Chapter 8

Seq-Well: A Sample-Efficient, Portable Picowell Platform for Massively Parallel Single-Cell RNA Sequencing

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Abstract

Seq-Well is a low-cost picowell platform that can be used to simultaneously profile the transcriptomes of thousands of cells from diverse, low input clinical samples. In Seq-Well, uniquely barcoded mRNA capture beads and cells are co-confined in picowells that are sealed using a semipermeable membrane, enabling efficient cell lysis and mRNA capture. The beads are subsequently removed and processed in parallel for sequencing, with each transcript's cell of origin determined via the unique barcodes. Due to its simplicity and portability, Seq-Well can be performed almost anywhere.

Key words Seq-Well, Single-cell RNA sequencing, Single-cell genomics, Systems biology, Transcriptomics, RNA-Seq, Picowells

1 Introduction

Single-cell RNA sequencing (scRNA-seq) is an emerging method that enables genome-wide expression profiling at cellular resolution. Population-level transcriptomic techniques, such as microarrays and bulk RNA-seq, average over a large number of cells and assume transcriptional homogeneity; yet even related cells of the same subtype can present dramatic heterogeneity in their transcriptional activities and states [1]. ScRNA-seq allows direct measurement of this variability, as well as analyses of expression covariation across cells. This information can be used to discover gene-expression patterns that define distinct cell types and states, as well as their molecular circuits and biomarkers, affording an unprecedented view of cellular phenotype. Over the years, technological progress and protocol improvements have resulted in a substantial increase

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Valentina Proserpio (ed.), *Single Cell Methods: Sequencing and Proteomics*, Methods in Molecular Biology, vol. 1979, https://doi.org/10.1007/978-1-4939-9240-9_8,

in the number of cells that can be processed in parallel [2–4], enhancing statistical power and providing opportunities to look at increasingly complex systems. Current methods used to prepare single-cell libraries include manual selection [5], FACS sorting [6], microfluidic circuits [7], droplet-based techniques [8–10], and picowells [11, 12].

Seq-Well, an example of the latter, is an easy-to-use, low-cost, sample-efficient and portable platform for massively parallel scRNA-seq [11]. Seq-Well utilizes PDMS arrays containing ~88,000 subnanoliter wells in which single cells and uniquely barcoded poly(dT) mRNA beads are co-confined with a semipermeable membrane. Crucially, well size ensures that only one barcoded mRNA capture bead can fit into each well, improving cell capture efficiency. Cells, meanwhile, are loaded at a low density to minimize cell doublets, ensuring single-cell resolution. Selective chemical functionalization allows reversible attachment of a semipermeable polycarbonate membrane with 10 nm pores, permitting buffer exchange for cell lysis while trapping larger macromolecules, such as nucleic acids, to minimize cross-contamination. The co-confined mRNA capture beads are covered in oligonucleotides that consist of a universal primer, a cell barcode (unique to each bead), a unique molecular identifier (UMI; unique to each primer), and a poly-T sequence that can capture cellular mRNA upon lysis and during hybridization [13]. Following these steps, the semipermeable membrane can be peeled off for bead removal. Finally, the barcoded beads can be pooled for reverse transcription, PCR amplification, library preparation, and sequencing, with a transcript's cell of origin and uniqueness determined via its cell barcode and UMI, respectively.

Importantly, implementing Seq-Well only requires a PDMS array, a polycarbonate membrane, a pipette, a clamp, an oven/heat source, and a tube rotator to produce stable cDNA product, making it functional in nearly every clinic and laboratory context.

2 Materials

All buffers and solutions are to be prepared with ultrapure water and stored at room temperature, unless otherwise indicated.

2.1 Array Processing Prior to Reverse Transcription

1. Bead loading buffer (BLB): 10% BSA, 100 mM sodium carbonate, pH 10. Add 2.5 mL BSA (100 mg/mL) to a 50 mL falcon tube. Add water to ~15 mL followed by 1.25 mL 2 M sodium carbonate. Add additional water to achieve a final volume of 25 mL. Titrate with glacial acetic acid to reach pH 10 (*see* **Note 1**).

2. Prelysis buffer: 5 M guanidine thiocyanate, 1 mM EDTA (*see* **Notes 2** and **3**).
3. Complete lysis buffer: 5 M guanidine thiocyanate, 1 mM EDTA, 0.5% sarkosyl, 1% β-mercaptoethanol. Combine 5 mL prelysis buffer with 25 μL 10% sarkosyl and 50 μL β-mercaptoethanol (*see* **Note 4**).
4. Hybridization buffer: 2 M NaCl, 4% PEG 8000 in PBS. Combine 10 mL 5 M NaCl with 13 mL of PBS, and 2 mL 50% (w/v) PEG 8000 (*see* **Note 5**).
5. Wash buffer: 2 M NaCl, 3 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.0), 4% PEG 8000. Combine 20 mL 5 M NaCl, 150 μL 1 M $MgCl_2$, 1 mL 1 M Tris–HCl (pH 8.0), and 4 mL 50% (w/v) PEG 8000. Add water to bring volume to 50 mL (*see* **Note 5**).
6. Polycarbonate membranes: 0.01 μm pores, 62 mm × 22 mm (*see* **Note 6**).
7. mRNA capture beads (*see* **Note 7**).
8. Seq-Well arrays (*see* **Notes 8** and **9**).
9. RPMI.
10. RP-10: RPMI with 10% FBS.
11. PBS for washing.

2.2 Array Storage

1. Array quenching buffer: 100 mM sodium carbonate, 10 mM Tris–HCl (pH 8.0). Combine 2.5 mL 2 M sodium carbonate with 500 μL 1 M Tris–HCl. Add water to bring total volume to 50 mL. Arrays can be stored in array quenching buffer for up to 1 month at 4 °C (*see* **Note 10**).
2. Aspartic acid solution: 20 μg/mL of L-aspartic acid, 2 M NaCl, and 100 mM sodium carbonate solution (pH 10.0). Arrays can be stored in the aspartic acid solution for up to 6 months at 4 °C (*see* **Note 10**).

2.3 Reverse Transcription

1. Maxima H-RT with Maxima 5× RT buffer.
2. 30% PEG 8000.
3. dNTP mix (10 mM each).
4. RNase Inhibitor.
5. Template Switch Oligo (*see* Subheading 2.5).
6. TE-TW: 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.01% Tween-20. Combine 49.5 mL water, 0.5 mL 1.0 M Tris pH 8.0, 100 μL 0.5 M EDTA, and 50 μL Tween-20.
7. TE-SDS: 10 mM Tris pH 8.0, 1 mM EDTA, 0.05% SDS. Combine 49.5 mL water, 0.5 mL 1.0 M Tris pH 8.0, 100 μL 0.5 M EDTA, and 250 μL 10% SDS.

2.4 PCR and Library Preparation

1. Exonuclease I (*E. coli*) with buffer (NEB Cat. No. M0293S).
2. 10 mM Tris–HCl (pH 8.0).
3. Thermocycler.
4. Microseal B adhesive seal.
5. Microseal F foil.
6. Qubit assay tubes.
7. Qubit 2.0 fluorometer.
8. 96-well PCR plates, skirted.
9. SMART PCR Primer (*see* below).
10. KAPA HiFi Hotstart Readymix PCR Kit.
11. Ampure DNA Spri beads.
12. 80% ethanol.
13. Agilent High Sensitivity DNA Kit.
14. Nextera XT kit.
15. Custom P5-SMART PCR hybrid oligo (*see* Subheading 2.5).

2.5 Primers

1. Template Switch Oligo: AAGCAGTGGTATCAACGCAGAG TGAATrGrGrG.
2. SMART PCR Primer: AAGCAGTGGTATCAACGCAGAGT.
3. Custom P5-SMART PCR hybrid oligo: AATGATACGGC-GACCACCGAGATCTACACGCCTGTCCGCGGAAGCAG TGGTATCAACGCAGAGT*A*C.
4. Custom Read 1 Primer: GCCTGTCCGCGGAAGCAGTGG TATCAACGCAGAGTAC.

3 Methods

3.1 Membrane Functionalization

1. Place a precut (22 × 66 mm) polycarbonate membrane onto a glass slide, using a gloved finger and tweezers to carefully separate the membrane and paper. Make sure the shiny side of the polycarbonate membrane is facing up. Discard any membranes that have creases or other large-scale imperfections (*see* **Note 11**).
2. Place membranes onto a shelf in the plasma cleaner (*see* **Note 12**).
3. Close the plasma cleaner door and make sure the three-way valve lever is in the closed position. Then turn on the main power and pump switch to form a vacuum (*see* **Note 13**).
4. Allow a vacuum to form for 2 min. Once the vacuum has formed, simultaneously turn the valve clockwise to 12:00 while turning the power to the high setting.

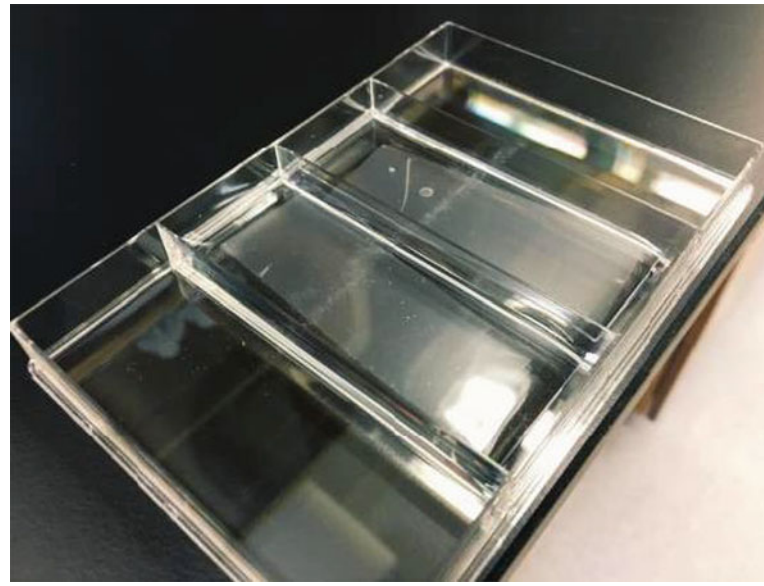

Fig. 1 Functionalized membranes can be stored in 1× PBS for 24 h

5. Treat membranes with plasma for 7 min.
6. After treatment, in the following order, turn the RF level valve from HIGH to OFF, then turn off the power followed by turning off the vacuum. Then slowly open the valve until you can just barely hear air entering the chamber. Allow the chamber to slowly fill with air until the door opens. This will take about 5 min (*see* **Note 14**).
7. Pipet 1 mL of 1× PBS into each well of a four-well plate. Transfer slides with treated membranes from the plasma cleaner to the four-well plate. Quickly pipet 4 mL of 1× PBS over the membrane, preventing the membrane from folding on itself (*see* **Note 15** and Fig. 1).
8. Remove any air bubbles underneath the membrane by gently pressing on the membrane using wafer forceps. Membranes are now functionalized and ready for use. Membranes solvated with 1× PBS should be used within 24 h.

3.2 Bead Loading

1. Aspirate storage solution and solvate each array with 5 mL of BLB (*see* **Note 16**).
2. Aliquot ~110,000 beads per array from bead stock into a 1.5 mL tube and spin on a tabletop centrifuge for 15 s to form a pellet (*see* **Note 17**).
3. Aspirate storage buffer and replace it with 500 μL of BLB. Invert the tube several times to wash the beads. Pellet the beads and then repeat the wash step with an additional 500 μL of BLB.

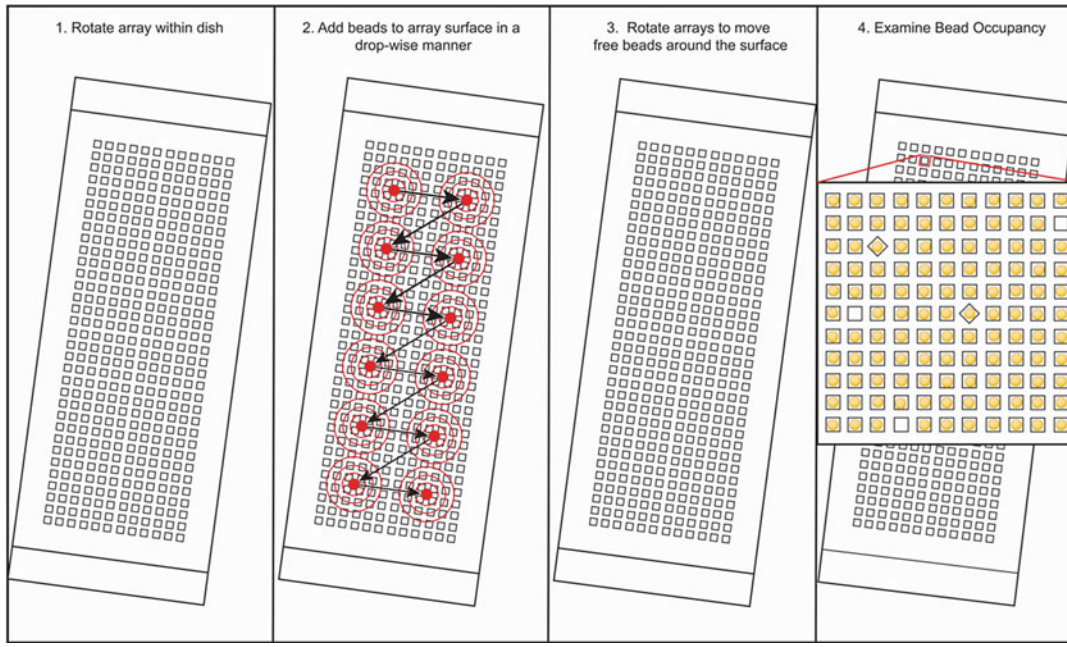

Fig. 2 Apply beads to the array in a dropwise fashion

4. Pellet beads, aspirate BLB, and resuspend in 200 μL of BLB per 110,000 beads.
5. Before loading beads, thoroughly aspirate BLB from the dish containing the array(s), being careful not to aspirate or dry the PDMS surface of the array(s). Center the array(s) so that there is no contact between the array(s) and the sides of the four-well dish.
6. Use a 200 μL pipette to apply 200 μL containing 110,000 beads, in a dropwise fashion, to the surface of each array. Your goal is to cover the surface of the entire array with beads (*see* Fig. 2).
7. Rock the four-well dish in the x and y directions for 10 min (*see* **Notes 18–20**).
8. Thoroughly wash array(s). Position each array so that it sits in the center of the four-well dish. Dispense 500 μL of BLB in the upper right corner of each array and 500 μL in the bottom right corner of the PDMS surface of each array. Be careful not to directly pipet onto the microwells, as it can dislodge beads. Using wafer forceps, push each array against the left side of the four-well dish to create a capillary flow—this will help remove excess beads from the surface. Aspirate the liquid from the bottom of the dish, reposition each array in the center of the four-well dish, and repeat, but this time pipetting BLB onto the opposite corners (*see* **Notes 21** and **22** and Fig. 3).

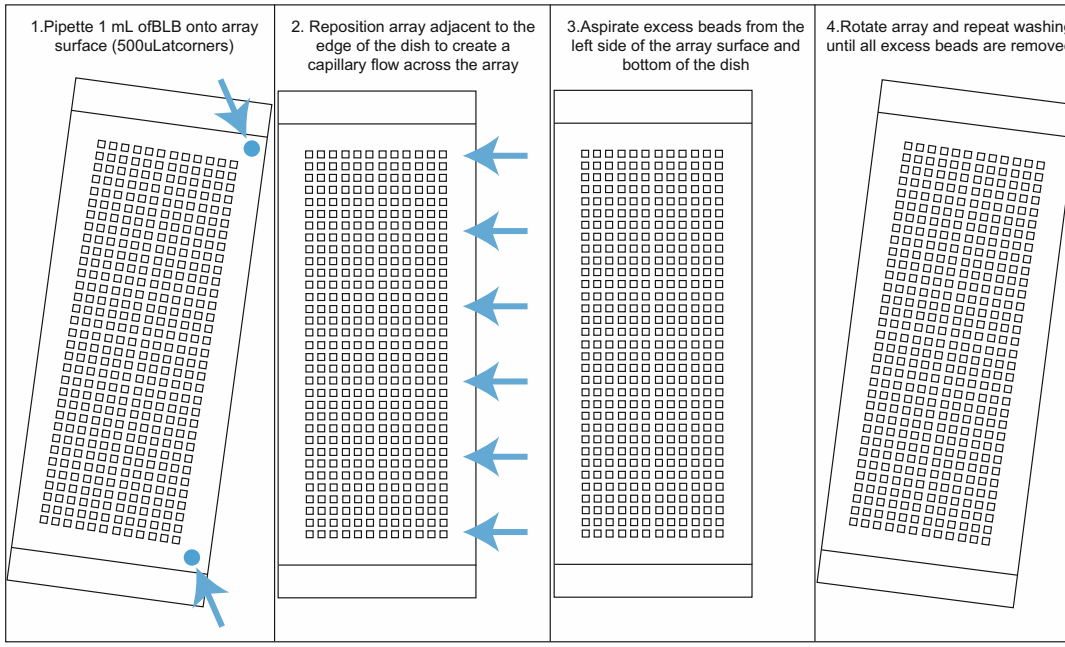

Fig. 3 Create capillary flow to draw excess beads from the center of the array

9. Repeat **step 8** as necessary. Periodically examine the array (s) under a microscope to verify that very few loose beads are present on the surface, as this will interfere with membrane attachment.
10. Once excess beads have been removed from the surface, solvate each array. If continuing to cell loading immediately (i.e., within 1–5 h), loaded arrays should be stored in 5 mL of BLB. Alternatively, loaded arrays can be stored for up to 2 weeks in Array Quenching Buffer.

3.3 Cell Loading

1. Obtain a cell or tissue sample and prepare a single-cell suspension using your preferred protocol.
2. Aspirate BLB from each array and soak in 5 mL of RPMI + 10% FBS (RP-10).
3. After obtaining a single-cell suspension, count cells using a hemocytometer and make a new solution of 15,000 cells in 200 μL of RP-10 (*see* **Note 23**).
4. Aspirate the RP-10 from the four-well dish, center each array in well, and then load the cell loading solution in a dropwise fashion onto the surface of each array.
5. Rock the array in the *x* and *y* directions for a total of 10 min—alternate between rocking for 20 s and letting the arrays sit for 30 s to let cells fall into wells.

6. Wash array(s) 4× with PBS to remove FBS in media (*see* **Note 24**). To wash, add 5 mL of PBS to the corner of the four-well dish and then aspirate.
7. Aspirate final PBS wash and replace with 5 mL of RPMI media (*no FBS*).

3.4 Membrane Sealing

1. Gather the follow materials before sealing the array(s): wafer forceps, paper towels, Agilent clamps, pretreated membranes, and clean microscope slides.
2. Use the wafer forceps, transfer the array from media to the lid of a four-well dish, being careful to keep the array as close to horizontal as possible (*see* **Note 25**).
3. Use the wafer forceps to remove a pretreated membrane from the four-well dish. Gently dab away excess moisture from the glass slide on the paper towel until the membrane does not spontaneously change position on the glass slide. Avoid touching the surface of the membrane that will be sealed to PDMS array as this may affect membrane sealing.
4. Carefully position the membrane in the center of the microscope slide leaving a small (2–3 mm) membrane overhang beyond the edge of the slide (*see* **Note 26** and Fig. 4).
5. Holding the membrane in your left hand, invert the microscope slide so that the treated surface is facing down.
6. Place the overhang of the membrane in contact with the PDMS surface of the array just above the boundary of the microwells (*see* Fig. 5).
7. Using a clean glass slide held in your right hand, firmly press down the overhang of the membrane against the PDMS surface of the array.

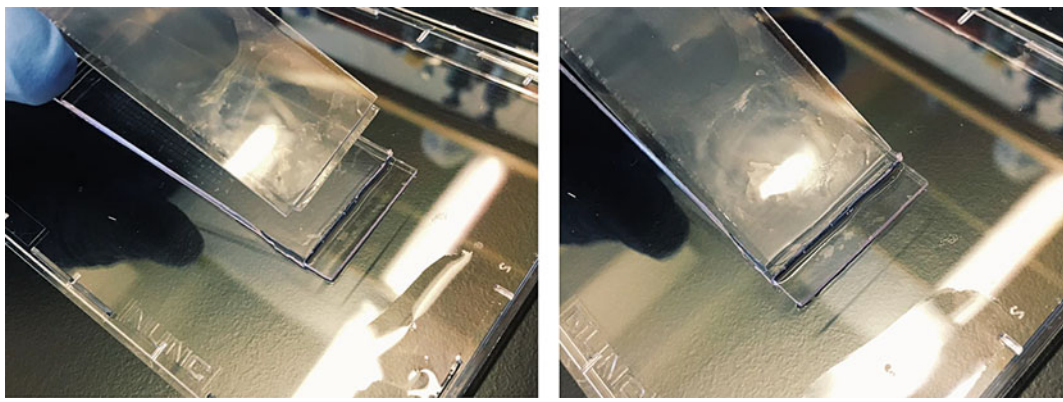

Fig. 4 Use tweezers to position the membrane on the glass slide so that there is a small overhang and touch it to the array just above the boundary of the wells

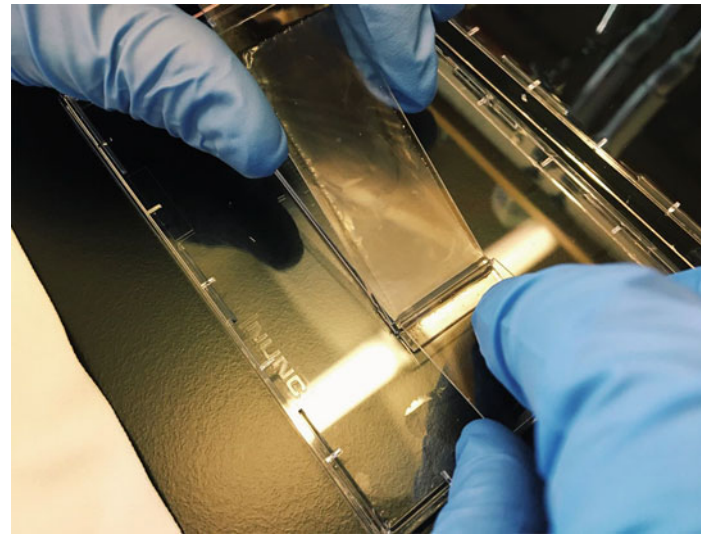

Fig. 5 Hold the membrane firmly against the array with a clean glass slide

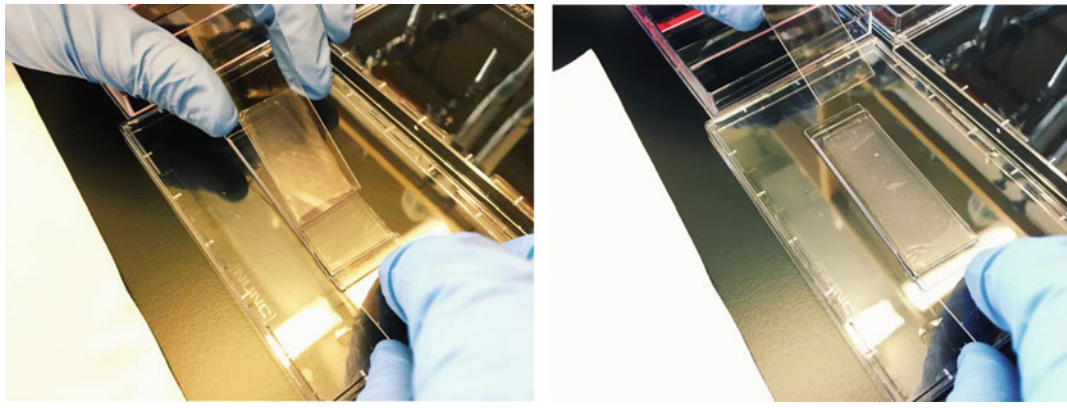

Fig. 6 Slide the hand holding the membrane across the array to apply the membrane

8. While maintaining pressure with your right hand to hold the membrane in place, gently apply the membrane by shifting your left hand across the array (*see* **Notes 27** and **28**, and Fig. 6).
9. After applying the membrane, carefully pry the array and membrane from the surface of the lid and transfer to an Agilent clamp (*see* Fig. 7).
10. Once the array is in the clamp, place a glass slide on top of the array and then assemble the clamp, tightening it just past the point of resistance. Be careful not to tighten too far so as not to break either of the glass slides.
11. Place the assembled clamp in a 37 °C incubator for 30 min (*see* **Note 29**).

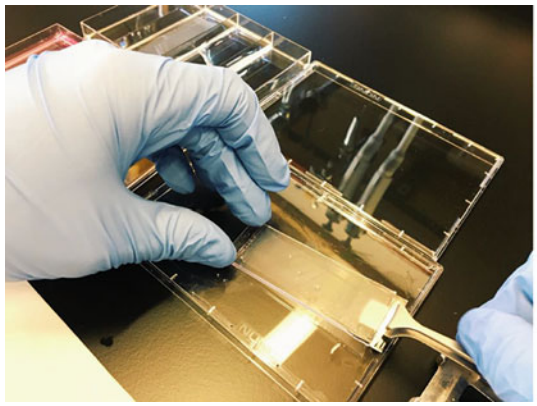
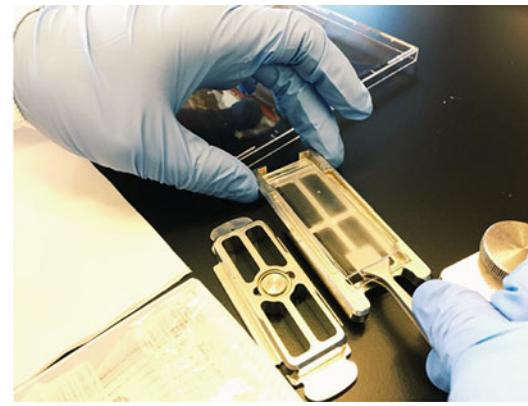

Fig. 7 Place the array in a clamp and heat it at 37 °C for 30 min to seal the membrane

3.5 Cell Lysis and Hybridization

1. Remove the clamp from the incubator and then remove the array(s) from the Agilent clamp(s) (*see* **Note 30**).
2. Submerge each array, with top slide still attached, in 5 mL of complete lysis buffer in a new four-well dish (*see* **Note 31**).
3. Gently rock the array(s) in lysis buffer until the top glass slide lifts off. Do not pry the top slide off as this can reverse membrane sealing. The time necessary for detachment of the top slide varies (10 s to 10 min). Just be patient.
4. Once the top slide has detached, let the array(s) rotate for 20 min at 50–60 rpm.
5. After 20 min, remove the lysis buffer and wash each array with 5 mL of hybridization buffer. *Use a container without bleach to collect lysis buffer waste because guanidine thiocyanate can react with bleach to create toxic gas.*
6. Remove hybridization buffer and add another 5 mL of hybridization buffer to each array. Rotate arrays for 40 min at 50–60 rpm. While arrays are rocking, prepare RT master mix (*see* **Note 32**).

3.6 Bead Removal

To remove beads from the array, either wash the arrays with a pipette or spin them down in a centrifuge with angled inserts.

3.6.1 Bead Removal by Pipette Washes

1. Aspirate hybridization buffer and replace with 5 mL of wash buffer.
2. Rock for 3 min. Fill 50 mL conical tube(s) with 48 mL of wash buffer (*see* **Note 33**).
3. Remove membranes with fine-tipped tweezers (*see* Fig. 8).
4. Carefully position the array over the 50 mL conical tube. Repeatedly wash (~15 times) beads from the surface of the array over the 50 mL falcon tube using 1 mL of wash buffer.

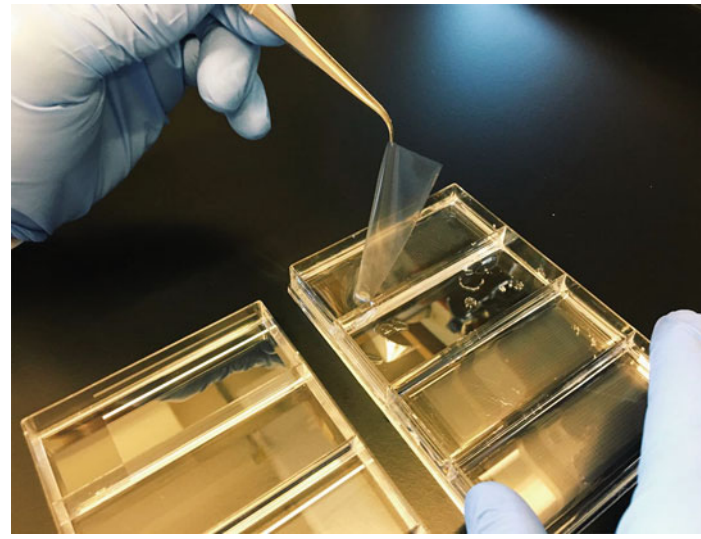

Fig. 8 After placing the array in wash buffer, remove the membrane with tweezers

Fig. 9 Carefully position the array over a conical of wash buffer and pipet on the array to dislodge beads

Flip the array and repeatedly wash (~15 times) the other end (*see* **Note 34** and Fig. 9).

5. Hold the array above the 50 mL conical and gently scrape the array ten times with a glass slide, dipping the glass slide into the wash buffer after every scrape. Flip the array and repeat.

6. Wash again using 1 mL of wash buffer (~10 times) and inspect the array underneath a microscope to check if there are any beads remaining. If so, take a glass slide and scrape more forcefully and continue to wash until all beads have been dislodged (*see* **Note 35**).
7. Spin the 50 mL falcon tube at 2000 × *g* for 5 min to pellet beads (*see* **Note 36**).
8. Aspirate all wash buffer except for ~1 mL. Be careful not to disturb the pellet of beads.
9. Transfer beads to a centrifuge tube and proceed to reverse transcription.

3.6.2 Alternative Method: Bead Removal with Inserts

1. Alternatively, you can remove beads using 3D-printed inserts.
2. Aspirate hybridization buffer and replace with 5 mL of wash buffer.
3. Fill a Falcon tube with 45 mL of wash buffer and label with sample name.
4. Remove membrane and place array into the Falcon tube with wash buffer.
5. Ensure that the array is angled within the tube as shown below.
6. Place the insert so the array is secured angled as shown in the image below.
7. Secure the lid and seal with parafilm, if necessary (*see* **Note 37**).
8. Put the sealed conical in a centrifuge, making certain the PDMS surface of the array is facing away from the rotor arm (*see* Fig. 10).
9. Centrifuge at 2000 × *g* for 5 min to remove the beads.

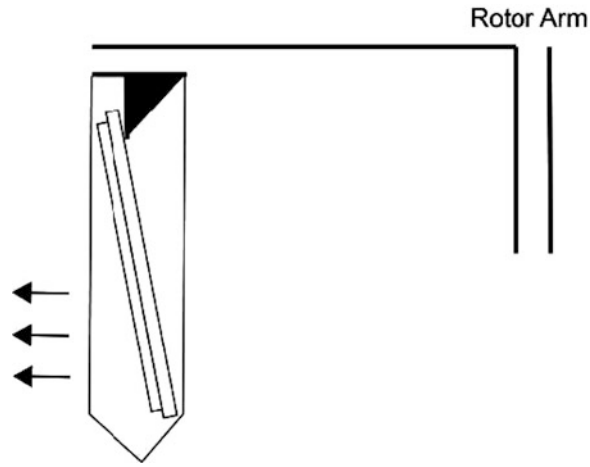

Fig. 10 Make sure the array faces outward so that the beads will fall out of wells during centrifugation

10. At this point you should see a small, but visible, pellet of beads at the bottom of the tube.
11. Aspirate 5–10 mL of wash buffer to enable easier removal of the array.
12. Remove the array and carefully position it over the top of the 50 mL tube.
13. Repeatedly wash any remaining beads from the surface of the array over the surface of the 50 mL falcon tube using 1 mL of wash buffer remaining in the tube.
14. Spin again at 2000 × *g* for 5 min to pellet beads.
15. Aspirate all wash buffer except for ~1 mL. Be careful not to disturb the pellet of beads.
16. Transfer beads to a 1.5 mL centrifuge tube and proceed to reverse transcription.

3.7 Reverse Transcription

1. Prepare the following Maxima RT Mastermix during the hybridization step (volumes provided are good for one array):
   40 μL H_2O.
   40 μL Maxima 5× RT buffer.
   80 μL 30% PEG 8000.
   20 μL 10 mM dNTPs.
   5 μL RNase inhibitor.
   5 μL 100 μM Template Switch Oligo.
   10 μL Maxima H-RT.
2. Centrifuge the 1.5 centrifuge tubes with beads for 1 min at 1000 × *g*.
3. Remove supernatant and resuspend in 250 μL of 1× Maxima RT Buffer (*see* **Note 38**).
4. Centrifuge beads for 1 min at 1000 × *g*.
5. Aspirate 1× Maxima RT buffer and resuspend beads in 200 μL of the maxima RT mastermix.
6. Incubate at room temperature for 30 min with end-over-end rotation.
7. After 30 min, incubate at 52 °C for 90 min with end-over-end rotation (*see* **Note 39**).
8. Following the RT reaction, wash beads once with 0.5 mL TE-TW, once with 0.5 mL TE-SDS, and twice with 0.5 mL of TE-TW (*see* **Notes 40** and **41**).

3.8 Exonuclease I Treatment

1. Prepare the following Exonuclease I Mix:
   20 μL 10× ExoI buffer.
   170 μL H_2O.

10 μL ExoI enzyme.

2. Centrifuge beads for 1 min at 1000 × *g* and aspirate the TE-TW solution.
3. Resuspend in 0.5 mL of 10 mM Tris–HCl pH 8.0.
4. Centrifuge beads again, remove supernatant and resuspend beads in 200 μL of Exonuclease I mix.
5. Place in a 37 °C incubator for 50 min with end-over-end rotation.
6. Wash the beads once with 0.5 mL of TE-SDS, then twice with 0.5 mL TE-TW (*see* **Note 42**).

3.9 Whole-Transcriptome Amplification (WTA)

1. Wash beads once with 500 μL of water, pellet beads, remove supernatant and resuspend in 500 μL of water.
2. Mix well (do not vortex) to evenly resuspend beads and transfer 20 μL of beads to a separate 1.5 mL tube to count the beads (*see* **Note 43**).
3. Pellet the small aliquot of beads, aspirate the supernatant, and resuspend in 20 μL of bead counting solution (10% PEG, 2.5 M NaCl) (*see* **Note 44**).
4. Count the beads using a hemocytometer (*see* **Note 45**).
5. Prepare the following PCR Mastermix (volumes provided are good for 2000 beads) (*see* **Note 46**):

   25 μL 2× KAPA HiFi Hotstart Readymix.

   24.6 μL H_2O.

   0.4 μL 100 μM SMART PCR Primer.

6. Pellet beads, remove supernatant, and resuspend in 50 μL of PCR Mastermix for every 2000 beads (*see* **Note 47**).
7. Pipet 50 μL of PCR Mastermix with beads into a 96-well plate, making sure to PCR the entire array (*see* **Note 48**).
8. Use the following cycling conditions to perform whole-transcriptome amplification (*see* **Note 49**).

	95 °C	3 min
4 Cycles	98 °C 65 °C 72 °C	20 s 45 s 3 min
9–12 Cycles	98 °C 67 °C 72 °C	20 s 20 s 3 min
Final extension	72 °C 4 °C	5 min infinite hold

3.10 Purification of PCR Products

1. Pool PCR products in a 1.5 mL microcentrifuge tube so that you have 7–8 PCR reactions per 1.5 mL microcentrifuge tube.
2. Purify the product by mixing thoroughly using Ampure SPRI beads at a 0.6× volumetric ratio (beads:PCR products (*see* **Note 50**).
3. Let the tubes sit in the rack off the magnet for 5 min, then place the rack on the magnet for 5 min.
4. Perform two washes with 80% ethanol.
5. After second wash, allow the beads to dry for 10 min on the magnet, then remove the rack from the magnetic, elute the beads in 100 μL, then place the rack back on the magnet and transfer the 100 μL to a new 1.5 mL microcentrifuge tube.
6. SPRI the 100 μL at 0.8× volumetric ratio, repeating **steps 6–8**.
7. After the second wash, allow the beads to dry for 5–10 min on the magnet, remove the rack from the magnetic, elute the beads in 15 μL, then place the rack back on the magnet and transfer the 15 μL to a new 1.5 mL microcentrifuge tube.
8. Run a High Sensitivity DNA D5000 ScreenTape on an Agilent 4200 Tapestation to determine the length distribution of your cDNA. The distribution should be fairly smooth with an average bp size of 900–1500 bp (*see* Fig. 11).
9. Proceed to library preparation or store the WTA product at 4 °C.

3.11 Nextera Library Preparation

1. Make certain that your thermocyclers are set up for Tagmentation (**step 5**) and PCR (**step 9**).
2. For each sample, combine 800 pg of purified cDNA with water in a total volume of 5 μL. It is ideal to dilute your PCR product in a separate tube/plate so that you can add 5 μL of that for tagmentation.

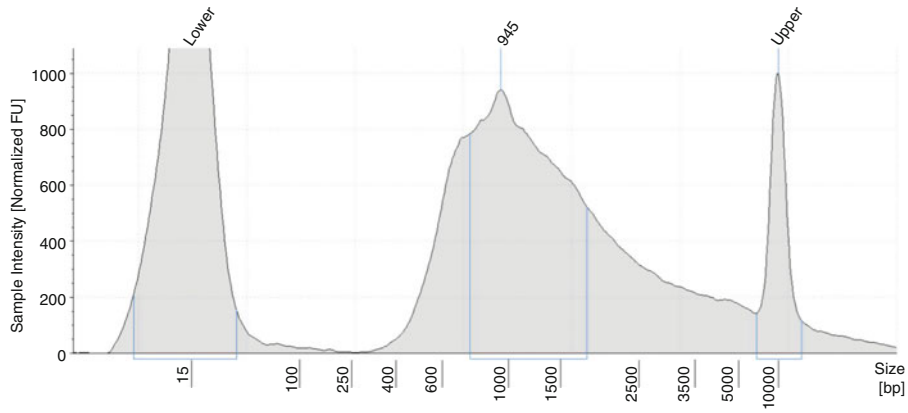

Fig. 11 An ideal WTA product distribution has a peak at 900–1100 bp and has a long tail reaching 5000 bp

3. To each tube, add 10 μL of Nextera TD buffer, then 5 μL of ATM buffer (the total volume of the reaction is now 20 μL).
4. Mix by pipetting ~5 times. Spin down.
5. Incubate at 55 °C for 5 min.
6. Let the thermocycler cool to 4 °C after incubation, and then immediately add 5 μL of Neutralization Buffer. Mix by pipetting ~5 times. Spin down for 1 min at 1000 × *g*. Bubbles are normal.
7. Incubate at room temperature for 5 min.
8. Add to each PCR in the following order:

   15 μL Nextera PCR mix.

   8 μL H_2O.

   1 μL 10 μM New-P5-SMART PCR hybrid oligo.

   1 μL 10 μM Nextera N700X oligo.
9. After sealing the reaction tubes and spinning them down (1 min at 1000 × *g*), run the following PCR program:

	95 °C	30 s
12 Cycles	95 °C 55 °C 72 °C	10 s 30 s 30 s
Final extension	72 °C 4 °C	5 min Infinite hold

10. Proceed to SPRI purification or store the WTA product at 4 °C.
11. SPRI at 0.6× volumetric ratio.
12. Let the tubes sit in the rack off the magnet for 5 min, then place the rack on the magnet for 5 min.
13. Perform two washes with 80% ethanol.
14. After the second wash, allow the beads to dry for 5–10 min on the magnet, remove the rack from the magnetic, elute the beads in 100 μL, then place the rack back on the magnet and transfer the 100 μL to a new 1.5 mL microcentrifuge tube.
15. Spri 100 μL at 0.8× volumetric ratio and repeat steps b and c.
16. After the second wash, allow the beads to dry for 5–10 min on the magnet, remove the rack from the magnetic, elute the beads in 15 μL, then place the rack back on the magnet and transfer the 15 μL to a new 1.5 mL microcentrifuge tube.
17. Run a High Sensitivity DNA D1000 ScreenTape on an Agilent 4200 Tapestation. Your tagmented library should be fairly smooth, with an average bp size of 600–750 bp (*see* **Note 51** and Fig. 12).

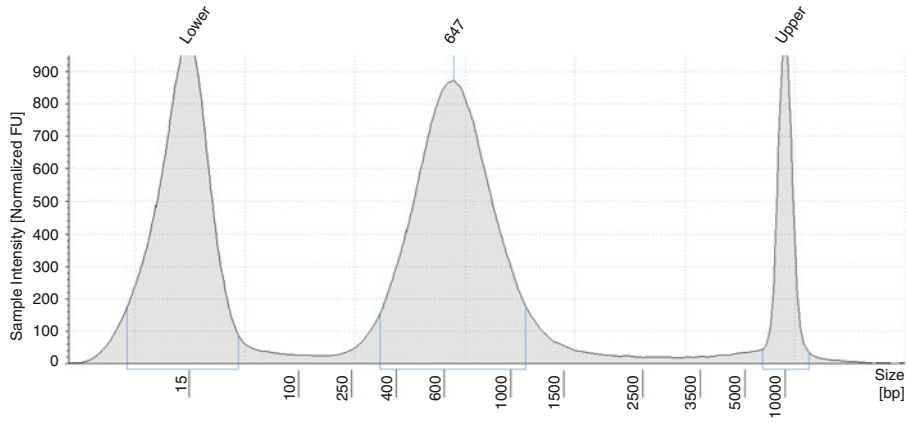

Fig. 12 An ideal NTA product distribution is a smooth bell curve with a peak between 600 and 750 bp

3.12 NextSeq500 Sequencing

1. Make a 5 μL library pool at 4 nM as input for denaturation.
2. To this 5 μL library, add 5 μL of 0.2 N NaOH (make this solution fresh from a 2 M NaOH stock).
3. Flick to mix, then spin down and let tube sit for 5 min at room temperature.
4. After 5 min, add 5 μL of 0.2 M Tris–HCl pH 7.5.
5. Add 985 μL of HT1 Buffer to make a 1 mL, 20 pM library (solution 1).
6. In a new tube (solution 2), add 165 μL of solution 1 and dilute to 1.5 mL with HT1 buffer to make a 2.2 pM solution—this is the recommended loading concentration.
7. Add 6 μL of Custom Read 1 primer to 1.994 mL of HT1 buffer to make 2 mL of 0.3 μM Custom Read 1 primer.
8. Follow Illumina's guide for loading a NextSeq500 kit. Seq-Well requires paired-end sequencing with a read structure of 20 bp read one, 50 bp read two, and 8 bp index one.

4 Notes

1. You will want ~25 mL of bead loading buffer for each array. It is important that you do not add the sodium carbonate directly to the BSA to avoid denaturing the BSA. This solution should be prepared fresh just before loading beads. The stock of BSA should be filtered prior to use with a 0.22 μm filter and should be kept at 4 °C.
2. It will take some time for the guanidine thiocyanate to dissolve. Be sure that this is prepared in advance.

3. Prelysis buffer is photosensitive so wrap the buffer's container with aluminum foil. Wrapped prelysis buffer can be stored at room temperature and has a shelf life of approximately 6 months.
4. Complete lysis buffer should be prepared immediately prior to use.
5. You will want 10 mL of hybridization buffer and 50 mL of wash buffer per array. Both solutions can be made in advance and stored at room temperature for 3 months.
6. We purchase membranes from Sterlitech Corporation.
7. We purchase the mRNA capture beads from Chemgenes (Cat. No. MACOSKO-2011-10). Currently, this is the only supplier manufacturing these beads.
8. You can transport arrays by placing them in 50 mL conical tubes filled with array quenching buffer or the aspartic acid solution. Two arrays will fit per conical if they are arranged back to back with their glass slides touching.
9. An alternative transportation method is to dry the arrays and transport them in a glass slide box. To dry the arrays, remove them from the storage buffer, use a paper towel to wick off excess liquid from the glass slide (while being careful not to touch the surface of the array), and then let them sit until air dried. Rehydrate the arrays by placing them in a four-well dish with either array quenching buffer or the aspartic acid solution. Place them under vacuum until there are no air bubbles remaining in the wells of the array. Alternatively, if a vacuum chamber is not available you can let the arrays soak overnight; they will be hydrated and ready to use the following day.
10. Use array quenching storage buffer for short-term storage for up to 1 month. If storing longer than 1 month, it is advisable to store in aspartic acid solution.
11. Prepare one extra membrane in case of a mistake during membrane application.
12. If your plasma oven has multiple shelves, place membranes on the bottom shelf to reduce the risk of them flying when vacuum is released and atmospheric pressure is restored.
13. The plasma should be a bright pink color. If not, adjust the air valve to increase or decrease the amount of oxygen you are letting into the chamber. Also check to see if vacuum has formed by gently pulling on the door of the plasma oven.
14. If membranes have slightly folded over, slowly flip the membrane back using sharp tweezers. If membranes have blown off the slide entirely, repeat membrane preparation procedure to ensure you know which side was exposed to plasma.

15. If transporting solvated membranes (e.g., between buildings), remove all but ~1 mL of PBS to prevent membranes from flipping within the dish. Alternatively, membranes can be solvated in 1× PBS, dried out, and stored for one week at room temperature. When ready to use membranes, they can be rehydrated with 1× PBS. This is helpful when traveling with membranes or when you want to run Seq-Well in a laboratory without access to a plasma cleaner.
16. Before bead loading, use a microscope to inspect wells for air bubbles. If air bubbles are present, place array(s) under vacuum with rotation (50 RPM) for 10 min to remove air bubbles in wells. The house vacuum in most laboratories should be sufficient to remove any air bubbles from the wells.
17. Never vortex beads, as this can fragment them and interfere with bead loading and transcript capture.
18. Place a black background behind the four-well dish to better visualize bead coverage of the array.
19. Be careful not to let the surface of the array dry. Sudden movements or tilting at too steep an angle can lead to spillage. If BLB falls off the surface of the array into the four-well dish, gently pipet the BLB back onto the corners of the PDMS surface of the array, being careful not to pipet directly onto wells.
20. It can be helpful to repeatedly (every ~30 s) rest the four-well dish level to allow beads to fall into wells. After tilting forward and backward for 10 min, tilt the four-well dish in whichever direction is needed to cover poorly loaded areas with beads.
21. You can save the excess beads by pipetting the liquid into a 50 mL conical instead of aspirating it. After collecting excess beads, wash them twice by spinning down at 1000 rcf and resuspending the pellet in TE-TW. Store the washed beads in TE-TW for future loading.
22. An alternative bead removal method is to add 3 mL of BLB and rock at ~30° angles six times to get beads to roll off the surface. Repeat this procedure three times.
23. You can also load cells in DMEM with 5% FBS or PBS with 0.05% BSA.
24. Washing with PBS is critical to ensure successful membrane attachment as FBS can interfere with membrane sealing.
25. Make sure the lid of the four-well dish is dry. Position the array in the corner of the lid so the array does not slide as you apply the membrane.
26. Occasionally, the membrane will fold back on the other side of the glass slide. Readjust the membrane until there is an

overhang. An alternative method to prevent this is to first invert the glass slide and then pull the membrane past the edge of the glass slide.

27. For optimal results, use little to no pressure while applying the membrane with the left hand. *See* Instructional Video (www.shaleklab.com/seq-well) for additional details. Attempts to manually seal the microwell device using pressure result in a "squeegee" effect, effectively removing moisture from the membrane while fixing membrane creases in place.
28. It is very important to avoid a rumpled membrane. If the membrane is creased, dip an edge of a glass slide in liquid and smooth over areas. However, there is a limited amount of remediation that is possible prior to decreasing the efficacy of sealing, so ideally create the smoothest membrane possible on the first pass.
29. This time is flexible and depends on the incubator. If you want to decrease this incubation time, please optimize on cell lines before proceeding with precious samples.
30. Sometimes the array will be stuck to the top piece of the clamp—this is fine, just carefully slide it off.
31. To make complete lysis buffer, combine 5 mL of prelysis with 25 μL of 10% sarkosyl and 50 μL β-mercaptoethanol.
32. The hybridization buffer may contain trace amounts of guanidine thiocyanate and therefore should be collected in the lysis buffer waste container.
33. Label the 50 mL conical tubes with sample names to avoid mixing up samples after removing beads from the array(s).
34. Make sure to pipet on the entire surface of the array, including the edges and corners.
35. Sometimes after scrapping empty wells will fill up with bubbles. Be careful not to mistake bubbles for beads when inspecting underneath a microscope.
36. You should see a small, but visible, pellet of beads at the bottom of the tube.
37. The array might move around at this point. This is not a problem.
38. Prepare 1× maxima by diluting 5× maxima buffer in RNAse free H_2O.
39. You can also let RT continue overnight at 52 °C and wash the beads the next day.
40. Salts in the RT buffer can cause SDS to precipitate, making it difficult to remove in subsequent washes, so it is best to begin with a single wash in TE-TW.

41. This is a stopping point; after the final TE-TW wash, beads can be resuspended in TE-TW and stored for up to 2 weeks at 4 °C.
42. This is another stopping point; after the final TE-TW wash, beads can be resuspended in TE-TW and stored for up to 2 weeks at 4 °C.
43. Do not vortex beads as this can result in bead fragmentation.
44. The bead counting solution aids in even dispersion of beads across a hemocytometer.
45. Sometimes the beads will not evenly disperse, making it difficult to count them. If this is the case, assume there are 60,000 beads for the following steps.
46. For instance, if you have 60,000 beads from an array, prepare a PCR mastermix with 750 μL of 2× KAPA HiFi Hotstart Readymix, 738 μL of H_2O, and 12 μL of 100 μM SMART PCR Primer.
47. For instance, if you have 60,000 beads resuspend in 1500 μL of PCR mastermix.
48. Periodically resuspend the beads to make sure they are evenly dispersed in the solution.
49. The total number of PCR cycles necessary for amplification depends on the cell type used. Approximately 16 cycles are optimal for primary cells (e.g., PBMCs) and approximately 13 cycles are optimal for cell lines or larger cells (e.g., macrophages). For experiments on dissociated human tissue, start with 16 cycles and optimize from there.
50. For instance, if you have 400 μL of product add 240 μL of SPRI beads and mix for a 0.6× volumetric SPRI.
51. We have successfully sequenced NTA product with average bp sizes from 350 to 800 bp.

Acknowledgments

R.G. was supported by the Intramural Research Program of the Division of Intramural Research Z01AI000947, NIAID, NIH; the UCLA-Caltech MSTP, and the NIGMS T32 GM008042. A.K.S. was supported by the Searle Scholars Program, the Beckman Young Investigator Program, the Pew-Stewart Scholars, a Sloan Fellowship in Chemistry, NIH grants 1DP2OD020839, 2U19AI 089992, 1U54CA217377, P01AI039671, 5U24AI118672, 2RM1HG006193, 1R33CA202820, 2R01HL095791, 1R01AI 138546, 1R01HL126554, 1R01DA046277, 2R01HL095791, and Bill and Melinda Gates Foundation grants OPP1139972, OPP1137006, and OPP1116944. J.C.L. was supported by NIH grants DP3DK09768101, P01AI045757, R21AI106025, and

R56AI104274, the W.M. Keck Foundation, Camille Dreyfus Teacher-Scholar program, and the US Army Research Office through the Institute for Soldier Nanotechnologies, under contract number W911NF-13-D-0001. This work was also supported in part by the Koch Institute Support (core) NIH Grant P30-CA14051 from the National Cancer Institute.

References

1. Kolodziejczyk AA, Lönnberg T (2018) Global and targeted approaches to single-cell transcriptome characterization. Brief Funct Genomics 17:209–219. https://doi.org/10.1093/bfgp/elx025
2. Svensson V, Vento-Tormo R, Teichmann SA (2018) Exponential scaling of single-cell RNA-seq in the past decade. Nat Protoc 13:599–604. https://doi.org/10.1038/nprot.2017.149
3. Kolodziejczyk AA, Kim JK, Svensson V et al (2015) The technology and biology of single-cell RNA sequencing. Mol Cell 58:610–620. https://doi.org/10.1016/j.molcel.2015.04.005
4. Ziegenhain C, Vieth B, Parekh S et al (2017) Comparative analysis of single-cell RNA sequencing methods. Mol Cell 65:631–643.e4. https://doi.org/10.1016/j.molcel.2017.01.023
5. Tang F, Barbacioru C, Wang Y et al (2009) mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods 6:377–382. https://doi.org/10.1038/nmeth.1315
6. Macaulay IC, Svensson V, Labalette C et al (2016) Single-cell RNA-sequencing reveals a continuous spectrum of differentiation in hematopoietic cells. Cell Rep 14:966–977. https://doi.org/10.1016/j.celrep.2015.12.082
7. Shalek AK, Satija R, Adiconis X et al (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498:236–240. https://doi.org/10.1038/nature12172
8. Klein AM, Mazutis L, Akartuna I et al (2015) Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell 161:1187–1201. https://doi.org/10.1016/j.cell.2015.04.044
9. Macosko EZ, Basu A, Satija R et al (2015) Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 161:1202–1214. https://doi.org/10.1016/j.cell.2015.05.002
10. Zheng GXY, Terry JM, Belgrader P et al (2017) Massively parallel digital transcriptional profiling of single cells. Nat Commun 8:14049. https://doi.org/10.1038/ncomms14049
11. Gierahn TM, Ii MHW, Hughes TK et al (2017) Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. Nat Methods 14:395–398. https://doi.org/10.1038/nmeth.4179
12. Bose S, Wan Z, Carr A et al (2015) Scalable microfluidics for single-cell RNA printing and sequencing. Genome Biol 16:120. https://doi.org/10.1186/s13059-015-0684-3
13. Kivioja T, Vähärautio A, Karlsson K et al (2012) Counting absolute numbers of molecules using unique molecular identifiers. Nat Methods 9:72–74. https://doi.org/10.1038/nmeth.1778