# Drop-Seq Laboratory Protocol

version 1.0 (5/21/15)

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The following is our current protocol for Drop-seq. It should also help you find all the equipment and reagents you will need to begin performing your own experiments.

We have continued to optimize this protocol since doing the experiments in the Macosko *et al.* manuscript. This is our current, fully optimized protocol. As a result, this protocol will not precisely match the methods section of the paper, which describes the specific experiments done in that work. This protocol also includes hints, suggestions and images that we cannot fit into the the methods section of the paper.

As people begin to adopt Drop-seq and we learn about their experiences with the technology, we will add additional hints and tips, and release updated protocols with a new date. We will increment the version number only when we change something substantive about the recommendation for a protocol step.

*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.* This protocol 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 analysis with a Drop-seq system.

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 them online). You can email us at dropseq@gmail.com.

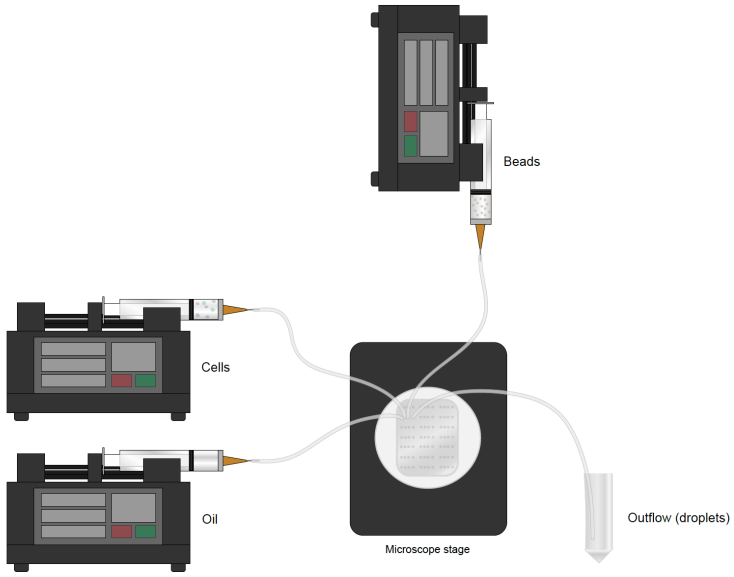
## Necessary start-up equipment:

- **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 enough 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 # 782N-6-150)
- **3 mL syringes** (we use BD #309657)
- **Tubing** to connect syringes to the device: Scientific Commodities, inc. (cat # BB31695-PE/2)
- **Luer lock 26-gauge 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 can be 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)

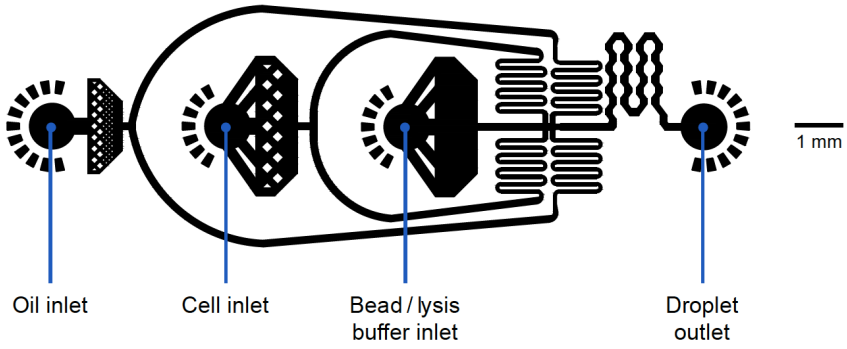
## Arranging and connecting your droplet generation set-up:

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**



## 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
  - 0.1% 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:** make from 20X SSC (Life Technologies, #15557-036)
- **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**

## 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) will make these 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 twice 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 >3 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 set too high.*

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## 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).

### Anticipating your yield of STAMPs

The number of STAMPs generated per hour is a product of two 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 less than 5% bead doublets.

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 and form 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, gently push oil through to the end of the tubing, and load the syringe into the pump as shown below (we use a 20 mL syringe for bigger experiments). Set the flow rate to 15,000 ul/hr. Insert the free end of the tubing into the left-most channel of a clean device (see figure on page 3 of this protocol).

   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 1 mL 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.

    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 farther 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 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 (but the triangle itself should be clear). *If the outflow to the right of the triangle looks like a clear stream and is not blurry, this means that you are not forming droplets!* Once you are sure that you are forming stable droplets (and see that they have made their way to the end of the outflow tube via the run down method), you can transfer the end of the outflow tubing into a 50 mL falcon tube to begin collecting usable droplets.

On occasion, if droplets are not being formed after a few minutes (a common example of this is when the flows all seem to be running but the outflow to the right of the junction is clear as mentioned above), it may be necessary to stop and re-start some of the flows. Stop the bead and cell flows for about 5 seconds while allowing the oil to continue running, then re-start the cells and beads.

If all flows seem to be running and droplets *are* being formed but you do not see a steady stream of beads flowing into the bead inflow chamber of the device, there is probably a clog in the needle of the bead syringe. Stop the flows, remove the bead syringe from the pump and pull back on the plunger - you should see a white cloud of beads fall back into the solution. Change the needle, and try again.

You will not always be able to see the cells passing through the cell inflow chamber of the device due to cell size and the speed at which they happen to be traveling (even slight backflows of oil into the cell inflow chamber can drastically alter the speed at which cells pass through this area), but so long as you see stable droplets forming you can safely assume that your droplets are getting cells.

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).

## Assessment of droplet quality and bead doublets

Place ~17 ul of oil into a Fuchs-Rosenthal hemocytometer chamber. Slowly add ~3 ul of your collected droplets (taken from the very top of the Falcon tube). 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 immediately once they came into contact with the lysis buffer.

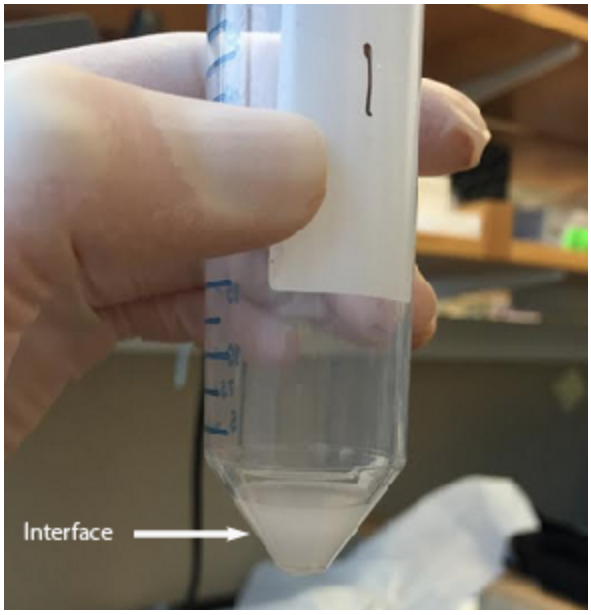
Your emulsion is of high quality if all of the droplets are uniform in size.

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

1. Remove most of the oil from the bottom of each Falcon tube using a P1000 pipette.

   Hint: the oil is at the bottom while the droplets form a slightly whiter layer on the top. Push the pipette to the first stop, insert its tip down into the bottom oil layer, push down to the second stop to eject any droplets that were caught on the way down, and then slowly start pulling up oil from the very bottom of the tube. As the volume decreases, it will become increasingly easier to see the separation between the oil layer and the droplet layer.




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

3. Add 1 mL of Perfluorooctanol (PFO) in a fume hood. Give the Falcon 3 firm shakes to break the droplets.

4. Spin at 1000xg for 1 minute.

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. (See page 17 for additional photos).

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.

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 to an eppendorf. Spin down briefly 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 molecules that are hybridized to the bead primers. One RT mix below is sufficient for the processing of ~ 90,000 beads (the maximum number of beads we have currently tested in a single RT reaction).

**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, in order to reduce mispriming during PCR. One 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, add 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 (above), spin down, remove the supernatant, and add another 1 mL of H2O. (The amount of H2O you resuspend in for counting will depend on the size of your bead pellet. As you perform more Drop-seq experiments, you will get a feel for how much to add.)

2. Count the beads. Mix well to evenly resuspend the beads, then quickly remove 20 uL into a Fuchs-Rosenthal hemocytometer chamber.

   Hint: it is helpful to use a 200 uL pipette (and not a 20 uL pipette) for this step, since it will eject the beads faster and with more force. You should have the pipette tip already loaded onto the 200 uL pipette, so that after mixing the beads you can remove and transfer the 20 uL in a rapid, uniform motion. By eye, the beads should appear evenly distributed within the counter, otherwise your count will not be accurate.

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. Typically the range falls between 14 and 16 cycles.

## Purification of the cDNA library and analysis on the BioAnalyzer

1. Vortex the bottle of AMPure beads to ensure that they are thoroughly mixed.

2. Add 30 ul of room temperature AMPure XP beads to each PCR tube of sample. Mix well. (This is a beads to sample ratio of .6x, which is our standard throughout this protocol).

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 cells/ul final cell concentration (in the droplets) should be 400-1000 pg/ul. Do not be too concerned if there is some yield variation from run-to-run. These differences are often due to either variability in counting the beads, or in the cell concentration itself.

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

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

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2. Add 30 ul of room temperature AMPure XP beads to each PCR tube of sample. Mix well.
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 and have an average bp size of 500-680 bp. Smaller-sized libraries will have more polyA reads; larger libraries may have lower sequence cluster density and cluster quality.

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 550 ul sample with 450 ul of HT1 buffer.

If using the NextSeq 500, make a 10 ul library pool at 3 nM (as quantified by the BioAnalyzer) as input for denaturation. For the final dilution, combine 100 ul of sample with 1180 ul of HT1 buffer.

**Sequencing specifications for the MiSeq and 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

## 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.**

## 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 the poor emulsion quality. Avoid storing cell suspension buffers on ice, because 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

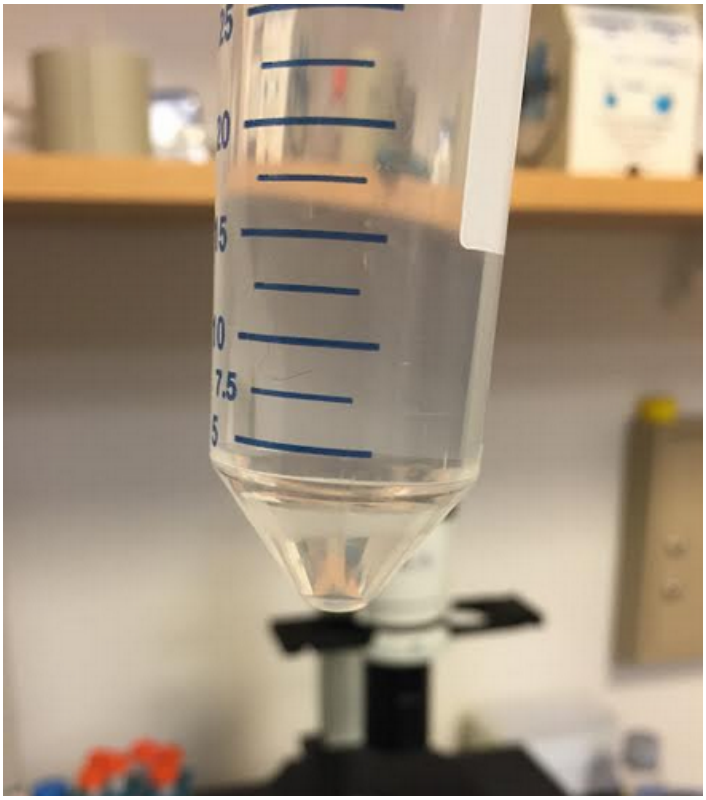
It is important to mix the beads to prevent them from settling; however, overly vigorous mixing will shear the beads. **We strongly recommend using the V&P Scientific Mixing System described in the protocol**, at the speeds described. A small amount of bead fragmentation during droplet generation is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

### Droplets break poorly/white 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.

**Additional tips for breakage:**

Below is a picture showing what we call a “clean break”. This was taken right after removing the Falcon tube from the centrifuge after the first spin (the tube currently contains 30mL of 6X SSX, although it may be difficult to see in this picture). Note how the break line is white and relatively thin vertically. Higher concentrations of BSA or serum in your sample may cause the white to extend a bit farther down, which would imply that the break isn’t as clean (meaning that not all of the droplets were broken). Adding additional PFO directly to the tube, briefly shaking, and spinning down again can help, but be cautious not to add too much PFO as it can lead to lower library quality downstream. 1.5mL of PFO per mL of sample collected (1mL of cell flow + 1mL of bead flow) should be your maximum. Typically, even breaks that do not look as clean as this are perfectly fine - when you remove the 6X SSC above the break line and then add another 30mL of 6X SSC to kick up the beads, it will be obvious whether or not most of your droplets were broken because you will see the beads floating around in solution. We have generated great data from breaks that were far less clean than this, so don’t be discouraged at this step!



Below is a picture showing what your tube should look like a few seconds after you've added the 30mL of 6X SSC to kick up the beads following the first spin. Notice how you can see the tiny white beads floating in solution, as well as a few droplets of falling oil. (For the first 5-10 seconds it will look far messier than this, since there will be a lot of oil sinking to the bottom of the tube). If your break was not as clean, the solution at this step will be more cloudy, which might mean that you'll need to do an additional wash with 6X SSC once you've transferred to the eppendorf tube.