 μL				

2. Mix gently by pipetting and incubate as follows. If using a thermal cycler, set the lid temperature to 85°C.

- a. 37°C for 60 min (or up to overnight)
- b. 65°C for 20 min
- c. 25°C for 5 min

3. Mix gently by inversion.

4. Ligation: Add 82 μL of master mix containing the following reagents:

A	B	C	D	E	F
Reagent	1 Rxn (μL)	w/10% extra	x	# Rxns	Final Volume
Buffer C	70 μL	77 μL	x		
Enzyme C	12 μL	13.2 μL	x		
Total	82 μL				

5. Mix gently by pipetting and incubate at room temperature for 15 min.
6. Samples can be stored at 4°C overnight or continue to FACS.

FACS

2h 30m

8 Prepare samples for FACS:

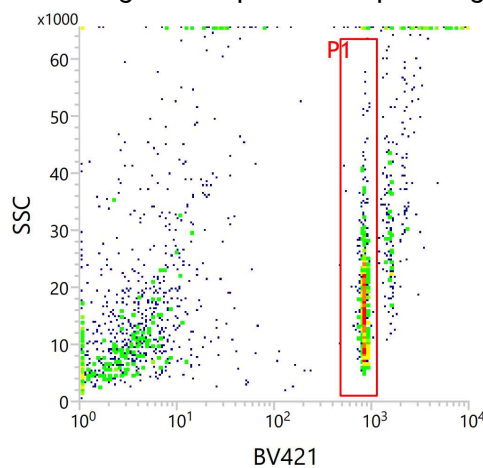
20m

1. Combine the reactions for each sample and add DPBS + 1% BSA up to a total volume of 1 mL.
2. Centrifuge at 1,000 x g for 10 min at 4°C in swinging bucket rotor.
3. Remove supernatant and gently resuspend in 200 µL DPBS + 1% BSA. Add another 800 µL DPBS + 1% BSA to bring total volume up to 1 mL.
4. Add 5 µL 1:10 diluted Hoechst 33342.
5. Add 1 µL of antibody to enhance labeling.
6. Incubate on ice for 5 min.
7. Filter the cells through a 40 µm cell strainer and transfer into a polypropylene tube for sorting. Keep on ice until ready to sort.

9 Single nuclei sorting:

2h

1. Thaw and spin down (5 sec at 1000 x g) 384-well plates containing 1 µL digestion buffer.
2. Sort single nuclei into wells using BD Influx or other sorter.
 - a. Use 2N gating for Hoechst stain to exclude debris and nuclei in cell replication stages. See example gate with Hoechst on the x-axis and side scatter on the y-axis. Debris is seen to the left of the gate and possible replicating nuclei are to the right.



- b. If NeuN 488 stained: Sort NeuN+ in col 1-22 and NeuN- in col 23-24
 - c. Sort using 1 drop single mode
3. After sorting spin down the sample plates for 5 sec at 1000 x g.
 4. Incubate plates for 20 min at 50°C to run proteinase K reaction.
 5. Plates can be stored at -20°C for up to 1 year.

Bisulfite Conversion

8h

10 Prepare the Zymo CT Conversion Reagent (3 bottles for 16 x 384-well plates)

20m

1. Add 7.9 mL Solubilization Buffer and 3 mL Dilution Buffer to each Conversion Reagent bottle and mix for 10 minutes.
2. Add 1.6 mL Reaction Buffer to each Conversion Reagent bottle and mix for an additional 5 minutes.

11 Bisulfite Reaction:

*This step can be automated using Biomek i7

1. Add 6 μL CT Conversion Reagent to each well.
2. Seal, vortex, and quick spin (500 rcf) plates.
3. Incubate plates using [DIRECT] method on PCR machines with 384-well plate block.
 - a. 98 °C for 8 minutes
 - b. 64 °C for 3.5 hours
 - c. Hold at 4 °C for up to 20 hours

12 Bisulfite Cleanup:

*Steps in this section are automated for up to 8 x 384-well plates using Biomek i7

12.1 Prepare MagBead master plate:

- a. Invert Magbead J stock tube repeatedly to ensure homogeneity.
- b. Using an 8-channel pipette, aliquot 58 μL Magbead J stock to each well of a 96-well plate (avoid bubbles and do not spin). Tap plate to remove bubbles.
- c. Distribute 14 μL beads to each well of a clean 384-well plate.
- d. Tap down 384-well plate and pipette out any bubbles from the bottom of the wells (do not spin).
- e. Distribute 1.5 μL to each well of sample plates.

12.2 Bind the free DNA to the Magbeads:

- a. Add 20 μL M-Binding Buffer to each well and mix by pipetting.
- b. Incubate at room temperature for 5 minutes.
- c. Move the plates onto the magnets.
- d. Remove supernatant and discard in the waste trough.

12.3 Wash the beads:

- a. With the plates still on the magnets, add 20 μL M-Wash Buffer to all plates and incubate at room temperature for 30 seconds.
- b. Remove supernatant and discard in the waste trough.

12.4 Add M-Desulphonation Buffer to the plates:

- a. Add 15 μL M-Desulphonation Buffer to all plates.
- b. Incubate at room temperature for 15 minutes.
- c. Remove supernatant and discard in the waste trough.

12.5 Wash the beads:

- a. Add 20 μ L M-Wash Buffer to all plates and incubate at room temperature for 30 seconds.
- b. Remove supernatant and discard in the waste trough.
- c. Repeat wash one additional time.

12.6 (Optional) Do an extra supernatant removal to further empty out wells if desired

12.7 Let the plates dry:

- a. Let plates sit for 5 minutes. Keep them on the magnets to avoid beads jumping due to static.
- b. Check that all the wash buffer has evaporated before moving on.

12.8 Elute the DNA from the beads using the RP elution plate:

- a. Add 5.5 μ L appropriate Random Primer solution to all plates (note the barcodes).
- b. Remove the plates from the magnets and seal well.
- c. DO NOT VORTEX; instead, throw the sealed plates onto the bench 10-15 times, until beads appear to be mixed into elution buffer. Repeat for all plates.
- d. Quick spin plates for 5s at 200xg
- e. Incubate at room temperature for 5 minutes. Place plates back onto the magnets.

12.9 Transfer eluted DNA into clean 384-well plates:

- a. Transfer 5 μ L sample elution into a clean, labeled 384-well plate
- b. Seal new sample plates and quick spin for 5s at 500xg.

12.10 (Optional) If running 16 plates, repeat steps 12:1-12:9 for the second set of 8 plates.

2h

Random Priming

3h

13 Random primed DNA synthesis:

****Steps in this section are automated for up to 16 x 384-well plates using Biomek i7**

2h

1. Prepare appropriate volume of Random Priming Master Mix (RP MM) in a 50-mL conical tube and invert to mix.

A	B	C	D
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A	B	C	D
Reagent	Volume per rxn	Vol for 8 plates	Vol for 16 plates
Nuclease-free H ₂ O	3.45 µL	12420 µL	24840 µL
Enzymatics Blue Buffer (10x)	1.025 µL	3690 µL	7380 µL
dNTPs (10 mM each)	0.50 µL	1800 µL	3600 µL
Klenow Exo- (50 U/µL)	0.025 µL	90 µL	180 µL

- Denature 8 sample plates at 98 °C for 3 minutes in thermocycler. Remove plates from PCR machines and immediately place on ice to prevent rehybridization. Proceed once bottom of each plate is cool to the touch.
- Add 5 µL RP MM to each sample well. Seal, vortex, and quick spin (5s at 500xg) sample plates.
- Incubate plates in thermocycler with the following program:
 - 4°C for 5 minutes
 - 25°C for 5 minutes
 - 37°C for 60 minutes
 - Hold at 4 °C
- Repeat steps 2-4 for second set of 8 plates (if applicable).

14

Inactivation of free primers & dNTPs:

1h

**Steps in this section are automated for up to 16 x 384-well plates using Biomek i7

- Prepare appropriate volume of Exo/rSAP Master Mix (E/S MM) in a 15-mL conical tube and invert to mix.

A	B	C	D
Reagent	Vol per rxn	Vol per 8 plates	Vol per 16 plates
Nuclease-free H ₂ O	1.15 µL	4140 µL	8280 µL
Enzymatics Blue Buffer (10x)	0.2 µL	720 µL	1480 µL
Exonuclease I (20 U/µL)	0.1 µL	360 µL	720 µL
rSAP (1 U/µL)	0.05 µL	180 µL	360 µL

- Add 1.5 µL Exo/Sap Master Mix to each sample well. Seal, vortex, and quick spin sample plates (5s at 500xg).
- Incubate plates in thermocycler with the following program:
 - 37°C for 30 minutes
 - Hold at 4°C
- Repeat steps 2-3 for second set of plates (if applicable).
- Either continue to Reformat and Cleanup or store at -20°C overnight.

- 15** Compress 16 x 384-well plates to 8 x 96-well plates and add SeraMagbeads
**Steps in this section are automated for 16 x 384-well plates using Biomek i7. Note that the bead cleanups for each set of plates will overlap to increase timing efficiency.
- 15.1** Thaw (if applicable) and quick spin (5s at 500xg) sample plates.
- 15.2** Compress 384-well plates 1-8 into four new 96-well plates 1-4. 8 wells will be combined into one well. One 384-well plate will be compressed to half a 96-well plate:
 - a. Combine samples from each quadrant of the top half of 384-plate 1 (Rows A-H) and dispense into the top half of a clean 96-well plate (Rows A-D).
Eg: A1,A2,B1,B2 of 384-well plate combine into A1 of the 96-well plate.
A3,A4,B3,B4 of 384-well plate combine into A2 of the 96-well plate.
 - b. Combine samples from each quadrant of the bottom half of 384-plate 1 (Rows I-P) and dispense into top half of the same 96-well plate (Rows A-D).
Eg: I1,I2,J1,J2 of 384-well plate combine into A1 of the 96-well plate along with the A/B row samples.
 - c. Continue this pattern of pooling with 384-well plate 2 going into the bottom half of the same 96-well plate. Plates 3-4 will go into a second 96-well plate, 5-6 into a third 96-well plate, and Plates 7-8 into a fourth 96-well plate.
- 15.3** Bind the free DNA to the Sera-Mag beads in 96-well plates 1-4:
 - a. Thoroughly mix Sera-Mag beads by repeatedly inverting bottle and pour Sera-Mag beads to the brim of a low-profile 96-well reservoir.
 - b. Add 73.6 μ L beads into sample plates and pipette up and down to mix.
 - c. Let plates incubate at room temperature for at least 5 minutes.
- 15.4** Compress 384-well plates 9-16 into four new 96-well plates 5-8:
 - a. Combine 384-well plates 9-16 following the same pattern of pooling in Step 15.2 above. Plates 9-10 will go into a fifth 96-well plate, 11-12 into a sixth 96-well plate, Plates 13-14 into a seventh 96-well plate, and Plates 15-16 into a eighth 96-well plate.

- 15.5** Bind the free DNA to the Sera-Mag beads in 96-well plates 5-8:
- Thoroughly mix Sera-Mag beads by repeatedly inverting bottle and pour Sera-Mag beads to the brim of a low-profile 96-well reservoir.
 - Place plates 1-4 onto 96-well magnet and incubate for at least 5 minutes.
 - Add 73.6 μ L beads to sample plates 5-8 and pipette up and down to mix. Incubate for at least 5 minutes.
- 16** Perform bead cleanups on 96-well plates 1-8
- 16.1** Carefully remove supernatant from plates 1-4 and discard in waste trough.
- 16.2** Wash beads in plates 1-4 with 80% EtOH:
- Keeping plates on the magnets, add 180 μ L of 80% EtOH to plates 1-4 and incubate at room temperature for at least 30 seconds. Subsequently remove and discard EtOH in the waste trough.
 - Repeat for a second wash.
 - Remove plates from the magnets and incubate until beads are dry. The beads should appear cracked and there should be no visible droplets of EtOH in the wells.
- 16.3** Remove supernatant and add EtOH to 96 plates 5-8.
- Carefully remove supernatant from plates 5-8.
 - Add 180 μ L EtOH to plates 5-8 and incubate for at least 30 seconds.
- 16.4** Elute DNA from plates 1-4 and perform second EtOH wash on plates 5-8:
- Once beads are dry, add 10 μ L EB to plates 1-4. Set a 5-minute timer upon completion of transfer.
 - Seal plates, vortex until beads no longer remain on the sides of the well, and quick spin 5s @ 150xg to avoid pelleting beads. When 5-minute timer is completed, replace plates on the magnets.
 - Remove EtOH from plates 5-8 and discard in the waste trough.
 - Repeat EtOH wash of plates 5-8.
 - Remove plates from the magnets and incubate until beads are dry.

16.5 Elute DNA from plates 5-8:

- a. Once plates are dry, add 10 μ L EB to plates 5-8. Start a 5-minute timer upon completion of transfer.
- b. Seal plates, vortex until beads no longer remain on the sides of the well, and quick spin 5s @ 150xg to avoid pelleting beads. When 5-minute timer is completed, replace plates on the magnets.

17 Compress 8 x 96-well plates to 1 x 96-well plate

17.1 Further compress plates 1-4 into a clean 96-well plate (Rows A-D). 4 wells will combine into 1 well of the new plate. Each original 384-well plate will be contained in one row of the 96-well plate.

- a. Carefully unseal plates 1-4, still on the magnets.
- b. Transfer eluted sample from plates 1-4 into a new 96-well plate.
- c. Combine rows A-D of plate 1 into row A of the new plate
- d. Combine rows E-H of plate 1 into row B of the new plate.
- e. Continue this pattern with A-D of plate 2 in row C of the new plate, etc, until rows E-H of plate 4 are in row H of the new plate.

17.2 Further compress 96-well plates 5-8 into new 96-well plate. 8 wells will combine into 1 well of the new plate. Each original 384-well plate will be contained in one row of the 96-well plate.

- a. Transfer eluted sample from plates 5-8 into a new 96-well plate.
- b. Combine rows A-D of plate 5 into row A of the new plate
- c. Combine rows E-H of plate 5 into row B of the new plate.
- d. Continue this pattern with A-D of plate 6 in row C of the new plate, etc, until rows E-H of plate 8 are in row H of the new plate.

17.3 Compress the two 96-well plates into single new 96-well plate.

- a. Transfer Columns 1-6 of first 96-well plate into Columns 1-6 of a new 96-well plate.
- b. Transfer Columns 7-12 of first 96-well plate into Columns 1-6 of the new 96-well plate to combine with previous transfer.
- c. Transfer Columns 1-6 of second 96-well plate into Columns 7-12 of the new 96-well plate.
- d. Transfer Columns 7-12 of second 96-well plate into Columns 7-12 of the new 96-well plate to combine with previous transfer.

17.4 *Note: final plate configuration will have six wells per original 384-well plate*

i.e., wells A1-A6 contain plate 1, wells B1-B6 contain plate 2, etc. for the rest of the left half of the plate. Wells A7-A12 contain plate 9, wells B7-B12 contain plate 10, etc. for the rest of the right half of the plate.

18 Perform bead cleanup on compressed 96-well plate.

18.1 Bind free DNA to the Sera-Mag beads:

- Thoroughly mix Sera-Mag beads by repeatedly inverting bottle and pour beads to the brim of the bead reservoir.
- Add 64 μ L beads to each well of the sample plate and pipette up and down to mix. Incubate at room temperature for 5 minutes.
- Move plate to plate magnet and wait until beads are settled to continue (approximately 3 minutes).
- Carefully remove supernatant and discard in waste trough.

18.2 Wash beads with 80% EtOH:

- Keeping plates on the magnets, add 180 μ L of 80% EtOH to plates 1-4 and incubate at room temperature for at least 30 seconds. Subsequently remove and discard EtOH in the waste trough.
- Repeat for a second wash.
- Remove plates from the magnets and incubate until beads are dry.

18.3 Elute DNA

- Add 10 μ L EB to each well of the sampleplate. Set a 5-minute timer.
- Seal plate, vortex until beads no longer cling to sides of wells, then quick spin for 5s at 100xg.
- After 5-minute timer is complete, unseal plate and place on magnet.
- Once beads have settled on the magnet (about 30 seconds), transfer elution to a clean 96-well plate.
- Check plate to ensure volume of wells appear even. Seal and quick spin (500xg).

DNA amplification

2h

19 Adaptase addition

*Steps in this section are automated for 8 or 16 original 384-well plates using Biomek i7

- Prepare appropriate volume of Adaptase Mix in a 1.5 mL microcentrifuge tube and keep on

ice.

A	B	C	D
Reagent	Vol per rxn	Vol per 8 plates	Vol per 16 plates
Elution Buffer (EB)	4.45 µL	239.2 µL	478.4 µL
Buffer G1	2.00 µL	112.5 µL	225.0 µL
Reagent G2	2.00 µL	112.5 µL	225.0 µL
Reagent G3	1.25 µL	70.3 µL	140.6 µL
Enzyme G4	0.50 µL	28.13 µL	56.26 µL
Enzyme G5	0.50 µL	28.13 µL	56.26 µL

2. Denature the 96-well plate in a thermocycler at 98 °C for 3 minutes. Immediately place on ice to prevent rehybridization. Plate is ready once bottom of plate is cool to the touch. Quick spin plate 5s at 500xg before unsealing.
3. Transfer 10.5 µL Adaptase mix to sample plate. Seal, vortex, and quick spin (5s at 500xg).
4. Incubate plate in a thermocycler with the following program:
 - a. 37°C for 30 minutes
 - b. 95°C for 2 minutes
 - c. Hold at 4 °C

20 PCR Reaction:

1. Add 5 µL PCR primers to sample plate. Be sure each set of 6 wells (the original 384-well plate) gets a uniquely indexed primer.
2. Transfer 25 µL KAPA to each well. Seal, vortex, and quick spin (5s at 500xg).
3. Incubate plate in a thermocycler using the following method:
 - a. 95°C for 2 minutes
 - b. 98°C for 30 seconds
 - c. 98°C for 15 seconds
 - d. 64°C for 30 seconds
 - e. 72°C for 2 minutes
 - f. Repeat steps c through e for a total of 13 cycles
 - g. 72°C for 5 minutes
 - h. Hold at 4°C

****Note:** 13 cycles is sufficient for mouse/human samples. More cycles should be run for smaller genomes (eg. Arabidopsis)
4. Plate can be held at 4°C overnight or stored at -20°C for longer times

21 Perform bead cleanup on PCR plate:

1. Thoroughly mix Sera-Mag beads by repeatedly inverting bottle and pour beads to the brim of the bead reservoir.
2. Add 40 μL beads to the sample plate and mix by pipetting. Upon addition of beads, start a 5-minute timer.
3. When 5-minute timer is complete, move plate onto magnet. Wait until the beads are settled to continue (approximately 3 minutes).
4. Carefully remove supernatant and discard in the waste trough.
5. Perform two 180 μL EtOH washes on plate.
6. After second EtOH wash is complete, remove plate from the magnet.
7. Once beads are dry, add 20 μL EB to each well of the plate. Start a 5-minute timer.
8. Seal plate, vortex until beads no longer cling to sides of wells, and quick spin for 5s at 100xg.
9. After 5-minute timer is complete, place plate onto magnet and carefully unseal.

22 Perform bead cleanup on compressed samples in tubes:

1. Using a 200 μL 8-channel pipette, compress wells such that samples from each original 384-well plate are contained in a single well.
 - a. Sample from A1-A6 should end up in A6, sample from B1-B6 should end up in B6, etc.
 2. Using a P-200 pipette, transfer sample from each well into an individually labeled, 1.5 mL microcentrifuge tube (16 tubes for 16 original 384-well plates).
 3. Add 96 μL Sera-Mag beads to each microcentrifuge tube. Set a 5-minute timer, then vortex and quick spin all tubes.
 4. After the 5-minute timer has completed, place each tube onto the DynaMag tube magnet. Allow beads to settle (approximately 3 minutes).
 5. Remove and discard supernatant, careful to not disturb the beads.
 6. Wash beads with 300 μL EtOH. Remove and discard EtOH.
 7. Repeat step 6.
 8. Using a P-10 pipette, remove any residual EtOH at the bottom of the tube and discard.
 9. Remove tubes from DynaMag tube magnet and allow to dry with lid open until beads are visibly cracked and there are no visible droplets of EtOH.
 10. Add 20 μL EB to each tube. Set a 5-minute timer, then vortex and quick spin all tubes.
 11. After 5-minute timer has completed, place each tube onto the DynaMag tube magnet.
 12. Transfer 20 μL from each sample tube into a new clean, labeled, 1.5 mL microcentrifuge tube. Try not to transfer any beads.
- *TIP: It helps to not push the tubes all the way down on the magnet. Slide them in about halfway so the bead pellet stays near the liquid.*

23 DNA Quantification and sample pooling:

1. To quantify the DNA in each sample tube, prepare an appropriate volume of Qubit reagent in a 15 mL microcentrifuge tube according to manufacturer's instructions.
 - a. Label one Qubit assay tube per sample. Label two additional tubes for the standards included in the Qubit kit. Also label tubes for any pools to be made.
 - b. Combine Qubit reagent and DNA in Qubit assay tubes according to manufacturer's specifications.
2. Measure the concentrations using the Qubit.
3. Create sequencing pool by combining normalized amounts of each sample tube.
4. Check the concentration of the pool using the Qubit. Pool is now ready for sequencing.