Drop-Seq Laboratory Protocol

version 3.1 (12/28/15)

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The following is our most current protocol for Drop-seq. It should also help you find all the equipment and reagents you need to get started.

We have continued to optimize this protocol since doing the experiments in the Macosko et al. *Cell* paper. What we are sharing here is our current, optimized protocol. As a result, this protocol will not precisely match the methods section of the paper, which is a description of the experiments done in the paper. Please feel free to use these optimizations without author consideration - just acknowledge "helpful advice from Evan Macosko, Melissa Goldman and Steve McCarroll", and please also include the URL and version number of the protocol in your methods section. This protocol also includes hints, suggestions and images that we could not fit into the the methods section of the paper.

As scientists begin to adopt Drop-seq and we learn about their experiences, we will add additional hints and tips to the protocol, and release updated protocols with a new date **(you are reading version 3.1, posted December 2015)**. We will increment the version number whenever we change something substantive about the recommendation for a protocol step. If you would like to be notified of future protocol updates, please send us an email at dropseq@gmail.com.

Starting with a species-mixing experiment is a critical first step for validating that your experimental setup is successfully producing libraries with high single-cell integrity. *It is critical to do a successful species-mixing experiment before doing any other single-cell experiment.* The protocol here includes step-by-step instructions for performing Drop-seq analysis of a human and mouse cell mixture (HEK and 3T3 cells). Successful demonstration that cell barcodes yield organism-specific libraries in such an experiment will ensure that you have all the experimental and computational components in place to produce high-quality single-cell libraries.

We will maintain a website with protocol updates and other information:

www.mccarrolllab.com/dropseq

We plan to start a question-and-answer forum as users get started; we will monitor the forum to answer new users' questions as they begin working with the technology.

Necessary start-up equipment:

- **An inverted microscope** to view the device (we use the Motic AE31)
- **Three syringe pumps** (we use KD Scientific Legato 100)
- **A highly controllable, powerful magnetic mixing system**; I have looked at options extensively and the VP Scientific (Part #710D2) magnetic stirrer is both sufficiently powerful and gentle to keep the beads suspended while not breaking them. You may consider buying a second mixer if you think it will be necessary to also mix your cells while they are sitting in the syringe, but typically we do not mix our cells. We load one **mixing disc** into the beads syringe to stir the beads, which you can also get from VP (VP cat # 772DP-N42-5-2). These mixing discs are thicker than the ones we had recommended previously, and therefore are better at keeping the beads uniformly suspended.
- **3 mL syringes** (we use BD #309657)
- **Tubing** to connect syringes to the device: Scientific Commodities, inc. (cat # BB31695-PE/2)
- **Luer lock 26-gage needles**. The connection between the tubing and the needle is intentionally quite snug; I do not use 27G needles (though the fit is easier) because the beads are more likely to clog in the smaller bore.
- **PDMS co-flow microfluidic droplet generation device.** We provide a CAD file with the *Cell* paper for the devices we used for all experiments in the paper. These devices were designed by our collaborator Anindita (Oni) Basu. Though many ideas and much optimization went into their design, their construction is straightforwardly accomplished in any academic or commercial microfluidics facility, as they are passive PDMS devices. Some commercial microfluidics companies that will make custom devices from CAD files include FlowJem, Nanoshift, and Dolomite Microfluidics.
- **100 micron cell strainers** for beads (VWR cat #21008-950)
- **40 micron cell strainers** for cells (VWR cat #21008-949)
- **Fuchs-Rosenthal hemocytometer** (Incyto # DHC-F01)

Required primers:

Name in this protocol	Name in Macosko, et. al	Sequence
Barcoded Bead SeqB	Barcoded Bead SeqB	5’ –Bead–Linker-TTTTTTTAAGCAGTGGTATCAACGCAGAGTACJJJJJJJJJJJJNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’
TSO	Template_Switch_Oligo	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
SMART PCR primer	TSO_PCR	AAGCAGTGGTATCAACGCAGAGT
New-P5-SMART PCR hybrid oligo	P5-TSO_Hybrid	AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C
Custom Read 1 primer	Read1CustomSeqB	GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC

Ordering the beads and what to do when they arrive:

We taught scientists at a local company, Chemgenes, how to do the split-and-pool synthesis described in the Cell paper. We have extensively tested these beads and in fact used them for all the experiments in the paper. Chemgenes (http://www.chemgenes.com) has made these “Barcoded Bead SeqB” beads available for purchase (you can ask them for the beads used in Macosko et al.)

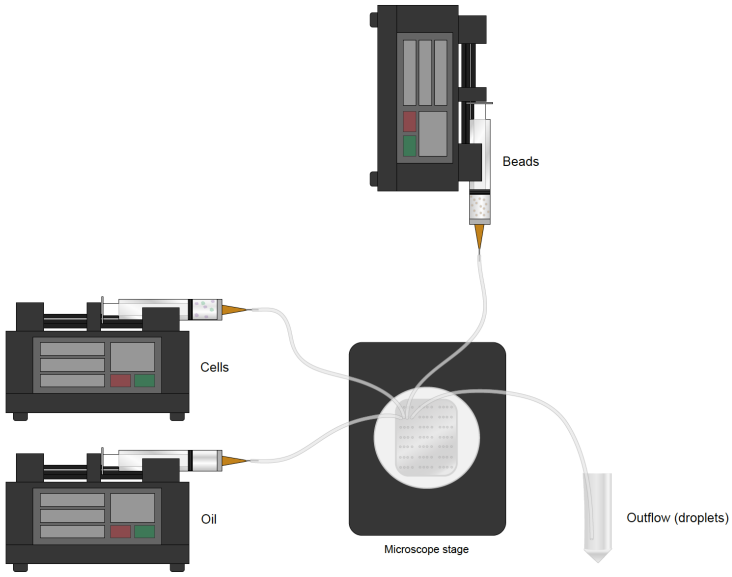
The beads arrive as a dry resin. Wash the resin once with 30 mL ethanol, then twice with 30 mL TE-TW. Resuspend in 20 mL TE-TW, pass through a 100 micron strainer, and count the beads using a Fuchs-Rosenthal hemocytometer. Store the counted beads at 4 C. We have stored beads in this way for >6 months without any apparent loss of activity.

> *Note: to pellet the beads when washing, we centrifuge at 1000xg for 1 min. Your centrifuge may require more time, or an adjustment of brake speed, which can kick up beads from the bottom if it is too high.*

Arranging and connecting your droplet generation set-up:

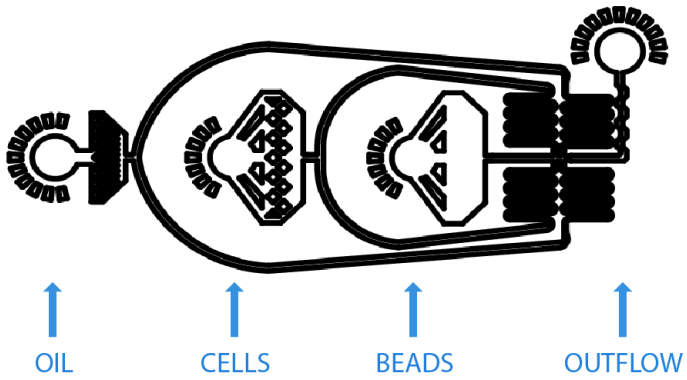
Set up the three syringe pumps next to the inverted microscope. It is best to have the bead pump resting on its side so that the syringe is angled down (rather than positioned horizontally, see figure below). We accomplish this by resting the bead pump on a shelf above the microscope. Your magnetic stirrer should be positioned close to the barrel of the bead syringe; once you find a good angle to get good mixing in the syringe you can fix the magnet in place, although it can be helpful to be able to move the magnet away from the syringe as the volume decreases in order to prevent the mixing disc from getting stuck in a vertical position.

Arrangement of components



Attachment of tubing into the device

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



Chemical reagents:

For cells

- TrypLE Express Enzyme (Life Technologies, #12604013)
- **BSA:** make a 10% solution using BSA powder (Sigma #A8806), store aliquots at -20 C
- **PBS-BSA:** make this fresh before each experiment
  - 1X PBS
  - 0.01% BSA (use the 10% stock)

For beads

- **Lysis Buffer (makes 1 mL):** *can store large stocks without DTT at room temperature*
  - 500 ul H2O
  - 300 ul 20% Ficoll PM-400 (GE healthcare)
  - 10 ul 20% Sarkosyl (Sigma #L7414))
  - 40 ul 0.5 M EDTA (Life Technologies)
  - 100 ul 2 M Tris pH 7.5 (Sigma)
  - 50 ul 1 M DTT → add this just prior to starting each Drop-seq experiment

Droplet generation oil

- Bio-Rad, catalog # 186-4006

Post droplet generation

- **6X SSC**
- **Perfluorooctanol (PFO)** (Sigma #370533)
- **TE-SDS (makes 50 mL):**
  - 10 mM Tris pH 8.0 + 1 mM EDTA
  - .5% SDS
- **TE-TW solution:**
  - 10 mM Tris pH 8.0 + 1 mM EDTA
  - 0.01% Tween-20
- **10 mM Tris pH 8.0**

Your first Drop-seq run:

The first experiment you should perform in your lab is with a mixture of intact human (HEK) and mouse (3T3) cells as we did in the *Cell* paper. Using a mixture of human and mouse cells will allow you to evaluate whether the loading concentration, droplet quality, and downstream library preparation are all working to give you data that is single-cell-resolution. It will also allow you to measure the single-cell purity of your libraries and your cell doublet rate. **We cannot emphasize strongly enough that it is critical to start with species-mixing experiments in order to evaluate the quality of the data you are generating and know whether it is truly single-cell.**

Using HEK and 3T3 cells (rather than a different mouse and human cell line) will also allow you to compare your transcript yield to the data in Macosko *et al.* 2015, to evaluate sensitivity (capture rates).

Once you have been successful, please email us a plot of your species-mixing results analogous to the plot in Figure 3A of the *Cell* paper. We would love to keep a gallery of people's successful Barnyard plots (and, with permission, to post it online). You can email us at dropseq@gmail.com .

Anticipating your yield of STAMPs

The number of STAMPs generated per hour is a product of three factors:

1. The concentration of cells used. A higher concentration of cells yields higher throughput, but also introduces more doublets and impurities (see Figure S3B in Macosko et al. 2015).

2. The concentration of beads used. We keep this fixed at around 120 beads/ul, which generates fewer than 5% bead doublets.

3. The size of droplets used. For the Cell paper, we used a microfluidic device that generates droplets that are 125-microns in diameter (almost exactly 1 nL). However, since the paper's publication, we have learned from our own experience, and from that of colleagues, that home-made microfluidic devices can have significant variations in droplet diameter, based on several factors related to microfluidic device master fabrication. ***We therefore recommend that you perform a test of droplet volume for devices fabricated with a particular master (the procedure for measuring droplet volume can be found at the end of this protocol).*** Please also note that if your droplet size differs significantly from 125-microns, you may need to adjust your flow rates from the numbers recommended in this protocol. We have used devices that produce as small as 90 micron droplets without observing any major change in Drop-seq data quality. We anticipate that droplet sizes in the range of 90-140 microns in diameter should produce perfectly fine data.

We recommend starting with a cell concentration of 100 cells/ul (final concentration in droplets will be 50 cells / ul when mixed 1:1 with lysis buffer and beads). At this concentration, ~5% of beads collected will have been exposed to a cell (a.k.a. STAMPs). If beads are flowed in at a concentration of 120 beads/ul, this yields 30,000 STAMPs (4 mL/hr * 120000 beads/mL * 0.05 = 24,000). Only 20-40% of beads are recovered after all washing and enzymatic steps, meaning that, practically speaking, ~10,000 STAMPs can be generated in 1-2 hours of droplet generation.

Pre-run setup

1. Load oil into a **10 mL syringe**. Affix needle to tubing, push up slightly on the plunger to expel all the air bubbles, then load the syringe into the pump as shown below (we use a 20 mL syringe for bigger experiments). Set the flow rate to 30,000 ul/hr, and press run until you see oil dripping out of the tubing. Set the flow rate to 15,000 ul/hr. Once you are certain that the oil is no longer dripping out of the tubing, insert the free end of the tubing into the left-most channel of a clean device (see figure on page 4).

Hint: cut the tubing on a sharp angle to facilitate its insertion into the device

2. Cut a shorter bit of tubing for an outflow channel, and insert it into the right-most channel of the same device. Let the free end hang into a designated waste container.

3. Prepare the beads**:**

    - Take out an aliquot of beads (remember that you want a final concentration of about 120,000 beads/mL). Spin down in a tabletop centrifuge, remove the TE-TW, and resuspend in Lysis buffer.

    - For a standard drop-seq requiring 1 mL of bead flow, resuspend the beads in 950uL Lysis buffer and mix in 50 ul of 1 M DTT just prior to starting droplet formation.

4. Prepare the cells: (the following is for HEK and 3T3 cells only)

    - Trypsinize for 5 min with TrypLE. Collect + spin down at 300xg for 5 min.

    - After spinning down post-trypsinization, resuspend in 1 mL of **PBS-BSA**.

        Hint: while non-stick tubes work best for later parts of the protocol, regular tubes perform better during this step.

    - Spin in microcentrifuge at 300xg for 3 minutes.

    - Remove supernatant, and resuspend in 1 mL of plain PBS. Pass through a 40 micron filter, and count.

    - Prepare a 1:1 mix of the two cells types (1:1 HEK to 3T3 cells) at a final combined concentration of 100 cells/ul. Use **PBS-BSA** to make this final dilution.

Loading cells and beads

1. Position your device on the microscope stage. Make sure you select a device that is clean and free of any defects or large particles of dust.

2. Draw up the cell suspension into a 3 mL luer-lock syringe. While holding the syringe in a vertical orientation, gently push out the air and bubbles. Affix a 26G needle, and cut a piece of tubing to connect the syringe to the device.

    Hint: to load the syringe, firmly press the tip of a 1mL pipet 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.

    Hint: when inserting the needle into one end of the tubing be careful not to pierce the tubing - even if the nick is in the part of the tubing that ends up being higher up on the needle, it is better to entirely cut off that portion of the tubing and try again.

3. Place a magnetic mixing disc into the barrel of a second 3 mL luer-lock syringe. Draw up the

bead suspension, push out the excess air and bubbles, and affix a 26G needle. Cut a piece of tubing to connect the bead syringe to the device.

4. The next step is to load the cell syringe into the device (the beads should always be loaded last). Place the cell syringe into its pump so that the plunger is flush with the moving pump surface (the same way you did with the oil). Adjust the flow rate to 30,000 ul/hr and briefly run to push all air out of the system until you see a small bead of liquid dripping from the free end of the tubing. Stop the pump, set it to **4,000 ul/hr**, and insert the free end of the tubing into the cell channel of the microfluidic device (see figure on page 2).

5. Turn on the magnetic mixer (for the VP mixer, use a speed of 25-30. Never go above 35 as this can lead to significant shearing of beads). Begin mixing the beads in the bead syringe so that they are evenly distributed. Then load the syringe into the syringe pump (remember that the orientation of this syringe will be facing vertically down), and again set the flow rate to 30,000 ul/hr to push out all air from the tubing. Once you see the bead of liquid, stop the pump, adjust the flow rate to **4,000 ul/hr**, and insert the free end of the tubing into the bead channel of the microfluidic device.

> Hint: be sure that the bead tube is not dripping when you insert it into the device - since this solution contains the lysis buffer, you do not want any of it flowing back into the cell channels prior to starting the run.

Flow rates

oil: 15,000 ul/hr
cells: 4,000 ul/hr
beads: 4,000 ul/hr

Starting your run

START order: cells → beads → oil
STOP order: beads → cells → oil

Begin by pressing start on the cell pump, then the beads, and then finally the oil. The logic behind this order is that you do not want any of the bead solution flowing back into the cell channels, because the bead solution contains lysis agents that could lyse incoming cells before they ever reach the droplet generation junction. For the same reason, if you ever need to stop the flows mid-experiment or are planning on reusing the device, stop the bead flow first. It is also helpful to simultanouesly pull out the oil, cells, and beads tubing from the device a few seconds after stopping the flows (while leaving the outflow tubing in) if you are planning on reloading more cells and beads for a larger experiment, or simply to help preserve it for reuse in the future.
It will take some time for the flow to stabilize (typically it takes about 10-40 seconds). The outflow tube

should be positioned in a waste container during this time. You can monitor the emulsion quality by eye by allowing the ejected droplets (coming out of the outflow tube) to run down the side of the container. When stable, this should appear as a hazy yet uniform line since all the droplets will be the same size (this line will usually be the width of about five columns of droplets). Under the microscope, droplet stability can be assessed by seeing a faint "flickering" pattern at the droplet generation junction (which looks like an elongated triangle). In addition, the flow to the right of this junction will appear "blurry" because the droplets are moving so quickly. *If the outflow to the right of the droplet generation looks like a clear stream and is not blurry, this means that you are not forming droplets. This clear stream will be very obvious - if you are set on a higher objective than 10x, you may see a narrow stream of oil flowing in the center, but the surroundings should be blurry if droplets are forming.* Once you are sure that you are forming stable droplets (and see that they have made their way to the end of the outflow tubing via the run down method), you can transfer the end of the outflow tubing into a falcon tube to begin collecting usable droplets.

On occasion, if droplets are not being formed after a few minutes, it may be necessary to stop and re-start some of the flows. I have empirically found that stopping the bead and cell flows while allowing the oil to continue flowing for a few seconds, and then re-starting cells and beads, can be very helpful in generating good quality emulsions.

Collect droplets in 50 mL falcon tubes. Collect 1 mL of aqueous flow into each falcon tube, which should take about 15 minutes. Note that the 1 mL refers to the amount of cells and beads you're flowing in (1 mL of cells and 1 mL of beads). *Collecting more than 1 mL in each falcon will negatively impact the breakage step.*

Assessment of droplet quality and bead doublets

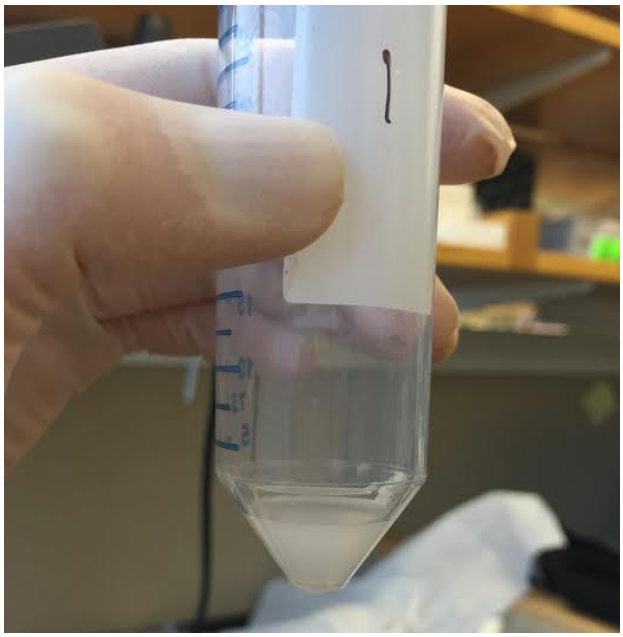
Place ~17 ul of oil into a Fuchs-Rosenthal hemocytometer chamber. Add ~3 ul of emulsion and gently rock back and forth a few times to distribute the droplets. Set a microscope to 10x and adjust the focus until you can clearly see the droplets with the beads inside. (The droplets will be transparent, and the beads will be smaller dark circles inside them). You will not be able to see any cells, since they were lysed once they came into contact with the lysis buffer upon droplet formation.

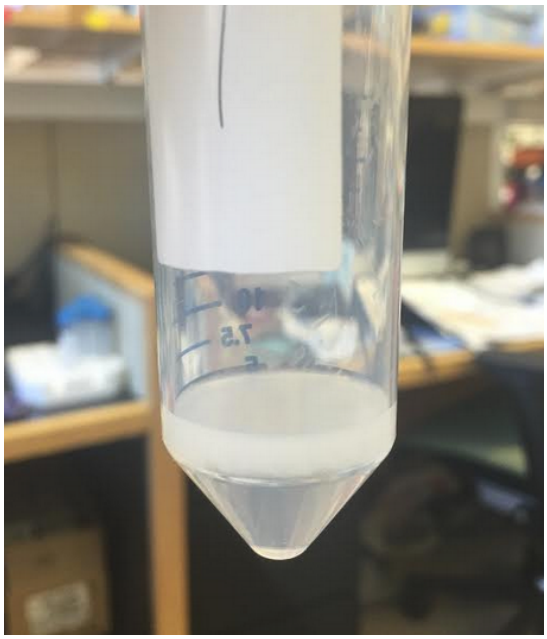
High quality emulsions are ones where all of the droplets will be the same size. You can also assess the overall quality of the droplets by holding the Falcon tube over a dark surface, tilting it slightly to the side, and then slowly returning it to a vertical position - you should see a uniform wall of droplets slowly falling down along the edge.

Count the number of bead singlets and doublets - droplets that contain only a single bead vs those that contain two beads. You should get a doublet rate that is 5% or lower.

Breakage

This is what your droplets should look like, prior to breakage. At one time the batches of oil we received were more opaque, causing the interface to look like the one pictured below to the left (the beads are in the whiter layer on top). The picture to the right is what your interface will most likely look like (with clear oil). Both types of oil work equally well.





1. Remove the oil layer from the bottom of the falcon by pressing a P1000 down to its first stop, pushing through the droplets to the bottom of the tube, pressing down to the second stop to expel any droplets, and then after waiting several seconds for the droplets to float back up to the droplet layer, sucking out the oil. You do not need to remove every last bit of oil - just remove most of it.

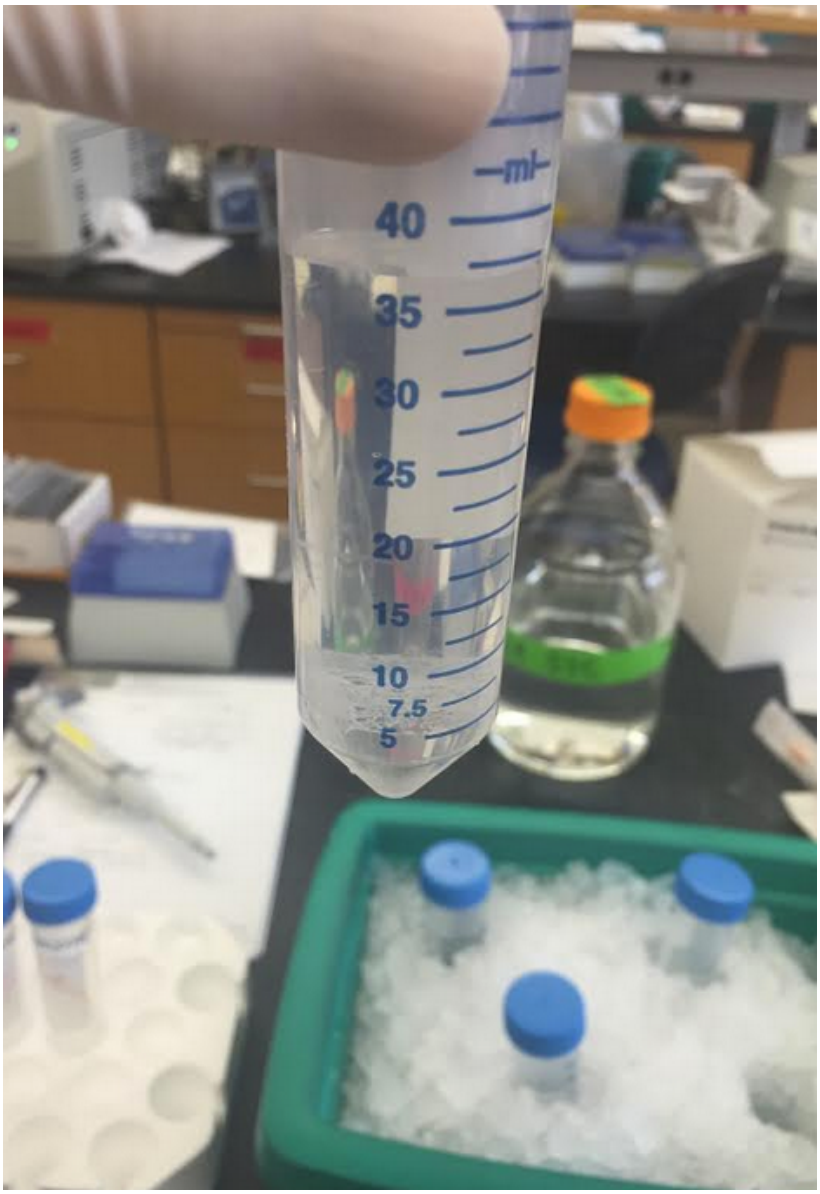
2. Add 30 mL of room temperature 6X SSC.

3. Add 1 mL of Perfluorooctanol (PFO) in a fume hood. Shake by hand to break the droplets (3-4 forceful vertical shakes).

4. Spin at 1000xg for 1 minute.

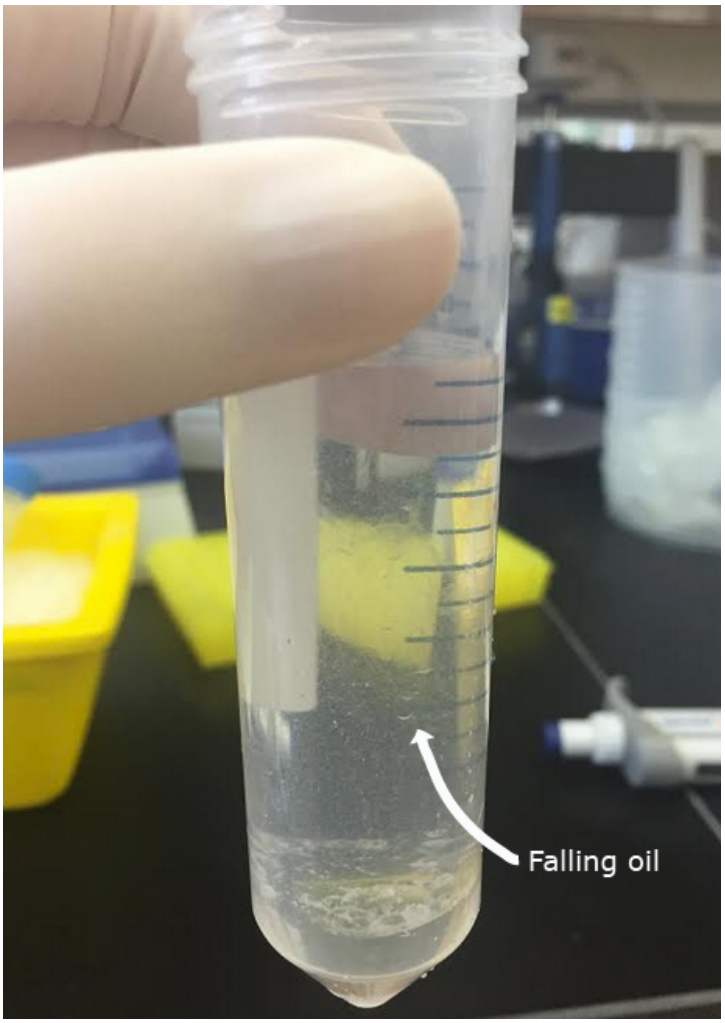
> Hint: to prevent the beads from floating up off the interface after spinning, it can be helpful to slightly loosen the caps of the tubes right before you place them into the centrifuge. It is also helpful to pre-punch holes in the ice with an empty Falcon tube.

A clean break: (the white beads are sitting on the interface)



5. Carefully remove the tube from the centrifuge into an ice bucket. Use a pipette to remove and discard the supernatant on top until there are only a few mL remaining above the interface.

6. Add 30 mL of 6X SSC to kick up the beads into solution. Wait a few seconds to allow the majority of the oil to sink to the bottom, then transfer the supernatant to a new Falcon tube. *Avoid transferring any oil or interface precipitate material.* You should be able to see the white beads floating around in the supernatant during this step (see image on next page).



7. Spin at 1000xg for 1 minute.

    Hint: this is a good time to add the maxima h- to your RT mix!

8. The beads are now pelleted to the very bottom of the Falcon tube, although you may not be able to see them yet. Carefully remove all but ~1 mL of liquid. With a pipette, mix this remaining ~1 mL a few times to kick up the beads, then transfer it to an eppendorf. Spin down in a tabletop centrifuge. Remove and discard the supernatant.

9. Wash 2x with 1 mL of 6X SSC, then once with ~300 ul of 5X RT buffer. Remove as much of the 5X RT wash as you can without taking up any beads. You are now ready to begin the reverse transcription.

    Hint: it can be helpful to set up two sets of eppendorf tubes per sample, since there will sometimes be some residual oil when you first transfer to an eppendorf. To get rid of it, simply add 1 mL of 6X SSC and watch as the clear oil rapidly falls to the bottom of the tube (this only takes about a second), then suck up the beads and transfer them to a clean tube.

Reverse transcription

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

> Note: 90,000 beads is the maximum number of beads that we've tested in a TechDev experiment.

RT mix recipe (makes 200 ul): *(can be prepared in advance without RTase)*

75 ul H2O
40 ul Maxima 5x RT Buffer
40 ul 20 % Ficoll PM-400
20 ul 10 mM dNTPs (Clontech)
5 ul RNase Inhibitor (Lucigen)
10 ul 50 uM Template Switch Oligo
10 ul Maxima H- RTase (add after you've begun the breakage portion of the protocol)

1. Add 200 ul of RT mix to the beads.
2. Incubate at room temperature for 30 minutes with rotation.
3. Incubate at 42 C for 90 minutes with rotation.
4. Wash the beads once with 1 mL TE-SDS, twice with 1 mL TE-TW*, and then if proceeding to exonuclease I treatment wash once more with 1 mL 10 mM Tris pH 8.0.

THIS IS A STOPPING POINT → beads can be stored at 4 C in TE-TW.

Exonuclease I treatment

This step chews back the excess bead primers that did not capture an RNA molecule. One RT mix below is sufficient for the processing of ~90,000 beads.

Exonuclease mix recipe (makes 200 uL):

20 ul 10x Exo I Buffer
170 ul H2O
10 ul Exo I

1. After washing once with 1 mL 10 mM Tris pH 8.0, resuspend in 200 ul of exonuclease mix.
2. Incubate at 37 C for 45 minutes with rotation.
3. Wash the beads once with 1 mL TE-SDS, twice with 1 mL TE-TW*, and then if proceeding to PCR, wash once more with 1 mL H2O.

THIS IS A STOPPING POINT → beads can be stored at 4 C in TE-TW.

Preparing for PCR

1. After washing once with 1 mL H2O, spin down, remove supernatant, and add another 1 mL of H2O.
2. Mix well to evenly resuspend the beads, then quickly remove 20 uL and pipette into a Fuchs-Rosenthal hemocytometer chamber.
3. Count all 16 boxes. The concentration (in beads/ul) is equal to: (# beads counted/16) * 5.
4. Apportion 2,000 beads into each PCR tube. This will yield ~100 STAMPs per PCR tube.
5. Spin down the tubes, and add the following PCR mix (per tube):

   24.6 ul H2O
   0.4 ul 100 uM SMART PCR PRIMER
   25 ul 2x Kapa HiFi Hotstart Readymix

6. Mix well and proceed to PCR.

STORE REMAINING BEADS AT 4 C IN TE-TW. We have stored beads successfully for >3 months without obvious cDNA degradation.

PCR program

95 C 3 minutes
4 cycles of:
98 C 20 s
65 C 45 s
72 C 3 min
9 cycles of:
98 C 20 s
67 C 20 s
72 C 3 min
Then:
72 C 5 min
4 C forever

> Hint: the recommendation of 13 cycles applies to the cells and conditions above. The number of cycles will need to be adjusted depending on the cell types assayed.

Purification of the cDNA library and analysis on the BioAnalyzer

1. Vortex the bottle of AMPure beads to mix.

2. Add 30 ul of room temperature AMPure XP beads to each PCR tube of sample. This is a .6x beads to sample ratio.

3. Purify according to manufacturer's instructions.

4. Elute in 10 ul H2O.

5. Run a BioAnalyzer High Sensitivity Chip according to the manufacturer's instructions. Use 1 ul of the purified cDNA sample as input.

Your cDNA library should be fairly smooth and have an average size of 1300-2000 bp.

The yield for 2000 beads generated from a 50 cell/ul final cell concentration should be 400-1000 pg / ul. Do not be too concerned if there is some yield variation from run-to-run. There is some variability in counting the beads, and cell concentration, which can lead to more or less cells per amplification.

Tagmentation of cDNA with Nextera XT

1. Preheat a thermocycler to 55 degrees.

2. For each sample, combine 600 pg of purified cDNA with H2O in a total volume of 5 ul.

3. To each tube, add 10 ul of Nextera TD buffer and 5 ul of Amplicon Tagment enzyme (the total volume of the reaction is now 20 ul). Mix by pipetting ~5 times. Spin down.

4. Incubate at 55 C for 5 minutes.

5. Add 5 ul of Neutralization Buffer. Mix by pipetting ~5 times. Spin down. Bubbles are normal.

6. Incubate at room temperature for 5 minutes.

7. Add to each PCR tube in the following order:

   15 ul of Nextera PCR mix
   8 ul H2O
   1 ul of 10 uM New-P5-SMART PCR hybrid oligo
   1 ul of 10 uM Nextera N70X oligo

8. Run this PCR program:

   95 C 30 sec
   12 cycles of:
   95 C 10 seconds
   55 C 30 seconds
   72 C 30 seconds
   Then:
   72 C 5 minutes
   4 C forever

Purification of the tagmented library and analysis on the BioAnalyzer

1. Vortex the bottle of AMPure beads to mix.

2. Add 30 ul of room temperature AMPure XP beads to each PCR tube of sample. This is a .6x beads to sample ratio.

3. Purify according to manufacturer's instructions.

4. Elute in 10 ul H2O.

5. Run a BioAnalyzer High Sensitivity Chip according to the manufacturer's instructions. Use 1 ul of the purified cDNA sample as input.

Your tagmented library should be fairly smooth, with an average bp size of 500-680bp. Smaller-sized libraries will have more polyA reads; larger libraries may have lower sequence cluster density and cluster quality. Note that we have successfully sequenced libraries from 420-700bp.

For a HEK/3T3 experiment, the expected yield will be in the range of 10-30 nM.

Sequencing your sample

If using the MiSeq, make a 10 ul library pool at 3 nM (as quantified by the BioAnalyzer) as input for denaturation. For the final dilution, combine 400 ul sample with 600 ul of HT1 buffer.

If using the NextSeq 500 - High Output, make a 10 ul library pool at 3 nM (as quantified by the BioAnalyzer) as input for denaturation. For the final dilution, combine 85 ul of sample with 1215 ul of HT1 buffer.

> Note: above are our most current standard dilutions, which produce optimal cluster density and % of clusters that pass filter. For the MiSeq, we tweak the sample input from 300 - 500 ul in 1000 ul total, depending on average tagmentation library size. For the NextSeq we vary the sample input from 70 ul - 100 ul in 1300 ul total. The smaller your library, the less sample you want to add to your final dilution.
>
> **Sequencing specifications for the MiSeq or NextSeq:**
>
> Read 1: 20 bp
> Read 2: 50 bp
> Read 1 Index: 8 bp ← **only necessary if you are multiplexing samples**
> Custom Read 1 primer

Troubleshooting:

Low-quality droplets

From time to time, we experience runs where droplet size is not uniform. We have identified three potential causes:

1) ***Failure to wait long enough for the flow to stabilize*.** It takes time to push all the destabilized flow through the outflow tube. You may need to wait longer to avoid having this volume in your sample.

2) ***Device imperfections*.** We generally use around 4,000 ul/hr as our flow rate for the cell and bead channels. However, we have seen batch-to-batch variation in droplet quality produced from individual devices. If you notice droplet quality problems, reduce the flow rate of cells and beads to 3,500 ul/hr, and decrease the oil flow rate proportionally. If this still does not produce good-quality droplets, consider switching to a new device.

3) ***Devices are old (>3 months)*.** Over time, our experience is that the Aquapel coating somehow degrades, which causes droplet quality to also decline. You may need fresh devices, or to re-treat your old devices with Aquapel.

4) ***Air bubbles in the microfluidic device*.** If you observed air bubbles accumulating in the microfluidic channels during droplet generation, this likely explains poor emulsion quality. Avoid storing cell suspension buffers on ice--when the buffer is brought up to room temperature, it can release gas (gas is more soluble in liquids at lower temperatures).

Beads breaking during droplet formation

It is important to mix the beads to prevent them from settling; however, overly vigorous mixing will shear the beads. **We strongly recommend using the V&P Scientific Mixing System described in the protocol**, at the speeds described. A small amount of bead fragmentation during droplet generation is expected, but these fragments should be nearly absent from the bead pool once you are counting beads for PCR amplification.

Droplets break poorly/precipitate at oil-aqueous interface

The amount of protein in your cell suspension strongly influences the “cleanliness” of the oil-aqueous interface after droplet breakage. For example, a lot of BSA (e.x. 0.2%) can produce a snow-like precipitate (though it does not appear to affect the library quality). **Serum is strongly inhibitory to Drop-seq and must be washed out completely before running cells.** Serum will also produce a lot of precipitate at the oil-aqueous interface.

Next steps:

Once you've gotten through to the sequencing step, you'll want to generate digital expression data, as well as basic metrics about the sequencing run. Jim Nemesh in our lab, with help from Alec Wysoker, has developed software for processing sequence data. Download the latest informatics guide on our webpage to learn more.