Apr 10, 2020

Single Nucleus Drop-seq (snDrop-seq)

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Human Cell Atlas Metho... KPMP

Zhang Lab

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External link: http://genome-tech.ucsd.edu/ZhangLab/

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Manuscript citation:
Lake, B.B., Chen, S., Hoshi, M. et al. A single-nucleus RNA-sequencing pipeline to decipher the molecular anatomy and pathophysiology of human kidneys. Nat Commun 10, 2832 (2019). https://doi.org/10.1038/s41467-019-10861-2

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

Created: March 29, 2019

Last Modified: April 10, 2020

Protocol Integer ID: 21909

Keywords: sequencing, single nuclei, DropSeq, seq protocol, seq, original drop, sndrop, mccarroll lab in the department, drop, protocol, mccarroll lab

Abstract

The protocol presented here is a Drop-Seq protocol modified for single nuclei.

The original Drop-Seq protocol comes from the McCarroll Lab in the Department of Genetics, Harvard Medical School.
http://mccarrolllab.org/download/905/

Materials

MATERIALS

- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0632
- 15 mL Falcon tubes
- PCR tubes
- SSC **Thermo Fisher Scientific** **Catalog #**AM9765
- 10x PBS **Thermo Fisher Scientific** **Catalog #**AM9624
- UltraPure Distilled Water **Invitrogen - Thermo Fisher** **Catalog #**10977-015
- TrypLE™ Express Enzyme **Thermo Fisher Scientific** **Catalog #**12604013
- TSO: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG **Integrated DNA Technologies, Inc. (IDT)**
- SMART PCR primer: AAGCAGTGGTATCAACGCAGAGT **Integrated DNA Technologies, Inc. (IDT)**
- New-P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTACACGCCT GTCCGCGGAAGCAGTGGTATCAACGCAGAGT∗ A∗C **Integrated DNA Technologies, Inc. (IDT)**
- Custom Read 1 primer: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAG AGTAC **Integrated DNA Technologies, Inc. (IDT)**
- BSA **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog ##**A8806
- Barcoded Bead SeqB: 5′ –Bead–Linker-TTTTTTTAAGCAGTGGTATCAAC GCAGAGTACJJJJJJJJJJJJNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′ **Chemgenes**
- Inverted Microscope **Catalog #**Motic AE31
- Syringe Pumps (3) **Catalog #**Legato 100
- Magnetic Stirrer **VP Scientific** **Catalog #**710D2
- Magnetic Mixing Discs **VP Scientific** **Catalog #**772DP-N42-5-2
- 3 mL syringes **Becton Dickinson (BD)** **Catalog #**BD #309657
- 10 mL syringes **Becton Dickinson (BD)** **Catalog #**BD 309695
- Tubing **Scientific Commodities** **Catalog #**BB31695PE/2
- Luer lock 26-gage needles **Becton Dickinson (BD)** **Catalog #**305111
- PDMS co-flow microfluidic droplet generation device **Catalog #**CAD file from McCarroll Lab
- 100 micron cell strainers (for beads) **VWR International (Avantor)** **Catalog #**21008-950
- 40 micron cell strainers (for cells) **VWR International (Avantor)** **Catalog #**21008-949
- Fuchs-Rosenthal hemocytometer **INCYTO** **Catalog #**DHC-F01
- Ficoll PM-400 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F5415-50ML
- Nuclease-free H2O **Life Technologies** **Catalog #**AM9930

Sarkosyl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L7414

EDTA 0.5 M **Life Technologies** **Catalog #**AM9260G

Tris 2M pH 7.5 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2944

Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533

30 um Uberstrainer **pluriSelect Life Science** **Catalog #**43-70030-03

50 mL centrifuge tubes **VWR International (Avantor)** **Catalog #**734-1876

Droplet Generation Oil **Bio-Rad Laboratories** **Catalog #**186-4006

Mineral Oil **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M5904

Tris 2M pH 8.0 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T3069

10% SDS **Teknova** **Catalog #**S0288

Tween-20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P9416

Maxima 5X RT Buffer **Thermo Fisher Scientific**

Maxima™ H Minus Reverse Transcriptase **Thermo Fisher Scientific** **Catalog #**EP0753

10 mM dNTPs **Takara Bio Inc.** **Catalog #**639125

RNase Inhibitor **Lucigen** **Catalog #**30281-1

10x Exo I Buffer **Thermo Fisher Scientific**

Exo I **Thermo Fisher Scientific** **Catalog #**FEREN0582

Kapa HiFi Hotstart Readymix **Kapa Biosystems** **Catalog #**KK2601

Agencourt AMPure XP beads **Beckman Coulter** **Catalog #**A63881

Qubit 4 Fluorometer **Thermo Fisher Scientific** **Catalog #**Q33238

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854

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Tubing **Scientific Commodities** **Catalog #**BB31695PE/2

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10 mL syringes **Becton Dickinson (BD)** **Catalog #**BD 309695

3 mL syringes **Becton Dickinson (BD)** **Catalog #**BD #309657

Magnetic Mixing Discs **VP Scientific** **Catalog #**772DP-N42-5-2

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- Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854
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- Ficoll PM-400 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F5415-50ML
- Exo I **Thermo Fisher Scientific Catalog #**FEREN0582
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- 3 mL syringes **Becton Dickinson (BD) Catalog #**BD #309657
- Tubing **Scientific Commodities Catalog #**BB31695PE/2
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- Tris 2M pH 8.0 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T3069
- 10% SDS **Teknova Catalog #**S0288
- EDTA 0.5 M **Life Technologies Catalog #**AM9260G
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- Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096
- Ficoll PM-400 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F5415-50ML

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- 30 um Uberstrainer **pluriSelect Life Science Catalog #**43-70030-03
- Luer lock 26-gage needles **Becton Dickinson (BD) Catalog #**305111
- Tris 2M pH 7.5 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T2944
- Maxima 5X RT Buffer **Thermo Fisher Scientific**
- Agencourt AMPure XP beads **Beckman Coulter Catalog #**A63881
- Barcoded Bead SeqB: 5′ –Bead–Linker-TTTTTTTAAGCAGTGGTATCAAC GCAGAGTACJJJJJJJJJJJJNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′ **Chemgenes**
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- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416
- Magnetic Stirrer **VP Scientific Catalog #**710D2
- Qubit 4 Fluorometer **Thermo Fisher Scientific Catalog #**Q33238
- Sarkosyl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L7414
- EDTA 0.5 M **Life Technologies Catalog #**AM9260G
- Fuchs-Rosenthal hemocytometer **INCYTO Catalog #**DHC-F01
- Inverted Microscope **Catalog #**Motic AE31
- BSA **Merck MilliporeSigma (Sigma-Aldrich) Catalog ##**A8806
- Droplet Generation Oil **Bio-Rad Laboratories Catalog #**186-4006
- Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096
- 100 micron cell strainers (for beads) **VWR International (Avantor) Catalog #**21008-950
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
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- TSO: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG **Integrated DNA Technologies, Inc. (IDT)**
- 100 micron cell strainers (for beads) **VWR International (Avantor) Catalog #**21008-950
- 10 mM dNTPs **Takara Bio Inc. Catalog #**639125
- RNase Inhibitor **Lucigen Catalog #**30281-1
- 10x Exo I Buffer **Thermo Fisher Scientific**

- Magnetic Mixing Discs **VP Scientific** **Catalog #**772DP-N42-5-2
- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P9416
- Nextera XT DNA Library Preparation Kit **Illumina, Inc.** **Catalog #**FC-131-1096
- PCR tubes
- 10% SDS **Teknova** **Catalog #**S0288
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0632
- Tubing **Scientific Commodities** **Catalog #**BB31695PE/2
- SMART PCR primer: AAGCAGTGGTATCAACGCAGAGT **Integrated DNA Technologies, Inc. (IDT)**
- Nuclease-free H2O **Life Technologies** **Catalog #**AM9930
- Maxima 5X RT Buffer **Thermo Fisher Scientific**
- Nuclease-free H2O **Life Technologies** **Catalog #**AM9930
- SSC **Thermo Fisher Scientific** **Catalog #**AM9765
- Qubit 4 Fluorometer **Thermo Fisher Scientific** **Catalog #**Q33238
- Exo I **Thermo Fisher Scientific** **Catalog #**FEREN0582
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10x PBS **Thermo Fisher Scientific** **Catalog #**AM9624

UltraPure Distilled Water **Invitrogen - Thermo Fisher** **Catalog #**10977-015

Troubleshooting

Prepare Buffers and Solutions

1 **BSA**

Make a 10% stock solution using BSA powder

BSA **Merck MilliporeSigma (Sigma-Aldrich) Catalog ##**A8806

2 **1X PBS**

Make a 1X stock solution using by diluting 10X PBS into nuclease-free distilled water

10x PBS **Thermo Fisher Scientific Catalog #**AM9624

UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #**10977-015

3 **Cell Loading Buffer** (makes 1 mL)

Prepare enough for the number of samples (1 mL per sample) that will be processed in one experiment

300 µL 20% Ficoll PM-400

Ficoll PM-400 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F5415-50ML

700 µL 1X PBS

4 **PBS-BSA**: make this fresh before each experiment

- Cell Loading Buffer
- 0.01% BSA (use the 10% stock)

5 **Lysis Buffer** (makes 1.2 mL)

Prepare enough for the number of samples (1 mL per sample) that will be processed in one experiment

960 µL H2O

Nuclease-free H2O **Life Technologies Catalog #**AM9930

12 µL 20% Sarkosyl

Sarkosyl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L7414

48 µL 0.5 M EDTA

EDTA 0.5 M **Life Technologies Catalog #**AM9260G

120 µL 2M Tris, pH 7.5

Tris 2M pH 7.5 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T2944

60 µL 1M DTT

Add this just prior to starting each DropSeq experiment

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632

6 **6X SSC**

Make 6X SSC working stock from 20X SSC

SSC **Thermo Fisher Scientific Catalog #**AM9765

7 **10 mM Tris pH 8.0**

Make 10 mM Tris pH 8.0 working stock from 2M Tris pH 8.0

Tris 2M pH 8.0 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T3069

8 **TE-SDS** (make 50 mL)

- 10 mM Tris pH 8.0 + 1 mM EDTA
- 0.5% SDS

EDTA 0.5 M **Life Technologies Catalog #**AM9260G

10% SDS **Teknova Catalog #**S0288

9 **TE-TW** (make 500 mL)

- 10 mM Tris pH 8.0 + 1 mM EDTA
- 0.01% Tween-20

EDTA 0.5 M **Life Technologies Catalog #**AM9260G

Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416

10 **RT mix** (makes 200 µL)

75 µL H2O

Nuclease-free H2O **Life Technologies Catalog #**AM9930

40 µL Maxima 5x RT Buffer

Maxima 5X RT Buffer **Thermo Fisher Scientific**

40 µL 20% Ficoll PM-400

Ficoll PM-400 20% in H2O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F5415-50ML

20 µL 10 mM dNTPs

10 mM dNTPs **Takara Bio Inc. Catalog #**639125

5 µL RNase Inhibitor

RNase Inhibitor **Lucigen Catalog #**30281-1

10 µL 50 uM Template Switch Oligo

TSO: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG **Integrated DNA Technologies, Inc. (IDT)**

10 µL Maxima H- RTase

Add after beginning breakage portion of the protocol (Step 27).

Maxima™ H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**EP0753

11 **Exonuclease Mix** (makes 200 µL)

20 µL 10x Exo I Buffer

10x Exo I Buffer **Thermo Fisher Scientific**

170 µL H2O

Nuclease-free H2O **Life Technologies Catalog #**AM9930

10 µL Exo I

Exo I **Thermo Fisher Scientific Catalog #**FEREN0582

Droplet Formation

12 Preparing the Barcoded Beads from Dry Resin

Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAGAGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo
Chemgenes Catalog #Macosko-2011- 10

The beads will arrive as a dry resin. To prepare the beads for an experiment from the dry resin, follow these steps:

1. Wash the resin with 30 mL ethanol
2. Wash twice with 30 mL TE-TW
3. Resuspend in 20 mL TE-TW
4. Pass through 100 micron strainer
5. Count the beads using a Fuchs-Rosenthal hemocyctometer
6. Store the counted beads at 4 °C

Note

To pellet the beads when washing, centrifuge at 1000xg for 00:01:00

Note

Do NOT vortex the barcoded beads. Vortexing will shear the beads and cause damage. To mix, simply invert tube gently a couple of times until mixed.

100 micron cell strainers (for beads) **VWR International (Avantor) Catalog #**21008-950

Fuchs-Rosenthal hemocytometer **INCYTO Catalog #**DHC-F01

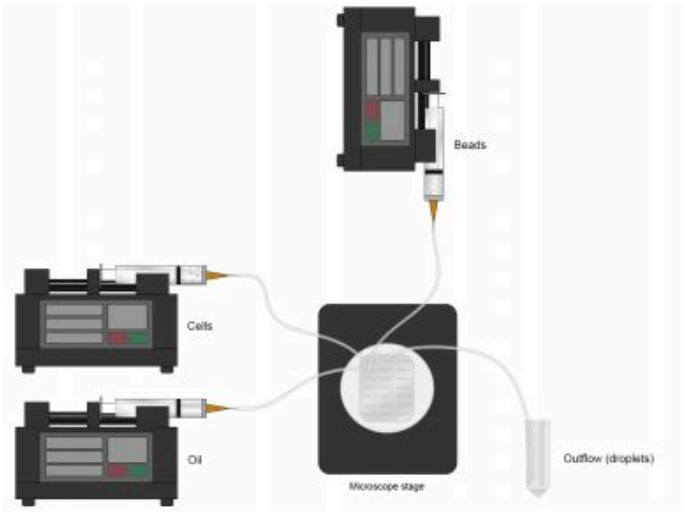
13 Arrangement of components

Syringe Pumps (3) **Catalog #**Legato 100

Inverted Microscope **Catalog #**Motic AE31

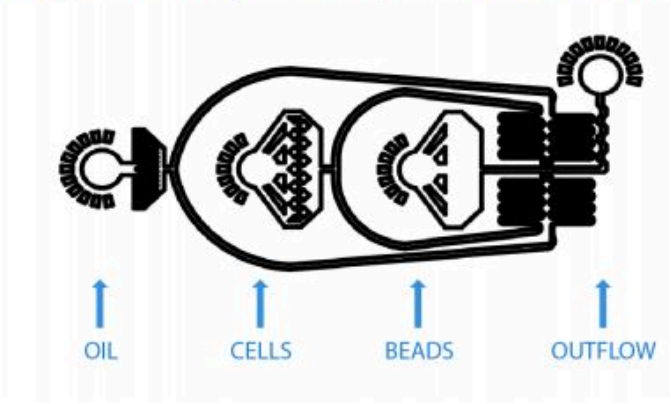
Magnetic Stirrer **VP Scientific Catalog #**710D2

Arrangement of components



Attachment of tubing into the device

Tubing is attached by pressing the tip of the tube into the circular punched holes.



Set up the syringe pumps next to the inverted microscope. Note: arrange the bead pump so that the syringe is pointing downward ratherly than horizontally. Place the magnet stirrer close to the barrel of the bead syringe.

Source: http://mccarrolllab.org/download/905/

14 Prepare Pump System

Syringe Pumps (3) **Catalog #**Legato 100

1. Power on the syringe pumps (switches located on the back left of the pump)
2. Set up correct flow rates using the screen on the pump

- Oil: 15,000 ul/hr
- Cells: 4,000 ul/hr
- Beads: 4,000 ul/hr

15 Prepare microfluidic cell

Inverted Microscope **Catalog #**Motic AE31

PDMS co-flow microfluidic droplet generation device **Catalog #**CAD file from McCarroll Lab

Tubing **Scientific Commodities Catalog #**BB31695PE/2

15 mL Falcon tubes

1. Cut and remove protective plastic top layer for one device on the chip
2. Cut the outflow tubing to proper length and connect it to the device so that the collection from the outflow can be safely collected
3. Use clean 15 mL tubes to catch the droplets
4. Use a waste container to catch waste

16 Prepare the beads

Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAGAGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo **Chemgenes Catalog #**Macosko-2011- 10

1. Take an aliquot of beads (final concentration: 120,000 beads/mL)
2. Spin down in a tabletop centrifuge
3. Remove the TE-TW storage buffer
4. Wash with DTT minus lysis buffer
5. Resuspend in Lysis buffer

17 Prepare nuclei according to the protocol "Isolation of single nuclei from solid tissues" steps 1-14.
dx.doi.org/10.17504/protocols.io.ufketkw

Dilute nuclei to 100 cells/ul. Use PBS-BSA for final dilution

18 Prepare syringes and lines for microfluidic device

Prepare enough syringes and lines for all samples at once (4-6 samples per experiment)

1. Oil syringe: 10 ml
2. Cell/Sample syringes: 3 ml
3. Bead syringes: 3 ml with magnetic mixing disc
4. Measure out tubing for the lines; make sure they are long enough to reach the microfluidic device from where the syringes will sit on the pumps
5. For each line cut one blunt end (for blunt end needle) and one angled end (insert into the device)
6. UV all tubing and needles

10 mL syringes **Becton Dickinson (BD)** **Catalog #**BD 309695

3 mL syringes **Becton Dickinson (BD)** **Catalog #**BD #309657

Magnetic Mixing Discs **VP Scientific** **Catalog #**772DP-N42-5-2

Tubing **Scientific Commodities** **Catalog #**BB31695PE/2

Luer lock 26-gage needles **Becton Dickinson (BD)** **Catalog #**305111

19 Load Syringes

Note

To load syringe, firmly press the tip of a 1000 ul pipette into the head of the syringe and slowly pull back on the plunger to draw in the solution. Pressing the tip in firmly helps reduce the introduction of bubbles. While holding the syringe in a vertical orientation, gently push out the air bubbles. Affix a 26G needle and tubing.

Note

Before droplet generation, coat connecting tubing and syringes with 1% BSA to prevent non-specific binding of nuclei to the surface, and then rinse with PBS

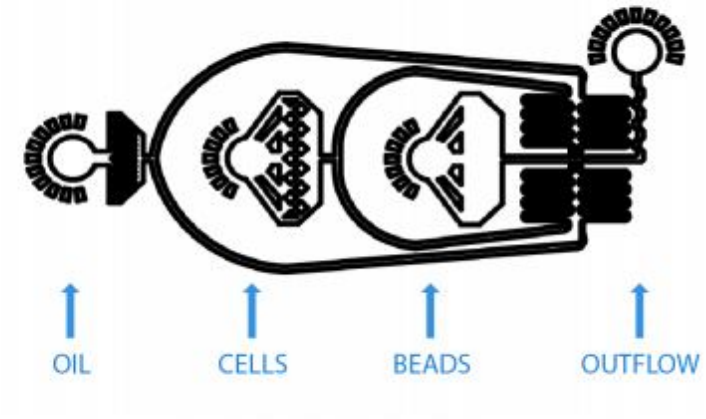
1. Flush the tubing with corresponding buffer; keep syringes vertical when flushing
2. Use 1 mL cell buffer to flush syringe/tube for each sample
3. Use 1 mL DTT minus lysis buffer to flush syring/tube for each bead setup
4. Load the syringes; keep syringes vertical when loading
5. Load cells/nuclei into cell syringes, making sure there are no air bubbles
6. Load beads into bead syringes, making sure there are no air bubbles
7. After beads are loaded in the syringe, keep horizontal to prevent clogging

20 System Assembly

1. Place oil syringe in pump and secure; using the screen, hold the free tube over the waste collection, then slowly move the pusher to the plunger until oil drips out
2. Push the angled end of the tubing into the device
3. Place cell/sample syringe in pump and secure; using the screen, hold the free tube over the wase collection, then slowly move the pusher to the plunger until buffer drips out
4. Push the angled end of the tubing into the device
5. Before placing the bead syringe, turn on the magnetic mixer for stirring disc (speed 25-30). Carefully rotate bead syringe by hand to mix up the beads in the lysis buffer.
6. Place bead syringe in pump and secure. Make sure the magnetic disc is circling up and down vertically in the syringe to keep the beads well mixed.
7. Using the screen, hold the free tube over the wase collection, then slowly move the pusher to the plunger until buffer drips out
8. Push the angled end of the tubing into the device

Attachment of tubing into the device

Tubing is attached by pressing the tip of the tube into the circular punched holes.



Source: http://mccarrolllab.org/download/905/

21 Run Pumps for Droplet Formation

1. Start oil pump
2. Start bead pump
3. Start cells/sample pump

22 Check for Droplet Formation

1. Have outflow line go to waste
2. Watch the beads move from the syringe to the device
3. Once the beads get to the bead chamber in the device, use a clean microscope slide to catch a drop of output
4. Check slide under a different microscope to see if droplets are uniform in size and shape
5. Once droplets are uniform begin collecting

23 Collect

1. Collect uniform droplets in 15 ml Falcon tubes until cells/sample runs out
2. The pump will sound an alarm once the cell/sample runs out; turn off alarm
3. STOP collecting in the 15 ml Falcon tube immediately (place a waste container under the outflow)

24 Stop pumps

1. Stop all pumps in any order
2. Place the 15 ml Falcon tube with droplets **on ice**

25 When running more than one sample go to step #20

Repeat Step 20- Step 24 for each sample

26 Clean up (**AFTER** Reverse Transcription is set up to incubate)

1. Turn off magnetic mixer
2. Remove syringes from pumps; remove tubing from microfluidic device
3. Power off pumps (switches located on the back left of the pump)
4. Cell/sample outflow tubing goes in biohazardous solid waste
5. All other lines can go in regular waste
6. Cell/sample syringes go in biohazardous solid waste
7. All other syringes can go in regular waste
8. Cell/sample needles go in biohazardous sharps waste
9. All other needles go in regular sharps waste

STAMP Collection

27 **S**ingle cell/nuclei **T**ranscriptomes **A**ttached to **M**icro**P**articles (STAMP)

Note

STAMPs are the barcoded beads with mRNA attached; for stability, work **on ice/keep cold** when working with STAMPs/RNA

Note

Add Maxima H- RTase to RT Mix (Step 10)

Prepare waste and collection tube

- Need one 50 mL Falcon tube for waste (can use same waste from preparing syringes)
- Need one new clean 50 mL Falcon tube for collection
- Need one 30 um Uberstrainer

- Autopipette, 5 mL pipette, and attachment for strainer

50 mL centrifuge tubes **VWR International (Avantor)** **Catalog #**734-1876

30 um Uberstrainer **pluriSelect Life Science** **Catalog #**43-70030-03

28 Breakage

1. Be careful not to disturb the oil/droplet band (direct buffer down the side of the 15 mL Falcon tube and control output so there is smooth flow)
2. Before droplet breakage, add 600 µL mineral oil and incubate in water bath at 72 °C for 00:05:00
3. Let sit on ice for 00:05:00
4. Remove the oil from the bottom of the 15 mL Falcon tube
5. Add 5 mL cold 6X SSC
6. Add 1 mL Perfluorooctanol (PFO) directly to the oil/droplet band to break the droplets; dispense in a circular motion directly over/onto the droplet bad
7. Gently roll/rotate the 15 mL Falcon tube on ice to help break the droplets

Mineral Oil **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M5904

Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533

29 Filter/Collection

Set up the 30 um Uberstrainer over the waste tube

1. Place strainer over waste tube (goal: save beads, discard supernatant)
2. Attach autopipette attachment to strainer
3. Angle/tilt tube so that no liquid will go up through the autopipette when using it for a vacuum
4. Use 1 mL cold 6X SSC to moisten the filter

Filter the beads (over waste tube)

1. Pass the aqueous (top layer) phase through the strainer
2. Pass the organic (bottom layer) phase through the strainer
3. Use a couple mililiters cold 6X SSC to rinse the 15 mL Falcon tube to try and get all the beads from the sides; pass the rinse through the strainer

4. Rinse strainer/filter twice with cold 6X SSC

Collect the beads (over new/clean 50 mL Falcon tube)

1. Carefully reverse the strainer over a new/clean 50 mL Falcon tube
2. Wash strainer with 1 mL cold 6X SSC . Repeat four more times (5 washes total)
3. Visually inspect the strainer to make sure all the beads have come off it

30 Spin Down

1. Using a 4 °C centrifuge , spin down the 50 mL Falcon tube at 1000xg for 00:01:00
2. Carefully remove, supernatant, leaving approximately 1 mL cold 6X SSC

31 Transfer to 1.5 mL tube

1. Resuspend beads in remaining 1 mL cold 6X SSC
2. Transfer to a new/clean 1.5 mL microfuge tube
3. Spin down (1000xg for 00:01:00)
4. Wash twice with 1 mL cold 6X SSC
5. Wash with 300 µL 5X RT buffer
6. Remove as much of the 5X RT buffer as possible without taking up any beads

Maxima 5X RT Buffer **Thermo Fisher Scientific**

Reverse Transcription

32 This step generates cDNA strands on the RNA hybridized to the bead primers. One RT mix is sufficient for processing approximately 90,000 beads.

1. Add 200 µL RT Mix to the beads
2. Incubate at Room temperature with rotation for 00:30:00
3. Incubate at 42 °C with rotation for 01:30:00
4. Wash beads once with 1 mL TE-SDS
5. Wash beads twice with 1 mL TE-TW

6. If proceeding to Exonuclease I treatment, wash once with
   1 mL 10 mM Tris pH 8.0

Note

Stopping point: if stopping, stop at TE-TW wash step. Beads can be stored at
4 °C in TE-TW

Exonuclease I Treatment

33 This step chews back the excess bead primers that did not capture an RNA molecule. One Exonuclease Mix is sufficient for processing approximately 90,000 beads.

1. After washing once with 1 mL 10 mM Tris pH 8.0 , resuspend in
   200 µL Exonuclease Mix
2. Incubate at 37 °C with rotation for 00:45:00
3. Wash beads once with 1 mL TE-SDS
4. Wash beads twice with 1 mL TE-TW
5. If proceeding to PCR, wash once with 1 mL H2O

Note

Stopping point: if stopping, stop at TE-TW wash step. Beads can be stored at
4 °C in TE-TW

PCR

34 This steps utilizes PCR to amplify the cDNA constructed during the Reverse Transcription step.

Bead Count

1. After washing once with 1 mL H2O , spin down to pellet beads
2. Remove supernatant

3. Add 1 mL H2O
4. Mix well by pipette to evenly resuspend the beads
5. Quickly remove 20 µL and pipette into a Fuchs-Rosenthal hemocytometer chamber
6. Count all 16 boxes

35 Calculate number of beads to split into PCR tubes

1. Calcuate the concentraion (beads/ul) = (#beads counts/16) x 5
2. Apportion 2,000 beads into each PCR tube (want approximately 100 STAMPs per PCR tube)

36 Prepare Master Mix

Master Mix (50 µL per PCR reaction)

24.6 µL H2O
Nuclease-free H2O **Life Technologies Catalog #**AM9930
0.4 µL 100 uM SMART PCR Primer
SMART PCR primer: AAGCAGTGGTATCAACGCAGAGT **Integrated DNA Technologies, Inc. (IDT)**
25 µL 2x Kapa HiFi Hotstart ReadyMix
Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2601

37 PCR

1. Spin down tubes
2. Add 50 µL PCR Master Mix to each reaction
3. Mix well by pipette
4. Run the following program on a thermocycler

95 °C for 00:03:00
4 cycles of
98 °C for 00:00:20
65 °C for 00:00:45
72 °C for 00:03:00
13 cycles of

98 °C for 00:00:20
67 °C for 00:00:20
72 °C for 00:03:00

Then

72 °C for 00:05:00
4 °C forever

Purification and Quantification

38 Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, add 30 µL room temperature magnetic beads to each PCR sample (this is a 0.6x beads to samples ratio based on volume)
2. Purify according to manufacturer's instructions
3. Elute in 10 µL H2O

Agencourt AMPure XP beads **Beckman Coulter** **Catalog #**A63881

39 Quantification

Can be performed by preferred method (qPCR, Qubit Assay, BioAnalyzer, Tapestation); we used Qubit Assay.

1. Use 2 µL sample input for this quantification
2. Quantify according to manufacturer's instructions

Qubit 4 Fluorometer **Thermo Fisher Scientific** **Catalog #**Q33238

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854

Note

The yield for 2000 beads generated from a 50 cell/ul total yield should be approximately 1-100ng.

QC Cutoff: > 1ng total

40 Amplified cDNA Gel

1. Confirm average size of cDNA library via gel electrophoresis
2. Average size should be between 1300-2000 bp

Tagmentation

41 This step simultaneously tags and fragments the cDNA library using transposomes

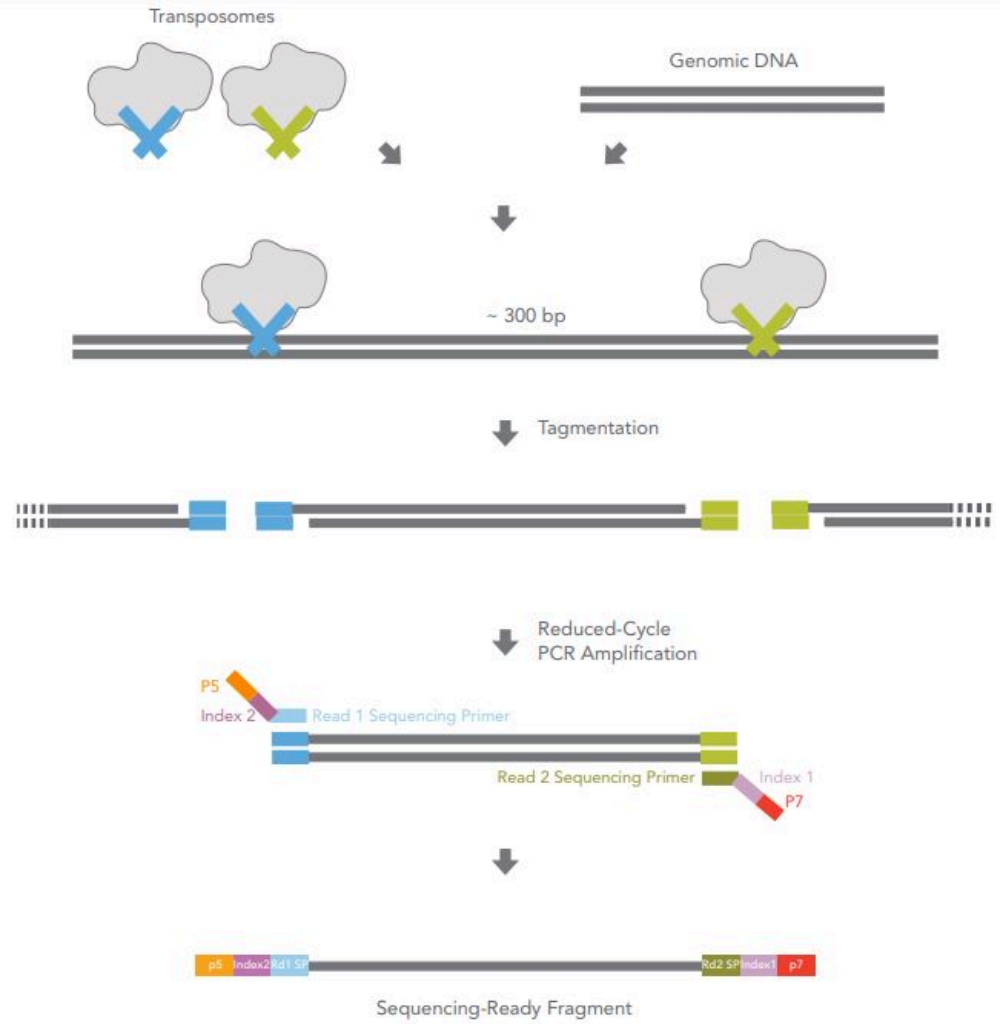


Figure 2: Nextera Library Preparation Biochemistry—Nextera chemistry simultaneously fragments and tags DNA in a single step. A simple PCR amplification then appends sequencing adapters and sample indexes to each fragment.

Source: https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/datasheet_nextera_dna_sample_prep.pdf

42 Nextera Kit

1. Preheat a thermocycler to 55 °C
2. Working in a cold rack/on ice: for each sample, combine 600 pg purified cDNA with **H2O** in a total volume of 5 µL

3. To each tube, add 10 µL Nextera TD buffer "Tagment DNA" and 5 µL Amplicon Tagment enzyme "Amplicon Mix" (the total volume of the reaction is now 20 µL)
4. Mix by pipette approximately 5 times
5. Spin down
6. Incubate at 55 °C for 00:05:00
7. Add 5 µL Neutralization Buffer (the total volume of the reaction is now 25 µL)
8. Mix by pipette approximately 5 times
9. Spin down (bubbles are normal)
10. Incubate at Room temperature for 00:05:00

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog** #FC-131-1096

43 PCR work up of tagmented library

Prepare PCR Master Mix (24 µL per PCR reaction)

15 µL Nextera PCR mix

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog** #FC-131-1096

8 µL H2O

Nuclease-free H2O **Life Technologies Catalog** #AM9930

1 µL 10 uM New-P5-SMART PCR hybrid oligo

New-P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTACACGCCT GTCCGCGGAAGCAGTGGTATCAACGCAGAGT* A*C **Integrated DNA Technologies, Inc. (IDT)**

44 PCR

1. Add 24 µL Tagmentation PCR Master Mix to each tube
2. To each individual tube, add 1 µL 10 uM Nextera N7XX oligo The total volume of the reaction is now 50 µL
3. Mix well and run the following program on a thermocycler

95 °C for 00:00:30

12 cycles of

95 °C for 00:00:10

55 °C for 00:00:30

72 °C for 00:00:30

Then

72 °C for 00:05:00

4 °C forever

Purification and Quantification

45 Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, add 30 µL room temperature magnetic beads to each PCR sample (this is a 0.6x beads to samples ratio based on volume)
2. Purify according to manufacturer's instructions
3. Elute in 12 µL to 15 µL H2O

Agencourt AMPure XP beads **Beckman Coulter Catalog #**A63881

46 2nd Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, perform a 0.6x purification
2. Purify according to manufacturer's instructions
3. Elute in 12 µL to 15 µL H2O

Agencourt AMPure XP beads **Beckman Coulter Catalog #**A63881

47 Quantification

Can be performed by preferred method (qPCR, Qubit Assay, BioAnalyzer, Tapestation); we used Qubit Assay.

1. Use 1 µL sample input for this quantification
2. Quantify according to manufacturer's instructions
3. Convert ng/ul to nM

Qubit 4 Fluorometer **Thermo Fisher Scientific Catalog #**Q33238

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854

Note

The yield for 2000 beads generated from a 50 cell/ul final cell concentration should be approximately 400-1000 pg/ul.

QC Cutoff >1ng

48 Sequencing Library Gel

1. Confirm average size of Sequencing library via gel electrophoresis
2. Average size should be between 500-680 bp

Note

Smaller-sized libraries will have more polyA reads; larger libraries may have lower sequence cluster density and cluster quality. Although the target size is 500-680 bp, the range can be as broad as 420-700 bp.

Sequencing

49 MiSeq Sequencing - **QC for estimation of library quality and number of nuclei captured**

1. Pool, denature, and dilute to loading concentration according to manufacturer's instructions
2. Sequencing specifications

- Read 1: 30 bp
- Read 2: 75~100 bp
- Read 1 Index: 8 bp
- Custom Read 1 primer

Hiseq 2500 Sequencing

1. Combine 8-12 snDrop-seq libraries to make a 10 µl library pool at 3 nM for denaturation.
2. After final dilution, load a combined library at 12 pM to the sequencer

- Read 1: 30 bp
- Read 2: 75~100 bp
- Read 1 Index: 8 bp
- Custom Read 1 primer

Custom Read 1 primer: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAG AGTAC **Integrated DNA Technologies, Inc. (IDT)**

MiSeq v3 (150 cycle) Kit **Illumina, Inc. Catalog #**MS-102-3001

Sequencing

50 snDrop-seq data processing

Mapping, demultiplexing and QC processing:
https://github.com/chensong611/Dropseq_pipeline

- Paired-end reads are removed if read 1 had more than four non-T bases in the last ten bases (to remove all non-poly(T)-captured contaminated reads)
- Paired-end reads are removed if read 1 had one or more bases with a poor quality score (<10)
- The right mate of each read pair is trimmed to remove any portion of the SMART adaptor sequence or any large stretches of poly(A) tails (6 consecutive bp or larger)
- The trimmed reads are aligned to the human genome (GENCODE GRCH38) with STAR (e.g. v2.5) with default parameter settings
- Reads that mapped to intronic or exonic regions as per the GENCODE gene annotation are included in gene counts
- Barcode synthesis errors are corrected by inserting N at the last base of the cell barcode for reads in which the first 11 bases of the cell barcode are identical and the last T base of the UMI is the same
- UMI counts for each gene of each nucleus are assigned by collapsing UMI reads that had only 1 edit distance to create a digital expression matrix (genes as rows, cells or nuclei as columns)