Implementing the Drop-Seq Protocol on Bio-Rad's ddSEQ Single-Cell Isolator

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Abstract

Studying cells on a single-cell level can provide greater resolution into complex biological systems than studying cells in bulk. Currently there are several different options for the types of chemistries that are used to obtain single-cell RNA-sequencing (RNA-Seq) libraries, as well as for the instrumentation used to capture single cells. Here we demonstrate the successful pairing of the Drop-Seq workflow with the Bio-Rad ddSEQ Single-Cell Isolator. Coupling these two technologies enables the selection of template-switching during the reverse transcriptase (RT) step, with an easy-to-use one-button instrument that encapsulates hundreds to thousands of cells in aqueous droplets.

Introduction

The recent development of single-cell sequencing has provided researchers new insights into gene expression profiles and enabled the discovery of new subpopulations of cells that were previously masked by bulk measurements. Pioneering methods, such as Drop-Seq, have allowed researchers to scale up the number of cells that can be interrogated by encapsulating single cells into aqueous-based droplets versus lower-throughput plate-based platforms (Macosko et al. 2015). Since the advent of the first "home-brew" solutions for single-cell RNA-Seq, new commercial platforms have been released. The Illumina® Bio-Rad Single-Cell Sequencing Solution uses commercially available SureCell® WTA 3' Library Prep Kits with the ddSEQ Single-Cell Isolator instrument to obtain single-cell RNA-Seq libraries (Sarkar et al. 2018). This end-to-end solution allows researchers to obtain high-quality single-cell RNA-Seq data in a fast, easy, and reproducible format.

Although both the Illumina Bio-Rad ddSEQ platform and the Drop-Seq protocol encapsulate cells into aqueous-based droplets, the RT chemistries of the two platforms are different. The Illumina Bio-Rad ddSEQ solution captures cells in droplets, where RNA transcripts undergo first-strand cDNA synthesis using barcoded primers in solution. Droplets are then broken open and second-strand synthesis is performed in bulk solution to make a double-stranded cDNA library in a 1:1 ratio. In contrast, in the Drop-Seq protocol RNA from single cells is hybridized to barcoded oligonucleotides, which are attached to beads (Figure 1). The droplets are broken open and first-strand cDNA synthesis is performed in bulk outside of droplets. A PCR is then performed to synthesize and amplify a double-stranded cDNA library using template-switching chemistry. At this point, both protocols utilize Illumina's Nextera chemistry to generate single-cell RNA-Seq libraries and are then sequenced on Illumina sequencers.

BIO-RAD

Here we demonstrate the ability to execute the Drop-Seq protocol on the Bio-Rad ddSEQ Single-Cell Isolator. This enables researchers the flexibility to choose the chemistry of their preference or historic choice while simplifying the experimental setup by using the single button–operated, commercially available ddSEQ instrument.

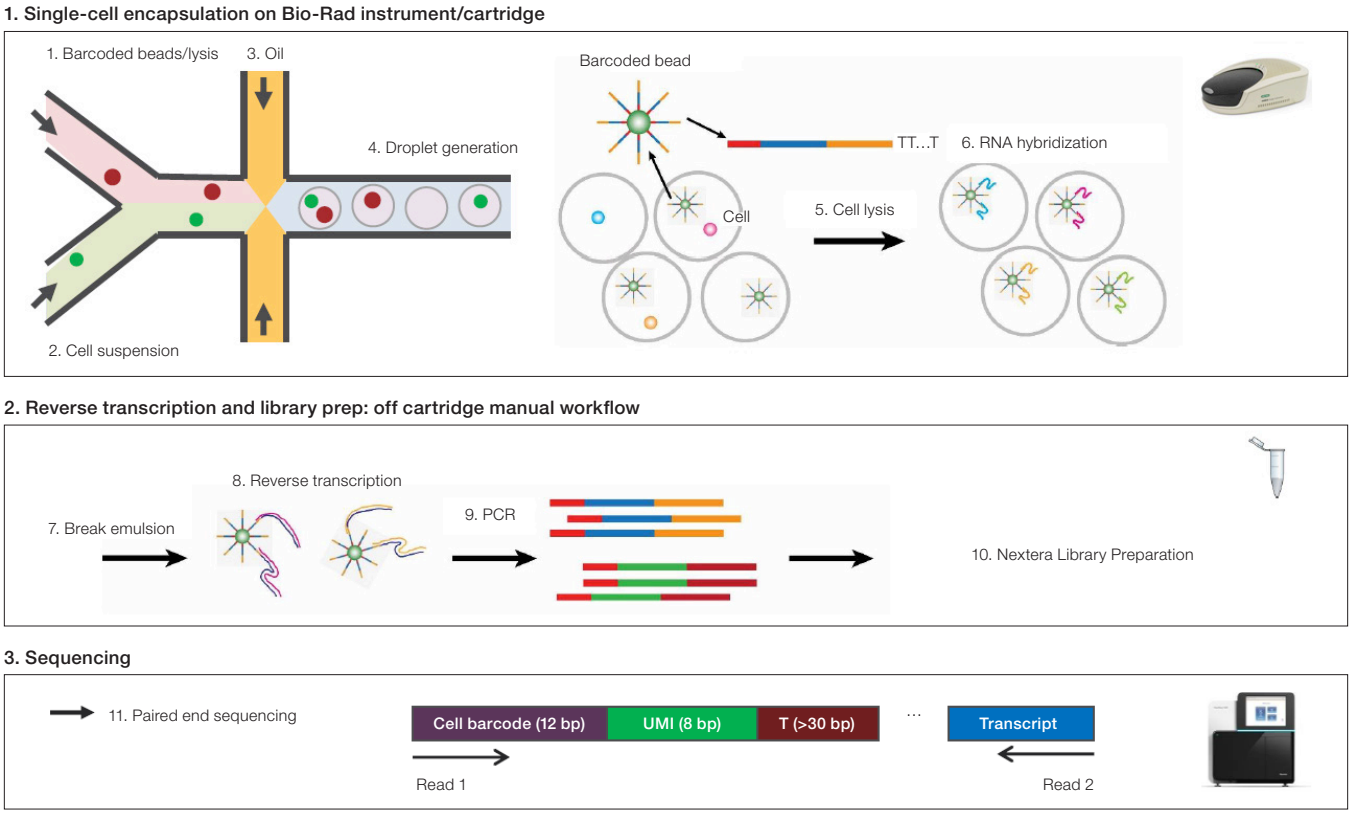

Fig. 1. Workflow for performing the Drop-Seq protocol on the Bio-Rad ddSEQ Single-Cell Isolator.

Materials and Methods

Preparation of Beads and Cells

Barcoded Bead SeqB Beads purchased from Chemgenes (www.chemgenes.com) were resuspended in lysis buffer made up of 0.2% sarkosyl, 20 mM EDTA, 60% OptiPrep, 200 mM Tris, pH 7.5, and 5% DTT. Barcoded Bead SeqB Beads were diluted to a final concentration of ~300 beads/µl. Human HEK293 and mouse NIH3T3 cells were mixed at a 1:1 ratio to a final combined concentration of ~300 cells/µl. Cells were resuspended in PBS + 1.0% BSA and 50% OptiPrep.

Cell Isolation, Library Prep, Sequencing, and Data Analysis

The bead suspension mix, cell suspension mix, and oil were loaded onto a ddSEQ Cartridge according to the SureCell WTA 3' Library Prep Reference Guide. Cells were encapsulated and barcoded with the ddSEQ Single-Cell Isolator (Bio-Rad Laboratories). From here, the Macosko et al. Drop-Seq protocol was followed. Briefly, droplets were broken open with perfluorooctanol (PFO), and the RNA transcripts hybridized to the bead primers were reverse transcribed to generate single-stranded cDNA in bulk solution. Exonuclease I treatment was then applied to chew back the excess bead primers that did not capture an mRNA molecule and undergo first-strand synthesis. PCR was performed using a template-switching PCR primer to amplify the cDNA library. The amplified cDNA libraries were purified and NGS libraries prepared using the Illumina Bio-Rad SureCell WTA 3' Library Prep Kit (Illumina). NGS libraries were sequenced on the Illumina NextSeq™ 550 System following a 20 bp, 8 bp, 50 bp paired-end reads cycling protocol. See Appendix for detailed protocol.

Results and Discussion

Detection of Genes in a Heterogeneous Population of Cells

A mixed species experiment was performed in which human HEK293 cells and mouse NIH3T3 cells were mixed at a 1:1 ratio. Cells were loaded onto a ddSEQ Cartridge with Drop-Seq beads, and the cells were encapsulated and barcoded with the ddSEQ Single-Cell Isolator. Single-cell RNA-Seq libraries were generated using the Drop-Seq protocol and its reagents. Libraries were sequenced on an Illumina NextSeq 550 System.

The knee plot in Figure 2 shows the cumulated fraction of genic transcripts assigned to cell barcodes. The inflection point (knee) is used to determine the number of barcoded cells detected. In graph A, barcodes to the left of the red line represent cells. Graph B shows the number of unique transcripts aligned to the mouse (●) and human (●) genome for each cell barcode. Unique transcripts mapping to both human and mouse genomes are shown in purple (●) and represent doublets.

Unbiased clustering of the mixed human and mouse cells revealed clustering driven by species by both principal components analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) plots (Figure 3). These results indicate the ability of the single-cell RNA-Seq experiments to determine distinct subpopulations from the starting mixed species population sample. The cells were further analyzed for cell cycle markers and showed subpopulations of cells in all five states of the cell cycle, including G1.S, S, G2.M, M, and M.G1.

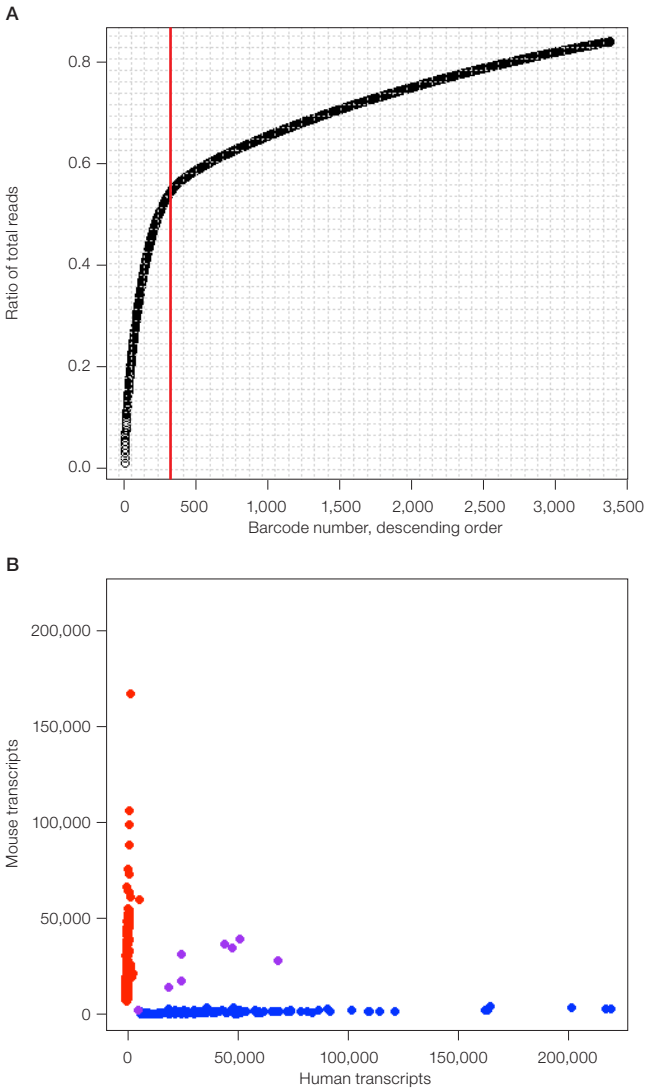

Fig. 2. Determination of cell number and cellular cross talk in a mixed species experiment. Human HEK293 cells and mouse NIH3T3 cells were mixed in a 1:1 ratio. **A**, the inflection point of the knee plot is used to determine the barcodes that correspond to cells in the experiment. **B**, cells are then plotted based on the number of mouse and human transcripts. **Results**: cross talk, 4.69%; purity, 96.18; average number of mouse transcripts, 26,007; average number of human transcripts, 43,519. Mouse, 193 (●); human, 140 (●); mixed, 8 (●).

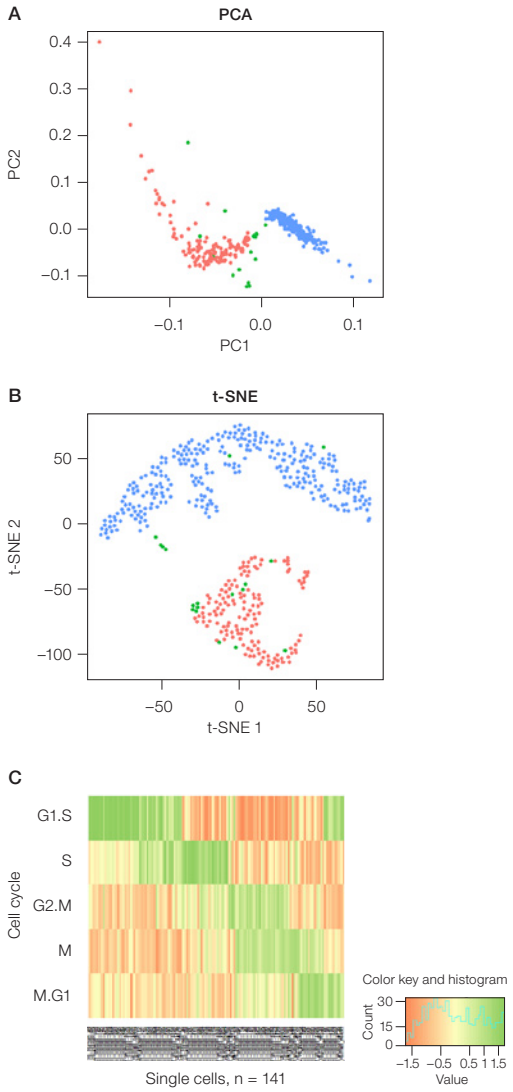

Fig. 3. Cluster analysis of individual cells. PCA (**A**) and t-SNE (**B**) analysis of the mixed species experiment. Heat map analysis (**C**) of all cells sorted by their expression of cell cycle markers. Human (●); mixed (●); mouse (●).

Crosstalk and Purity as a Function of Input Cell Concentration

The number of cells that were loaded onto a single chamber of a ddSEQ Cartridge was titrated down to 2.5% and 1.25% of the starting cell density. Figure 4 shows that when the cell number is reduced, the number of doublets goes down to 0%. High cellular purity of mouse or human transcripts was still maintained (>95%) at lower cell numbers, as well as a high average number of mouse or human transcripts. Moreover, this experiment demonstrates the ease with which fewer cells can be interrogated from a single ddSEQ Cartridge well.

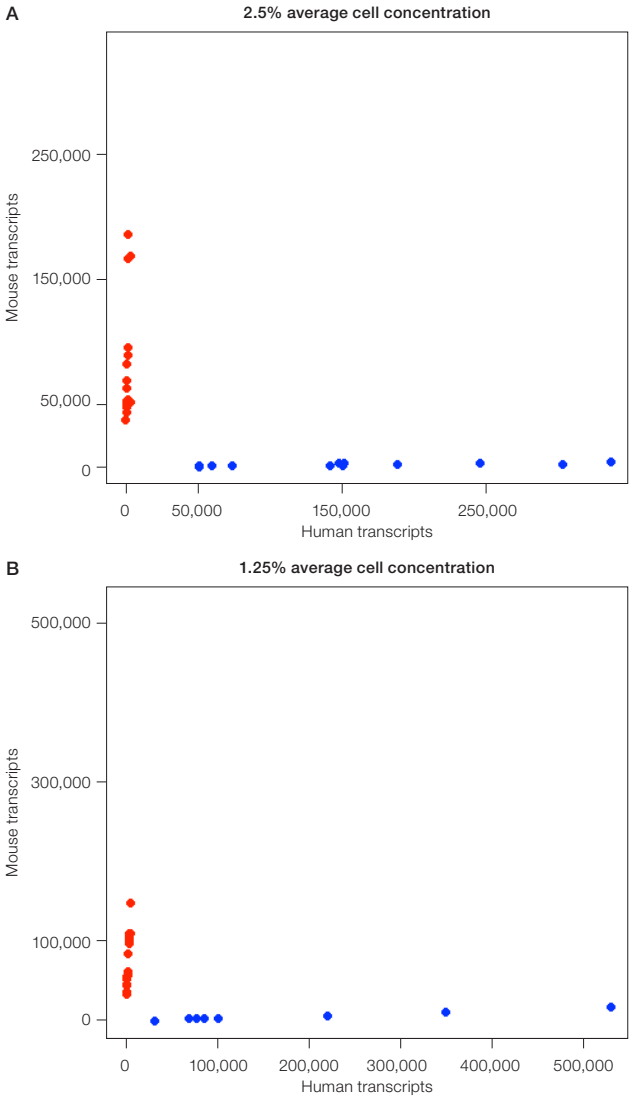

Fig. 4. Cross talk plots with fewer cells in the mixed-species experiment. **Results A**: cross talk, 0%; purity, 97.95; average number of mouse transcripts, 82,045; average number of human transcripts, 158,480. Mouse, 16 (●); human, 12 (●); mixed, 0 (●). **Results B**: cross talk, 0%; purity, 95.85; average number of mouse transcripts, 71,779; average number of human transcripts, 183,382. Mouse, 21 (●); human, 8 (●); mixed, 0 (●).

Single-Cell RNA-Seq Data Correlate to Bulk RNA-Seq Controls

As a control (Figure 5), RNA controls from the External RNA Controls Consortium (ERCC) were spiked in and recovered at an 8% conversion efficiency with a 96% correlation to ERCC's expected counts. Single-cell RNA-Seq data were then pooled together and compared to data from a bulk RNA-Seq experiment. There was a 96% correlation in both the human and mouse cell samples between single and bulk RNA-Seq data.

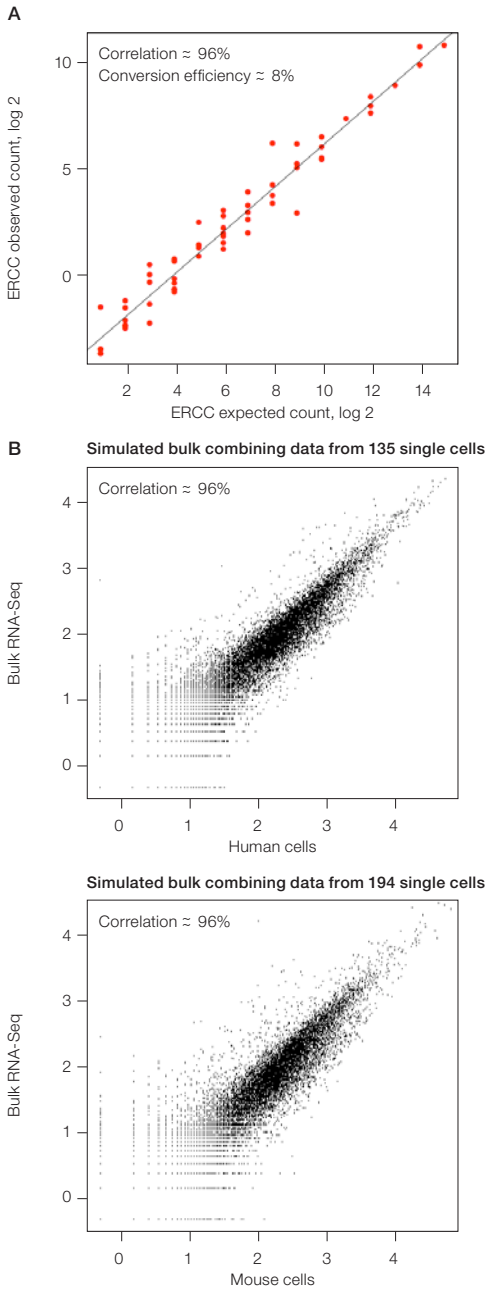

Fig. 5. Experiments from ERCC spike-in controls and bulk controls. **A**, ERCC controls were spiked into a single-cell ddSEQ experiment. **B**, single-cell RNA-Seq data were compared to data obtained from bulk controls.

Conclusion

We have demonstrated that Drop-Seq beads and their chemistry can be paired with the Bio-Rad ddSEQ Single-Cell Isolator to perform single-cell RNA-Seq experiments. The benefits of pairing the two platforms are a one-touch, easy-to-use instrument coupled with the use of template-switching chemistry. In the mixed species experiment, there was low cross talk, high purity, and distinctive clustering between the human and mouse cells, showing the ability to separate the two species using single-cell RNA-Seq. We demonstrated that the number of cells can be titrated down for experiments requiring fewer cells. Moreover, there was excellent correlation between Drop-Seq single-cell RNA-Seq data and bulk RNA-Seq data, as well as effective recovery of expected ERCC reference sequences. This workflow shows a simplified method for executing Drop-Seq experiments with the Bio-Rad Single-Cell Isolator.

References

Macosko EZ et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 161, 1202–1214. cell.com/cell/pdf/S0092-8674(15)00549-8.pdf. Accessed Oct. 24, 2018.

Sarkar A et al. (2018). Efficient Generation of CA3 Neurons from Human Pluripotent Stem Cells Enables Modeling of Hippocampal Connectivity In Vitro. Cell Stem Cell 22, 684–697. dx.doi.org/10.1016/j.stem.2018.04.009. Accessed Oct. 24, 2018.

Appendix

Carrying out the Drop-Seq protocol on the Bio-Rad ddSEQ Single-Cell Isolator enables researchers to use the chemistry of their choice while simplifying the experimental setup.

Required Primers:

Primer Name	Sequence
Template-switch oligo	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
SMART PCR primer	AAGCAGTGGTATCAACGCAGAGT

Required Beads:

Beads are ordered from Chemgenes (**www.chemgenes.com**) and are called Barcoded Bead SeqB Beads. The beads arrive as a dry resin. Wash the resin once with 30 ml of 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 TC20 Automated Cell Counter (Bio-Rad Laboratories). Store the counted beads at 4°C.

Prepare Solutions

Consumables Required

Supplier	Reagent	Part Number
Any	10 mM Tris, pH 8.0	N/A
Thermo Fisher Scientific	0.5 M EDTA	15575020
Thermo Fisher Scientific	10% SDS	AM9823
Sigma-Aldrich	10% Tween-20	11332465001

Procedure

1. Prepare TE/SDS solution by adding the reagents in the order shown below.

Component	Final Concentration	Volume, µl
10 mM Tris, pH 8.0	1x	4,740
0.5 M EDTA	1 mM	10
10% SDS	0.5%	250
Total		**5,000**

2. Prepare TE/TW solution by adding the reagents in the order shown below.

Component	Final Concentration	Volume, µl
10 mM Tris, pH 8.0	1x	4,985
0.5 M EDTA	1 mM	10
10% Tween-20	0.01%	5
Total		**5,000**

Prepare, Count, and Assess Viability of Cells in Single-Cell Suspension

Procedure

1. Prepare cells according to the verified protocol: Mixed Species Control in the SureCell WTA 3' Library Prep Reference Guide.

Prepare Cell and Barcode Suspension Mixes

Consumables Required

Supplier	Reagent	Part Number
Thermo Fisher Scientific	Gibco PBS, pH 7.4	10010023
Any	BSA, cell culture grade	N/A
Sigma-Aldrich	OptiPrep	D1556
Sigma-Aldrich	20% sarkosyl	L7414
Thermo Fisher Scientific	0.5 M EDTA	15575020
Sigma-Aldrich	2 M Tris, pH 7.5	T2944
Any	DTT	N/A

Procedure

1. Create the cell suspension mix by combining the following components in a new 1.7 ml tube on ice. Before adding the filtered cells to the cell suspension mix, vortex the cells for 1 sec. Repeat three times.

Component	Final Concentration	Volume per Sample, µl	Volume for 1 Cartridge, µl (4 Samples)	Volume for 2 Cartridges, µl (8 Samples)
1x PBS + 1.0% BSA	1x	9.5	38	76
OptiPrep	50%	12.5	50	100
Filtered cells (2,500 cells/µl)	~300 cells/µl	3.0	12	24
Total		**25.0**	**100**	**200**

2. Create the barcode suspension mix by combining the following components in a new 1.7 ml tube on ice. Pipet the barcode suspension mix with a P200 single-channel pipet 10–15 times while on ice and then centrifuge briefly.

Component	Final Concentration	Volume per Sample, µl	Volume for 1 Cartridge, µl (4 Samples)	Volume for 2 Cartridges, µl (8 Samples)
20% sarkosyl	0.2%	0.25	1	2
0.5 M EDTA	0.02 M	1.0	4	8
OptiPrep	60%	15.0	60	120
1 M Tris, pH 7.5	0.2 M	2.5	10	20
H_2O		5.0	20	40
1 M DTT	5%	1.25	5	10
Total		**25.0**	**100**	**200**

3. Pipet an appropriate volume of Drop-Seq beads, depending on the concentration of the stored aliquots, to achieve a final concentration of ~300 beads/µl. Spin down Drop-Seq beads in a tabletop centrifuge at 1,000 x g for 1 min. Discard supernatant and resuspend beads in the appropriate volume of barcode suspension mix, depending on the number of samples being processed.

   Example: If processing one cartridge of cells, prepare enough beads to resuspend in 100 µl of barcode suspension mix. For a final concentration of 300 beads/µl, spin down 300 beads * 100 µl, or 30,000 total beads, and resuspend in 100 µl of lysis buffer.

Isolate Single Cells

Procedure

1. Isolate beads and cells into droplets with the ddSEQ Single-Cell Isolator according to the SureCell WTA 3' Library Prep Reference Guide.
2. After droplet generation, use a P50 multichannel pipet set to 43 µl to gently and slowly aspirate the droplets from the output well and transfer them into a new 15 ml conical tube on ice. Repeat with the second well for the same sample to combine the two into the same 15 ml conical tube. For each sample, combine the 2 wells of droplets for that sample into a new 15 ml conical tube on ice.

Break Emulsion

Consumables Required

Supplier	Reagent	Part Number
Any	6x SCC	N/A
Sigma-Aldrich	Perfluorooctanol (PFO)	370533
Thermo Fisher Scientific	Maxima 5x RT Buffer	EP0751

Procedure

1. Per the Macosko et al. protocol, remove the oil layer from the bottom of the 1.7 ml tube by pressing a P200 down to its first stop, pushing through the droplets to the bottom of the tube, and pressing down to the second stop to expel any droplets. After waiting several seconds for the droplets to float back up to the droplet layer, suck out the oil. You do not need to remove every last bit of oil, just remove most of it.
2. Add 3 ml of room-temperature 6x SSC.
3. Add 100 µl of perfluorooctanol (PFO) in a fume hood. Shake by hand to break the droplets (3–4 forceful vertical shakes).
4. Spin at 1,000 x g for 1 min.
5. Carefully remove the tube from the centrifuge into an ice bucket. Add 3 ml of 6x SSC to bring the beads back 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 15 ml 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.
6. Spin at 1,000 x g for 1 min.
7. The beads are now pelleted to the very bottom of the Falcon tube, although you may not be able to see them. Carefully remove all but ~1 ml of liquid. Mix this remaining ~1 ml a few times with a pipet to bring the beads back into solution then transfer to a clean 1.7 ml tube. Spin at 1,000 x g for 1 min in a tabletop centrifuge. Discard the supernatant.
8. Wash twice with 1 ml of 6x SSC, then once with ~300 µl of 5x RT buffer. Remove as much of the 5x RT wash as you can without taking up any beads.

Reverse Transcription

Consumables Required

Supplier	Reagent	Part Number
Thermo Fisher Scientific	Maxima H Minus Reverse Transcriptase	EP0751
Thermo Fisher Scientific	Maxima 5x RT Buffer	EP0751
Sigma-Aldrich	20% Ficoll PM-400	F5415
Any	10 mM dNTPs	N/A
Lucigen	NxGen RNase Inhibitor	30281-1
Any	Template-switch oligo	N/A

Procedure

1. Prepare the Reverse Transcription Mix by adding the reagents in the order shown below.

Component	1 Reaction + 10% excess, µl	4 Reactions + 10% excess, µl	8 Reactions + 10% excess, µl
H_2O	8.25	33	66
Maxima 5x RT Buffer	4.4	17.6	35.2
20% Ficoll PM-400	4.4	17.6	35.2
10 mM dNTPs	2.2	8.8	17.6
NxGen RNase Inhibitor	0.55	2.2	4.4
50 µM template-switch oligo	1.1	4.4	8.8
Maxima H Minus Reverse Transcriptase	1.1	4.4	8.8
Total	**22**	**88**	**176**

2. Add 20 µl of the RT mix to the beads.
3. Incubate at room temperature for 30 min with rotation.
4. Incubate at 42°C for 90 min with rotation.
5. Wash the beads once with 100 µl TE/SDS, then twice with 100 µl TE/TW. If proceeding to Exonuclease I treatment, wash once more with 100 µl 10 mM Tris, pH 8.0.

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

Exonuclease I Treatment

Consumables Required

Supplier	Reagent	Part Number
NEB	10x Exo I Buffer	M0293S
NEB	Exo I	M0293S

Procedure

1. Prepare the exonuclease mix by adding the reagents in the order shown below.

Component	1 Reaction + 10% excess, µl	4 Reactions + 10% excess, µl	8 Reactions + 10% excess, µl
10x Exo I Buffer	2.2	8.8	17.6
H_2O	18.7	74.8	149.6
Exo I	1.1	4.4	8.8
Total	**22**	**88**	**176**

2. After washing once with 100 µl of 10 mM Tris, pH 8.0, resuspend in 20 µl of exonuclease mix.
3. Incubate at 37°C for 45 min with rotation.
4. Wash the beads once with 100 µl TE/SDS, twice with 100 µl TE/TW. If proceeding to PCR, wash once more with 100 µl H_2O.

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

PCR to Amplify cDNA

Consumables Required

Supplier	Reagent	Part Number
Kapa Biosystems	Kapa HiFi HotStart ReadyMix	KR0370

Procedure

1. Prepare the cDNA PCR Mix by adding the reagents in the order shown below.

Component	1 Reaction + 10% excess, µl	4 Reaction + 10% excess, µl	8 Reaction + 10% excess, µl
H_2O	23.1	92.4	184.8
10 µM SMART PCR Primer	4.4	17.6	35.2
2x Kapa HiFi HotStart ReadyMix	27.5	110	220
Total	**55**	**220**	**440**

2. Wash once with 100 µl of H_20.
3. Spin down the tubes and remove supernatant.
4. Resuspend in 50 µl of cDNA PCR mix to each tube and transfer to a 96-well PCR plate.
5. Mix well and proceed to PCR.

Lid Temperature	Reaction Volume
105°C	50 µl

Step	Temperature, °C	Time
1	95	3 min
2	98	20 sec
3	65	45 sec
4	72	3 min
5	Go to step 2, 3x	
6	98	20 sec
7	67	20 sec
8	72	3 min
9	Go to step 5, 8x	
10	72	5 min
11	4	Hold

Clean Up cDNA, Tagment cDNA, Amplify Tagmented cDNA, Clean Up Libraries, Assess Libraries, and Prepare for Sequencing

Procedure

1. After cDNA synthesis, follow the SureCell WTA 3' Library Prep Reference Guide, starting at **Clean Up cDNA**, for all subsequent steps.
2. For sequencing, use the Sequencing Primer in the SureCell WTA 3' Library Prep Kit and the following sequencing specifications:
   - Read 1: 20 bp
   - Read 2: 50 bp
   - Read 1 index: 8 bp ← necessary only if samples are multiplexed

Visit **bio-rad.com/dropseq** for more information.

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