Chapter 3

Profiling Transcriptional Heterogeneity with Seq-Well S^3: A Low-Cost, Portable, High-Fidelity Platform for Massively Parallel Single-Cell RNA-Seq

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Abstract

Seq-Well is a high-throughput, picowell-based single-cell RNA-seq technology that can be used to simultaneously profile the transcriptomes of thousands of cells (Gierahn et al. Nat Methods 14(4):395–398, 2017). Relative to its reverse-emulsion-droplet-based counterparts, Seq-Well addresses key cost, portability, and scalability limitations. Recently, we introduced an improved molecular biology for Seq-Well to enhance the information content that can be captured from individual cells using the platform. This update, which we call Seq-Well S^3 (S^3: Second-Strand Synthesis), incorporates a second-strand-synthesis step after reverse transcription to boost the detection of cellular transcripts normally missed when running the original Seq-Well protocol (Hughes et al. Immunity 53(4):878–894.e7, 2020). This chapter provides details and tips on how to perform Seq-Well S^3, along with general pointers on how to subsequently analyze the resultant single-cell RNA-seq data.

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

1 Introduction

Single-cell RNA-seq (scRNA-seq) is a powerful approach for examining the cellular composition of healthy and diseased tissues [3–8]. Several high-throughput scRNA-seq methodologies have been developed enabling the characterization of thousands of cells from complex systems in a single experiment. Most rely upon early barcoding of cellular mRNA—for example, during reverse transcription using barcoded mRNA capture beads—to allow ensemble processing while retaining single-cell resolution.

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Seq-Well, like its predecessor Drop-seq, is one such method [1, 4]. Seq-Well was designed to overcome key cost, portability, and scalability limitations associated with reverse-emulsion droplets-based cell capture and barcoding methods like Drop-seq through combining the throughput and cost-effectiveness of Drop-seq with the simplicity and sampling efficiency of picowells. In Seq-Well, cells are efficiently loaded by gravity into picowells where each cell's transcripts are uniquely barcoded using the aforementioned bead reagent. Engineered surface chemistry allows sealing of the picowell array with a selectively permeable membrane, facilitating rapid buffer exchange and parallel cell lysis, while preventing mRNA escape. Seq-Well works with low inputs (e.g., sorted populations of targeted cells [9] like antigen-specific T or B cells, fine-needle aspirates [10], tissue pinches) and fragile cells (e.g., granulocytes [11], hepatocytes), and it can be implemented in challenging environments with limited peripherals—e.g., the clinic, BSL-3/4 facilities [12]. This alleviates the need to ship samples and associated artifacts/concerns, and enables work across diseases and geographies.

In the original Seq-Well and Drop-seq protocols, as in SMART-seq, a template-switching mechanism was used to append a second PCR priming site during reverse transcription, facilitating subsequent whole transcriptome amplification by PCR [1, 4, 13]. Through a series of experiments, we found that this process had inefficiencies, limiting our ability to characterize cells with high fidelity. To address these issues, we recently developed a new variant of Seq-Well, Seq-Well S^3 (S^3: Second-Strand Synthesis), which incorporates a second-strand-synthesis step after reverse transcription to add a second PCR priming site. This modification allows for the recovery of complementary DNA (cDNA) that is reverse transcribed but for which the template switch reaction failed, enhancing the capture of key transcripts, such as transcription factors, affinity receptors, and signaling molecules by up to tenfold [2].

In this chapter, we provide a step-by-step breakdown of the Seq-Well S^3 (Second-Strand Synthesis) protocol, along with tips and tricks that leverage our collective knowledge to help prevent common pitfalls.

2 Materials

All buffers and solutions are to be prepared with sterile ultrapure water (Gibco Cat. No. 15230170) 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 screwtop conical 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 (Sigma Cat. No. AM9422), 1 mM EDTA (*See* **Note 2, 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 (Fisher Scientific Cat No. BP337-100ML).
5. Wash buffer: 2 M NaCl, 3 mM MgCl2, 20 mM Tris–HCl (pH 8.0), 4% PEG 8000. Combine 20 mL 5 M NaCl, 150 μL 1 M MgCl, 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. 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 (Millipore Sigma Cat. No. P9416-50ML).
7. Wafer-Handling Tweezers for 4 in. wafers (Excelta, Cat. No.17-467-333).
8. Fisherbrand Filter/Membrane Forceps (Fisher Scientific, Cat. No. 09-753-50).
9. Polycarbonate (PCTE) Membrane Filters, 0.01 Micron, 62 mm × 22 mm (Sterlitech Corp. Cat. No. PCT00162X22100).
10. Plasma Cleaner (Harrick Plasma Cat. No. PDC-001-HP).
11. Pump for Plasma Cleaner (Harrick Plasma Cat. No. PDC-VPE).
12. VWR Sterile Disposable Serological Pipets, Plugged, Polystyrene 10 mL (VWR Cat. No. 89130-898).
13. VWR Sterile Disposable Serological Pipets, Plugged, Polystyrene 25 mL (VWR Cat. No. 89130-900).
14. mRNA capture beads (*See* **Note 6**). (Chemgenes Cat. No. MACOSCKO-2011-10(V+)) (*See* **Note 7**).
15. Vacuum desiccator (VWR Cat. No. 24988-164).
16. Vacuum tubing, for connecting vacuum desiccator to house vacuum or vacuum pump.

17. Surgical tubing, for connecting disposable aspirators to liquid trap.
18. Sterile 4-well dishes (Thermo Fisher Scientific Cat. No. 267061).
19. VWR Sterile Disposable Aspirating Pipets, Polystyrene 1 mL (VWR Cat. No. 414004-264).
20. Fisherbrand Fine Point High Precision Forceps (Fisher Scientific, Cat. 22-327379).
21. Seq-Well arrays (*See* **Note 8, 9**)
22. RPMI (Gibco Cat. No. 11875093).
23. FBS, Heat Inactivated (Gibco Cat. No. A3840002).
24. RP-10: RPMI with 10% FBS.
25. Neubauer Improved Disposable Hemocytometer (VWR Cat. No. 82030-468).
26. Trypan Blue Solution, 0.4% for Cell Counting (Thermo-Fisher Cat. No. 1520061).
27. X-Y Rotator (Thermo-Fisher Cat. No. 88880025).
28. Platform for X-Y Rotator (Thermo-Fisher Cat. No. 88880106).
29. 1× PBS for washing (Thermo-Fisher Cat. No. 10010049).
30. Glass Slides, precleaned (Thermo-Fisher Cat. No. 12-550-A3).
31. Hybridization Clamp (*See* **Note 10**) (Agilent Cat. No. G2534A).
32. Incubator with a range of 37C-52C.

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 the total volume to 50 mL. Arrays can be stored in array quenching buffer for up to 1 month at 4C.
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. If storing arrays longer than 1 month, they should be stored in the aspartic acid solution.
3. Sterile 50 mL Conical Tubes.

2.3 Reverse Transcription

1. VWR cleanroom SpecWipers, for RNA-clean work (VWR Cat. No. 21913-214).
2. RNAse-Zap (Thermo-Fisher Cat. No. AM97800).
3. Safe-Lock Lo-Bind 1.5 mL microcentrifuge tubes, Biopur grade (Eppendorf Cat. No. 022600028).

4. Maxima H-RT with Maxima 5× RT buffer. (Thermo-Fisher Cat. No EP0753).
5. 30% PEG 8000 (mixed from 50% PEG 8k (Fisher Scientific Cat No. BP337-100ML).
6. dNTP mix, 10 mM each (Thermo-Fisher Cat. No. R0193).
7. SuperaseIN RNase Inhibitor (Thermo-Fisher Cat. No. AM2696).
8. Template Switch Oligo (*see* Subheading 2.6).
9. TE-SDS: 10 mM Tris pH 8.0, 1 mM EDTA, 0.05% SDS. Combine 49.5 mL water, 0.5 mL 1 M Tris pH 8.0, 100 μL 0.5 M EDTA, and 250 μL 10% SDS.
10. HulaMixer Sample Mixer (Thermo-Fisher 15920D).

2.4 Exonuclease Treatment and Second Strand Synthesis

1. Exonuclease I (E. coli) with buffer (New England Biolabs Cat. No. M0293).
2. 10 mM Tris–HCl, pH 8.0.
3. Safe-Lock Lo-Bind 1.5 mL microcentrifuge tubes, Biopur grade (Eppendorf Cat. No. 022600028).
4. Maxima 5× RT buffer. (Thermo-Fisher Cat. No EP0753).
5. 30% PEG 8000 (mixed from 50% PEG 8k (Fisher Scientific Cat No. BP337-100ML).
6. dNTP mix, 10 mM each (Thermo-Fisher Cat. No. R0193).
7. SuperaseIN RNase Inhibitor (Thermo-Fisher Cat. No. AM2696).
8. dN-SMRT Oligo (*see* Subheading 2.6).
9. TE-SDS: 10 mM Tris pH 8.0, 1 mM EDTA, 0.05% SDS. Combine 49.5 mL water, 0.5 mL 1 M Tris pH 8.0, 100 μL 0.5 M EDTA, and 250 μL 10% SDS.
10. HulaMixer Sample Mixer (Thermo-Fisher 15920D).

2.5 WTA, Library Preparation and Sequencing

1. 10 mM Tris–HCl, pH 8.0.
2. Thermocycler, 96-well plate compatible.
3. Microseal B adhesive seal (Bio-rad Cat. No. MSB1001).
4. Microseal F foil (Bio-rad Cat. No. MSF1001).
5. Qubit assay tubes (Thermo-Fisher Cat. No. Q32856).
6. Qubit 4.0 fluorometer (Thermo-Fisher Cat. No. Q33238).
7. Qubit DNA High Sensitivity Kit (Thermo-Fisher Cat. No. Q32854).
8. 96-well PCR plates, fully skirted (Thermo-Fisher Cat. No. AB2396).
9. SMART PCR Primer (*see* Subheading 2.6).

10. KAPA HiFi Hotstart Readymix (Roche Cat. No. KK2602).
11. Ampure XP beads (Beckman-Coulter A63881).
12. 80% ethanol (mix fresh day-of: combine ultrapure water with 200-proof ethanol (VWR Cat. No. TX89125-170).
13. Agilent TapeStation 4200 (Agilent Cat. No. G2991BA).
14. Agilent D5000 High Sensitivity DNA Tapes (Agilent Cat. No. 5067-5592).
15. Agilent D5000 High Sensitivity DNA Buffer and Ladder (Agilent Cat. No. 5067-5593).
16. Custom P5-SMART PCR hybrid oligo (*see* Subheading 2.6).
17. Nextera XT kit (Illumina Cat. No. 20024906).
18. NextSeq500 v2.5 Kit (Illumina Cat. No. 20024906).

2.6 Primers

1. Template Switch Oligo: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG. (HPLC purification, diluted to working concentration of 100 uM).
2. dN-SMART randomer AAGCAGTGGTATCAACGCAGAGTGANNNGGNNNB. (HPLC purification, diluted to working concentration of 1 mM).
3. SMART PCR Primer: AAGCAGTGGTATCAACGCAGAGT. (HPLC purification, diluted to working concentration of 100 uM).
4. Custom P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C. (HPLC purification, diluted to working concentration of 10 uM).
5. Custom Read 1 Primer: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC. (HPLC purification, diluted to working concentration of 100 uM).

3 Methods

3.1 Membrane Functionalization

1. Use membrane forceps or plastic tweezers (supplied with the Agilent Hybridization clamp) to place a precut (22 × 66 mm) polycarbonate membrane onto a glass slide. Be careful not to touch the surface of the membrane, manipulate it by touching the edges only. Using a gloved finger and tweezers, carefully separate the membrane from the paper—be careful to only touch the edges of the paper (Fig. 1).
2. Make sure the shiny side of the polycarbonate membrane is facing up. Discard any membranes that have creases or large visible imperfections. Repeat this process for the number of arrays that you plan to process, plus one extra, in case of error during membrane application (Figs. 2, 3, 4, and 5).

Fig. 1 Removing the separator paper using membrane forceps

Fig. 2 Using membrane forceps to center the membrane on the slide

Fig. 3 Membrane centered on a slide, shiny side up. This is *correct*

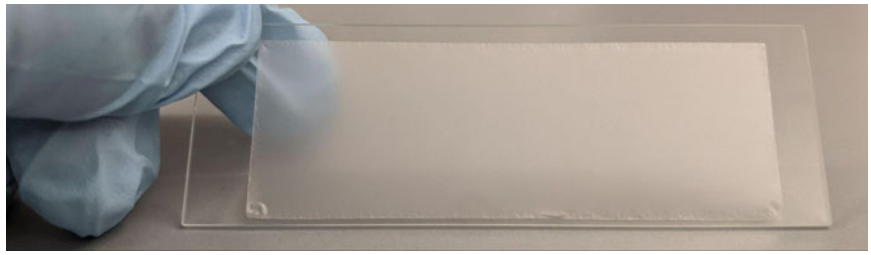


Fig. 4 Membrane centered on slide, dull side up. This is *incorrect.* If you are having difficulty distinguishing between the dull and shiny side, hold the membrane on a side under a light source and gradually change the angle of the slide—the shiny side will catch the light

Fig. 5 Membrane with a significant crease; membranes with large imperfections like this should be discarded

3. Place membranes on slides onto a shelf (*See* **Note 11**) in the plasma cleaner (Fig. 6).
4. Close the plasma cleaner door, and make sure that the (three-way main valve) lever is pointed to the left, this is the closed position. Turn on the main power and pump switch to form a vacuum (Fig. 7).

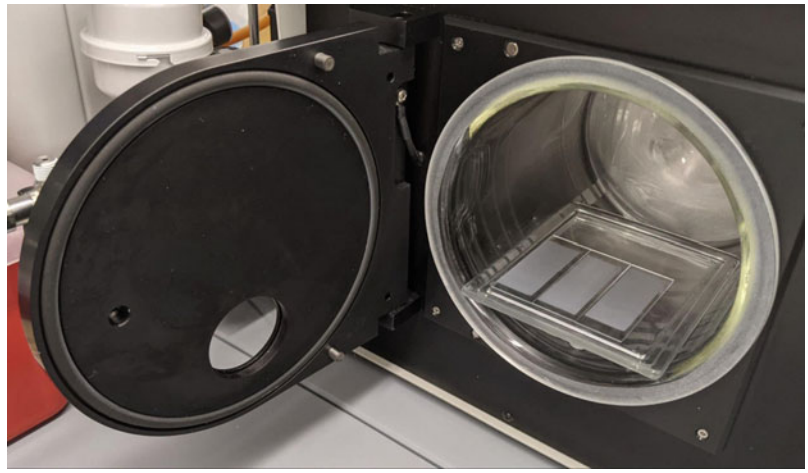


Fig. 6 Place membranes on slides onto a shelf in the plasma cleaner

Fig. 7 Turn on the main power and pump switch to form a vacuum

5. Allow a vacuum to form for 2 min. Once the vacuum has formed, simultaneously turn the three-way valve clockwise to 12:00 (pointing up), while turning the power to "High." The plasma should be a bright pink in color (*See* **Note 12**) (Fig. 8).
6. Treat membranes with plasma for 7 min (*See* **Note 13**), start the timer when you see the plasma appear.
7. After treatment, in the following order, turn the RF level valve from HIGH to OFF, and then turn off the power, then turn off the vacuum.

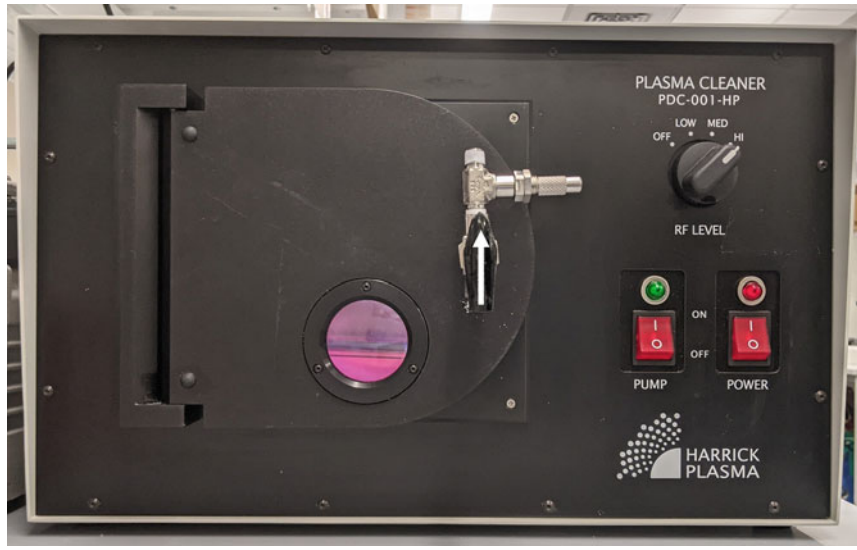


Fig. 8 The plasma should be a bright pink in color

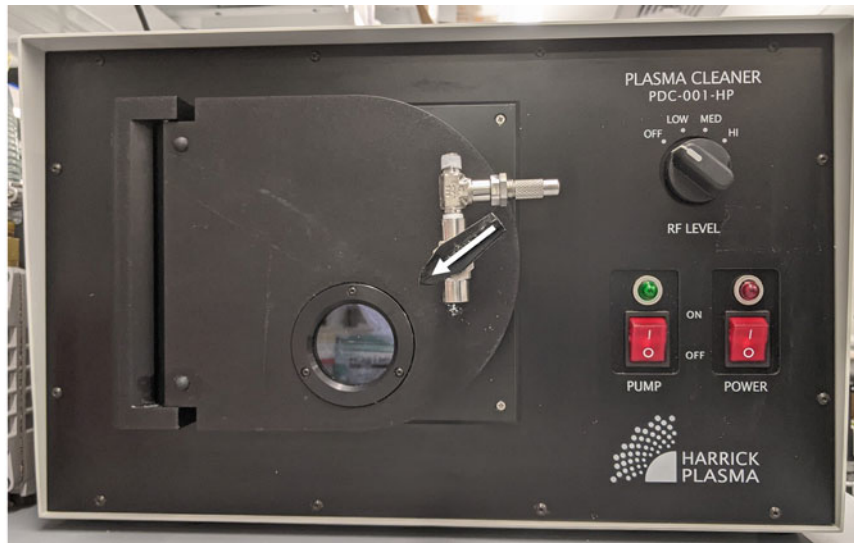


Fig. 9 Allow the chamber to slowly fill with air until the door opens on its own

8. Slowly turn open the air valve until you can just barely hear the air entering the chamber (*See* **Note 14**). Allow the chamber to slowly fill with air until the door opens on its own (*See* **Note 15**) (Fig. 9).
9. Prefill a 4-well dish with 1 mL of PBS in each well. Tilt the dish so that the PBS collects in the bottom right corner of each well.
10. Carefully transfer the membranes-on-slides to individual wells of a 4-well dish (Fig. 10).

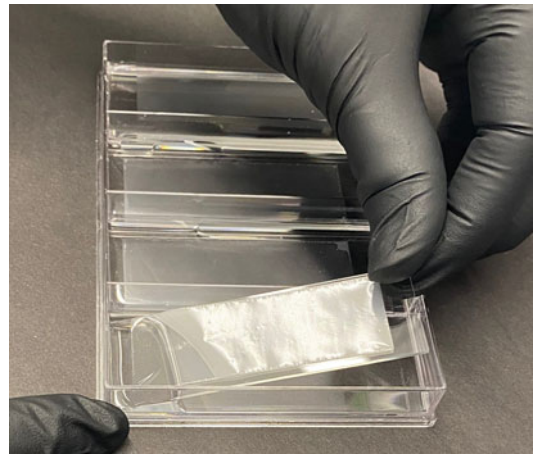


Fig. 10 Carefully transfer the membranes-on-slides to individual wells of a 4-well dish

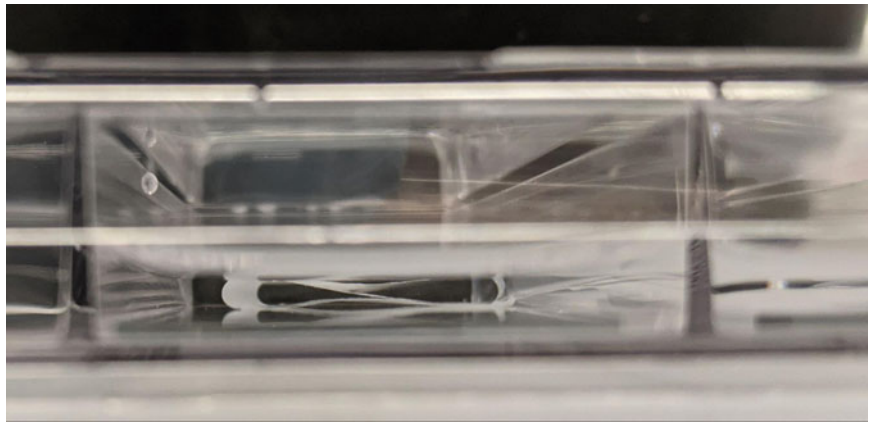


Fig. 11 Side view of 4-well dish showing a fully hydrated membrane (you can tell because it is not floating) that folded over during transport. Because the membrane has fully folded over, it is impossible to know which side was functionalized—membranes like this should be discarded

11. Quickly pipette 5 mL of 1× PBS over the membrane (*See* **Note 16**)—you may need to use tweezers to secure the membrane and prevent the membrane from folding over on itself (*See* **Note 17**).
12. Remove any air bubbles underneath the membrane by gently pressing on the membrane with membrane forceps or the tip of serological pipette used to dispense the PBS (only touch the edges of the membrane, avoid any contact with the center of the membrane) (*See* **Note 18**). Inspect the membranes (*See* **Note 19**). Membranes are now functionalized and ready for use, and solvated membranes should be used within 24 hrs of solvation (Figs. 11 and 12).

Fig. 12 An over-functionalized membrane will usually turn opaque white, and may also shrivel

3.2 Bead Loading

1. Aspirate storage solution and solvate each array with 5 mL of BLB. Inspect arrays (*See* **Note 20**).
2. Set up the house vacuum with an aspirator trap.
3. Aliquot 110,000 beads/array from a bead stock. Do not vortex beads, as this action can fragment them and interfere with bead loading and transcript capture. Resuspend beads by gently rocking and/or flicking the tube. Pellet beads by spinning for 15 s on a tabletop centrifuge (*See* **Note 21**).
4. Resuspend beads (previously in storage buffer) by aspirating the storage buffer and adding 500 μL of BLB. Invert the tube several times to wash the beads, pellet the beads, and then repeat this wash step with an additional 500 μL of BLB.
5. Pellet beads in 1.5 mL microcentrifuge tube, aspirate BLB, and resuspend in 200 μL of BLB per 110,000 beads.
6. Use the vacuum aspirator to aspirate *all* bead loading buffer (*See* **Note 22**). Minimal liquid on the array is crucial for the following steps: work quickly and periodically inspect arrays to ensure that they do not dry out completely.
7. Use a clean P1000 pipette tip, wafer forceps, or membrane forceps to center the array in the 4-well dish, making sure that the two long sides are not touching the sides of the dish (*See* **Note 23**).
8. Before the arrays dry out (*See* **Note 24**), add 200 μL of beads, dropwise, onto each array, in the pattern shown below (*See* **Note 25, 26**), Be very careful not to get bubbles on the surface of the array (*See* **Note 27**).
9. Gently agitate the arrays (*See* **Note 28**) or place on a flat rocker moving very slowly (<50 RPM).
10. Rock the arrays for 5 min, and then leave the arrays stationary for 5 min. Check the arrays frequently during both incubations to ensure that they have not completely dried out (*See* **Note 29**), and that the beads have not fallen off the side of the array (*See* **Note 30**).

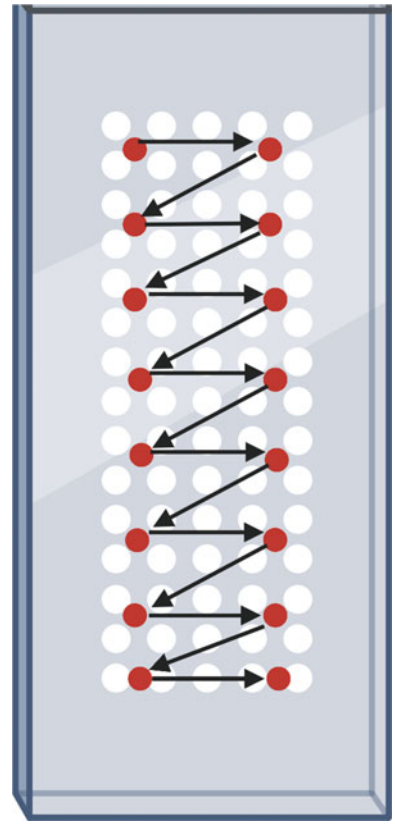


Fig. 13 A very large number of beads on the top of the array (not in wells) is expected and in most fields of view nearly all microwells will be filled with beads

11. Inspect the beads under a microscope. Here, we expect to see a very large number of beads on the top of the array (not in wells) and we also expect that in most fields of view nearly all microwells will be filled with beads (*See* **Note 31**) (Fig. 13).
12. Start washing the array—first, use a 1 mL pipette to deposit 500 μL of BLB on two opposite corners of the array (Fig. 14, Panel a). Then, reposition the array so that the long side of the slide is entirely in contact with the side of the 4-well dish (Fig. 14, Panel b, c). Use a 1 mL pipette to aspirate excess beads from the array surface and the dish and collect them in a 1.5 mL microcentrifuge tube. Rotate the arrays and repeat this washing step with 1 mL pipette (Fig. 14, Panel d).
13. Rotate the arrays again and repeat the washes twice more, using a serological pipette to add the BLB and the aspirator connected to the house vacuum. Do not touch the aspirator on top of the actual wells, stay on the borders of the array.
14. Check the arrays under a microscope to determine whether further washing is necessary. If you can scan across the entire array and find ~10–20 beads still on the surface, this array does

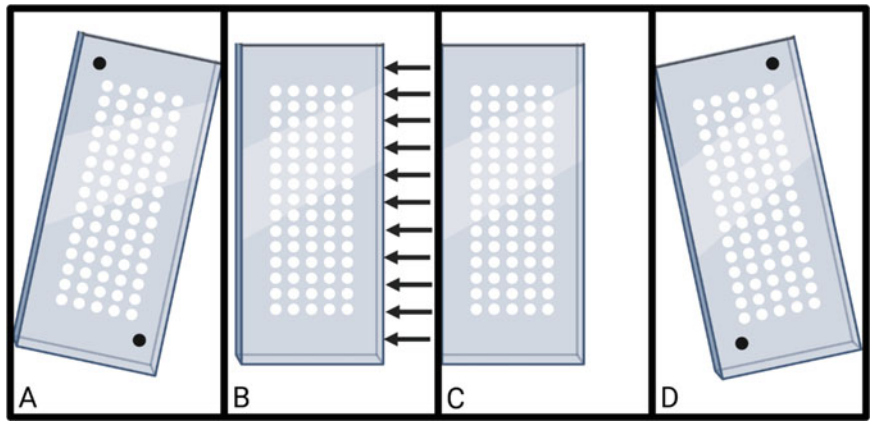


Fig. 14 Start washing the array on two opposite corners of the array (Panel A). Then, reposition the array so that the long side of the slide is entirely in contact with the side of the 4-well dish (Panel B, C). Rotate the arrays and repeat this washing step (Panel D)

not require further washing. Otherwise, repeat the wash steps with a serological pipette and vacuum aspirator until you have removed almost all beads from the surface of the array.

15. Once an array has been sufficiently washed, use a serological pipettor to add 5 mL of BLB to the well. Do not pipette directly over the wells—as this action can displace beads—instead, add liquid from the corner of the 4-well dish (*See* **Note 32**).
16. Spin down the beads collected in a 1.5 mL microcentrifuge tube during the first two washes using a tabletop centrifuge (or for 1 min at 800G). Use a 1 mL pipette to wash the beads twice in TE-TW. Resuspend the beads in 200 μL of TE-TW and store at 4 C (*See* **Note 33, 34**).

3.3 Cell Loading

1. Keep cells on ice until you are ready to load. Before proceeding with cell loading, cells should be counted (*See* **Note 35**). Generally, cells should be loaded in a certified biosafety cabinet in accordance with your approved biosafety protocols.
2. Remove arrays from 4 C. Mark the top and bottom of the dish so that you know which way the lid goes (Fig. 15).
3. Aspirate the bead loading buffer (or array quenching buffer, if left overnight).
4. Add 5 mL of RPMI (*See* **Note 36**) + 10% FBS (*See* **Note 37**) to each array.
5. The RPMI will slowly turn purple, this is because the bead loading buffer (or array quenching buffer) are both very basic, allow this to proceed for ~1 min.
6. Aspirate RPMI + 10% FBS.
7. Add 5 mL Fresh RPMI + 10% FBS.

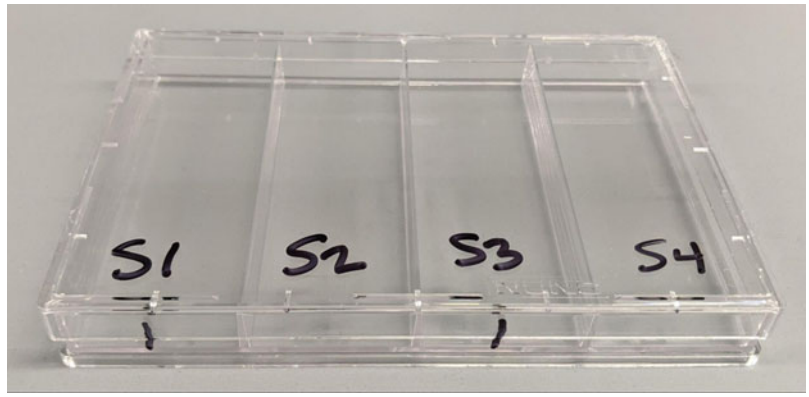


Fig. 15 Mark the top and bottom of the dish so that you know which way the lid goes

8. Incubate arrays room temperature with RPMI + 10% FBS for 5–10 min.
9. While this incubation is happening, prepare a 1.5 mL microcentrifuge tube containing cells at a dilution of 75 k/mL in RPMI + 10% FBS (*See* **Note 38**).
10. Use the vacuum aspirator to aspirate all the liquid in one well. Aspirate both off the top of the array and also from the well. Only perform this action for one array at a time, leaving the rest fully hydrated.
11. Use a P1000 pipette tip to center the array, ensure no side of the array is touching the 4-well dish.
12. Keeping in mind that there are beads in the wells, tip the 4-well dish as little as possible (~1–2 degrees off the table) to make the small amount of liquid on the array pool on the bottom border of the array. Gently aspirate this small remaining volume of liquid.
13. Use a P200 pipette to add 200 μL of cells to the array, dropwise. Again, be sure not to hold the pipette too far above the wells, as the falling drops can displace both beads and also result in uneven cell loading. Watch the pipette tip as you are dispensing liquid, be careful to stop dispensing liquid before you hit a bubble. Refer to the pattern illustrated in **Step 8** of Subheading 3.2.
14. Once all the arrays are loaded, take the arrays out of the biosafety cabinet for 5 min (*See* **Note 39**). This is a critical step, do not leave the arrays unattended (*See* **Note 40**). Instead, watch the arrays to make sure they are not touching the sides of the dish (*See* **Note 41**) or drying out (*See* **Note 42**).
15. After 10 min, bring the arrays back into the hood.

16. Add 5 mL of 1× PBS (*See* **Note 43**) to each array. Be very careful to pipette not on the top of the array, but into the side of the dish.
17. Very, very gently rock the entire dish back and forth; here we are trying to remove all of FBS from the top of the array, without letting the beads or cells pop out of the wells.
18. Use the vacuum aspirator to aspirate the PBS carefully from the sides of the well. Also aspirate the PBS from the very bottom (border) of the array—not from any wells—this procedure can be accomplished by touching the aspirator to the very bottom (border) of the array, quickly, once or twice.
19. Repeat PBS wash 4×.
20. Add 5 mL of RPMI on top of the array (if you accidentally add FBS here the membrane will not seal).

3.4 Membrane Sealing

1. Membranes should be in PBS, fully hydrated, and plasma-treated on the same day. If membranes were hydrated previously and are stored at 4 °C, allow them to come to room temperature.
2. Gather the following additional items: Hybridization Clamps, Clean Slides, VWR SpecWipers, 1000 μL Pipette tips. Ensure that the hybridization clamp is clean, and open it, as below, before you begin the membrane sealing process (Fig. 16).
3. Take off the top cover of a 4-well dish, keep it in the same orientation as it was sitting on the dish.

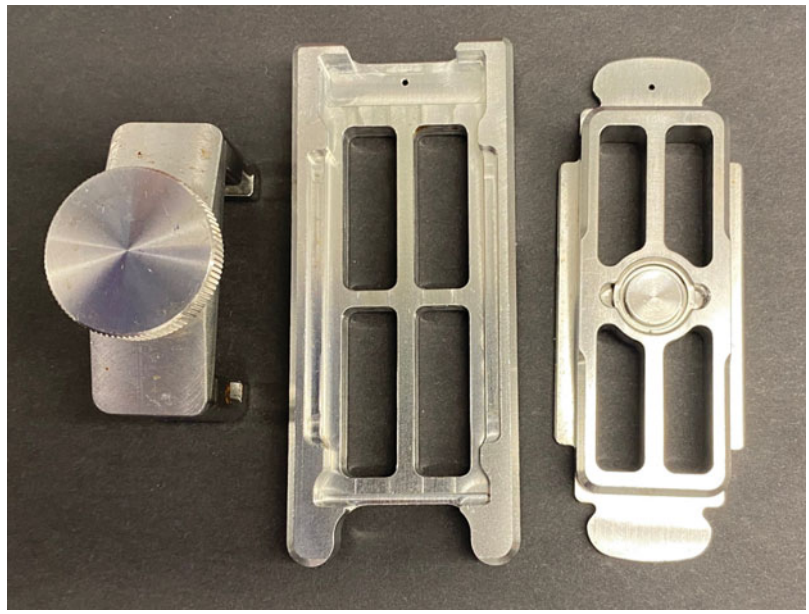


Fig. 16 Ensure that the hybridization clamp is clean

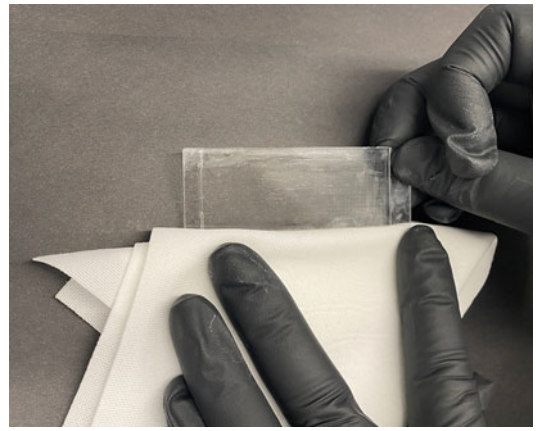


Fig. 17 Blotting excess liquid off the array, loaded with beads and cells, onto a SpecWiper. Note that the array is angled to aid in the removal of liquid, but tilted no more steeply than about 20–30 degrees

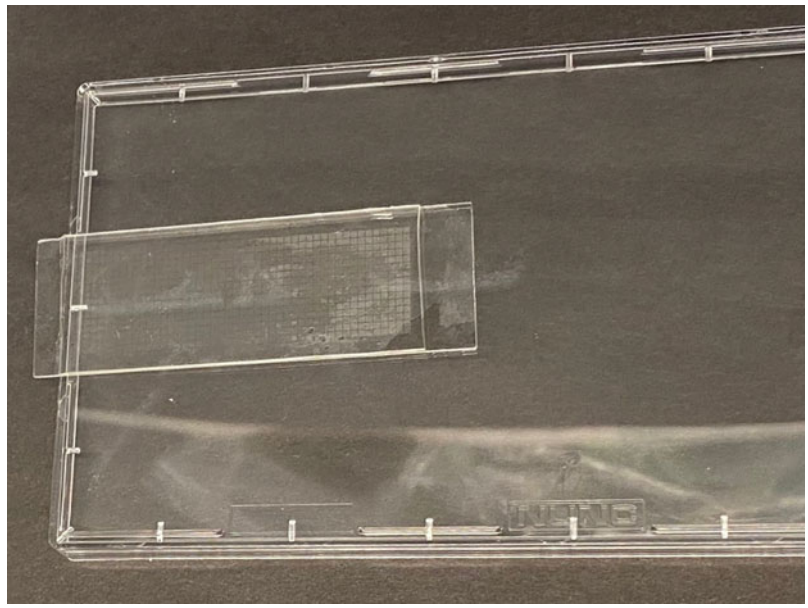


Fig. 18 Blotted array positioned at the edge of 4-well dish

4. Keeping the array as flat as possible, remove the array out from the 4-well dish. Blot excess liquid off the array using a SpecWiper. Place the array on the top of the 4-well dish—leave a little bit of the array overhanging so it does not get stuck on the top (wet glass on wet plastic) (Figs. 17 and 18).
5. Work very quickly—take the slide with the membrane on top of it out of the PBS dish, tap the sides of the slide with the membrane on a SpecWiper to remove excess liquid (Fig. 19).

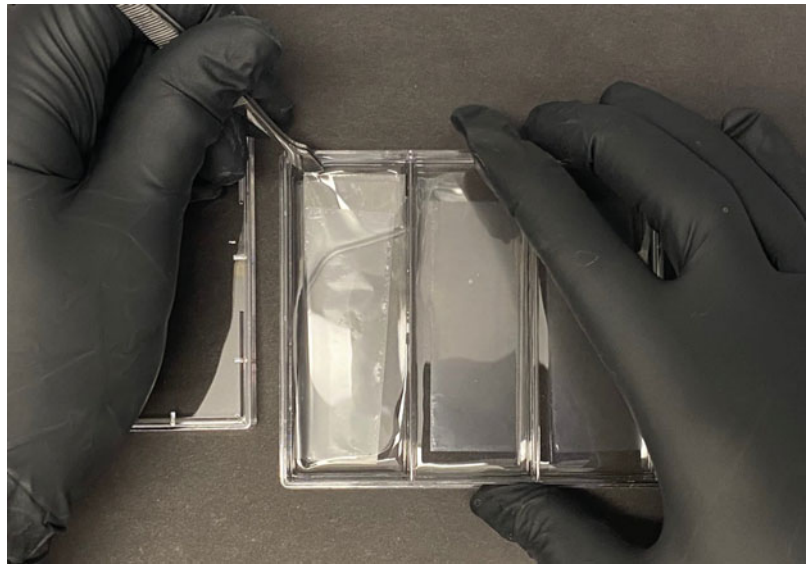


Fig. 19 Using membrane forceps (Wafer forceps can also be used for this step) to remove the solvated functionalized membranes from the 4-well dish

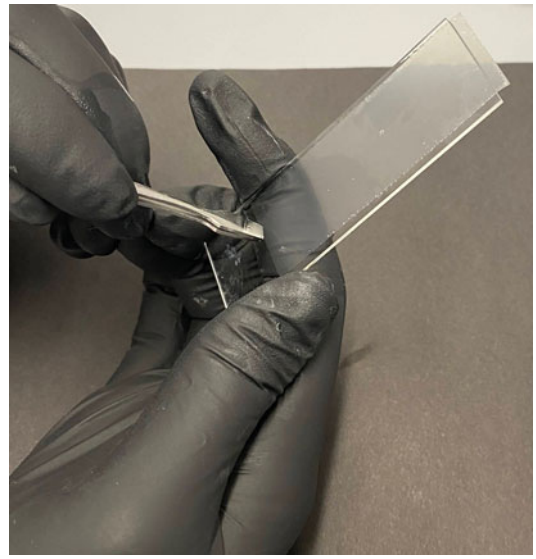


Fig. 20 Turn the glass slide with the membrane over so that the membrane is facing down. Center the slide with the membrane over the array

6. Use membrane forceps to manipulate the membrane so that it is centered on the slide with a 2 mm overhang on the end (Fig. 20).
7. Turn the glass slide with the membrane over so that the membrane is facing down. Center the slide with the membrane over the array (Fig. 21).

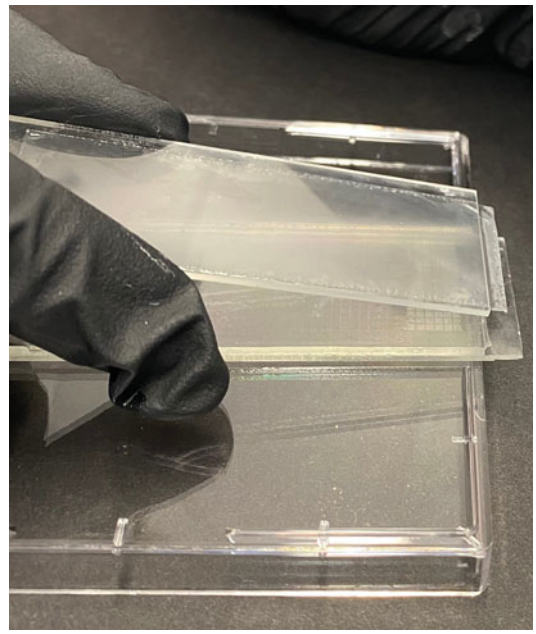


Fig. 21 Use another slide to pin the overhang of the membrane to the left side of the array

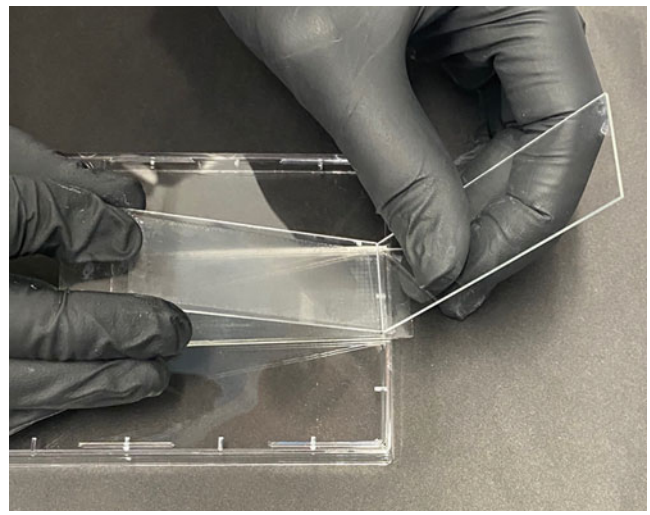


Fig. 22 Gently drag the slide with the membrane on it across the surface of the array, do not press down on the slide

8. Use another slide to pin the overhang of the membrane to the left side of the array (Fig. 22).
9. Gently drag the slide with the membrane on it across the surface of the array, do not press down on the slide. If you see imperfection in the membrane, take note. Do not try to flatten it manually (Figs. 23 and 24).

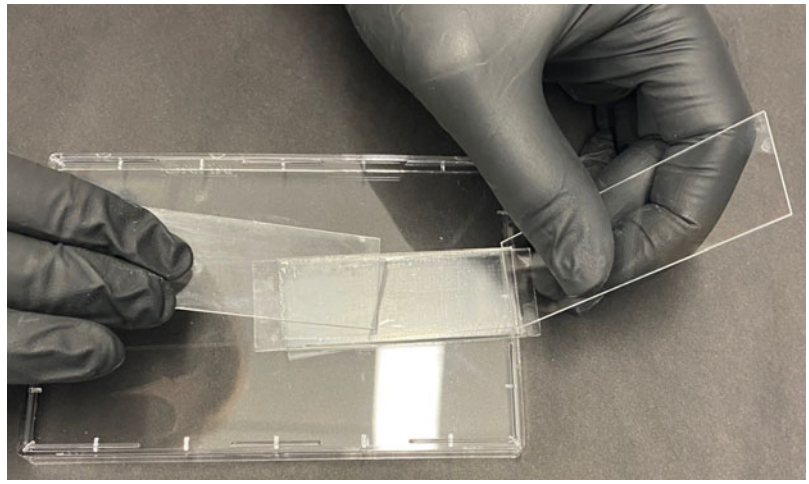


Fig. 23 Gently drag the slide with the membrane on it across the surface of the array, do not press down on the slide

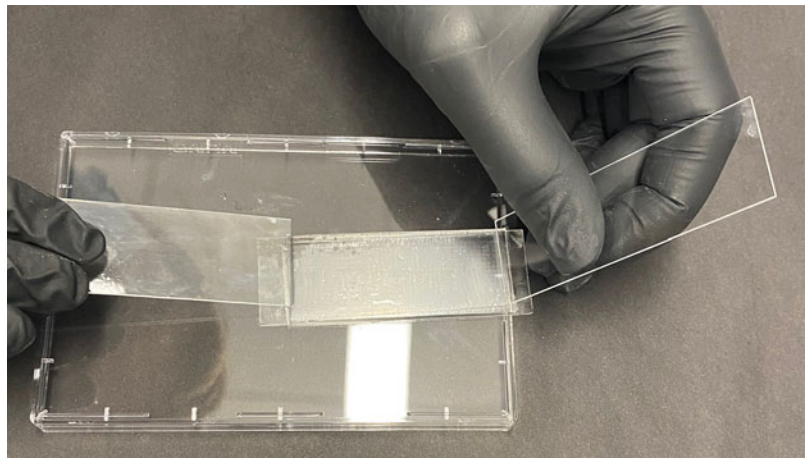


Fig. 24 If you see imperfection in the membrane, take note

10. Keeping the array relatively flat, place the array and membrane in a hybridization clamp (Fig. 25).
11. Gently add another clean slide on top of the membrane (Fig. 26).
12. Assemble the clamp, tighten the screw to the point of resistance (Figs. 27, 28, and 29).
13. Label the lab tape on the array screws.
14. Place clamp in 37 C incubator for 40 min.

3.5 Lysis and Hybridization

1. Prepare lysis buffer plates—in a fresh 4-well dish, add 5 mL of Complete-Lysis buffer to every well of the 4-well dish that will be occupied by an array.

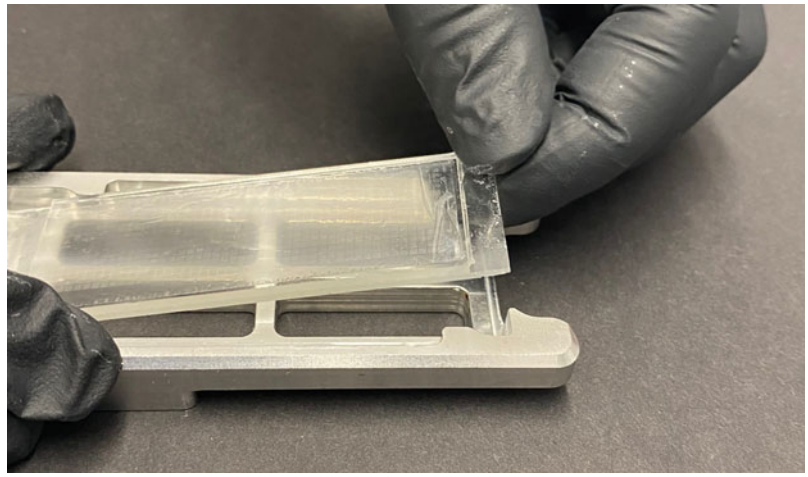


Fig. 25 Keeping the array relatively flat, place the array and membrane in a hybridization clamp

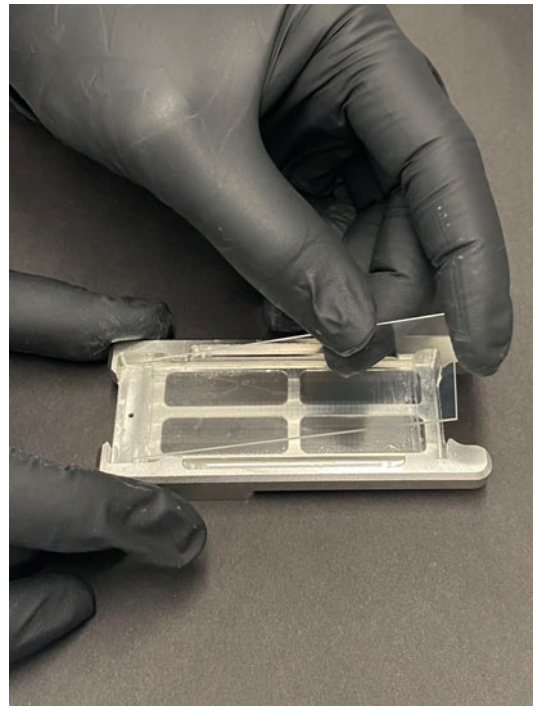
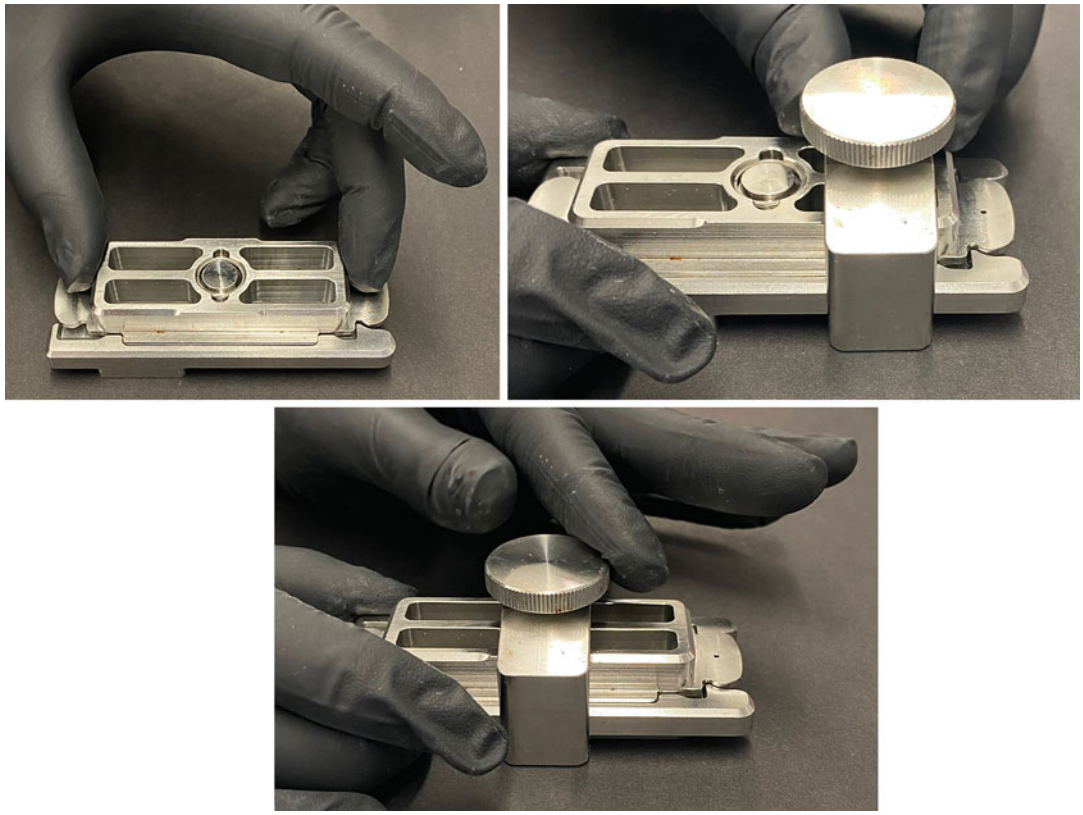


Fig. 26 Gently add another clean slide on top of the membrane

2. Remove the clamp(s) from the incubator. Then, carefully remove the array from the clamp (*See* **Note 44**). Lifting the arrays sandwich by the long sides of the slide, carefully transfer the arrays into the lysis buffer plates (*See* **Note 45**). Do not attempt to remove the top slide (Fig. 30).



Figs. 27, 28, 29 From left: Placing the top on the clamp, sliding the locker bar on the clamp, and tightening the hybridization clamp screw. The screw on the clamp should be tightened to "finger tightness"—the amount of tightness that can be easily achieved using one finger to tighten the screw (If using two fingers, tighten the screw just to the point of resistance. Be conservative here: overtightening can crack the slides and may also prevent membrane sealing)

3. Let the arrays rock on an X-Y rotator (50 RPM) for 5 min. The top slide should slide off on its own. If any top slide remains stuck, do not pry off the top slide, as this can reverse membrane sealing. Instead, continue rotation for 10 more minutes. If the slide has still not moved, use a P200 pipette tip to gently nudge the top slide (Fig. 31).
4. Once all the top slides have detached, incubate in lysis buffer while rocking for 20 min.
5. Check to make sure the aspirator you are using is connected to a liquid trap that *does not contain bleach. SAFETY:* This step is very important as the interaction between lysis buffer and bleach will create cyanide gas. Aspirate the lysis buffer into a specific, appropriately labeled, waste container.
6. Add 5 mL of hybridization buffer to each well.

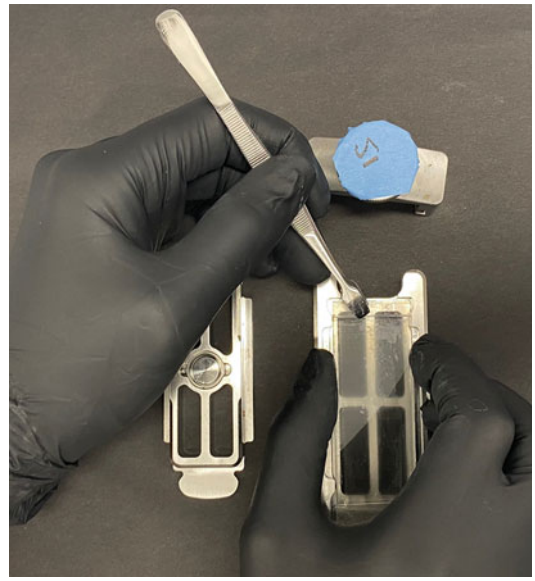


Fig. 30 Using wafer forceps or membrane forceps, remove the sealed array from the hybridization clamp. During this transfer step, it is very important to avoid putting different (or opposing) forces on the top and bottom slide, as this may reverse membrane sealing. A common pitfall here is tightly clamping the end of the top and bottom slides, forcing the slides apart. To avoid this outcome, gently clamp the forceps on the end of the slides, and slowly lift the array until it is high enough to grab the long ends of the slide sandwich with your thumb and forefinger

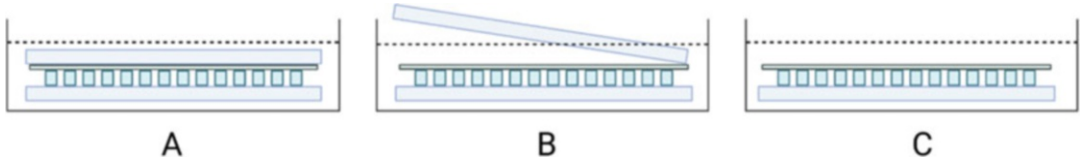


Fig. 31 Illustration showing a sealed array with membrane and top slide (**a**), top slide being removed after sliding off during rocking (**b**), array with sealed membrane in lysis buffer (**c**)

7. Aspirate the hybridization buffer into the same dedicated waste receptacle (*See* **Note 46**).
8. Add 5 mL of hybridization buffer to each well.
9. Rotate arrays for 40 min at 50 RPM. While arrays are rotating, prepare RT mastermix, as described in Subheading 3.6.

3.6 Bead Recovery

1. Create an RNA-clean workspace: Spray down your working area and tools (pipettes, plate sealers etc.) 2× with 70% ethanol, 1× with RNAse Zap (allow the Zap 30 s to react, do not wipe it away until it visibly foams) and 2× with 70% ethanol.

2. Gather one 50 mL conical per array, clean slides, a new 4-well dish, at least 40 mL of wash buffer per array and wafer forceps.
3. Label the conicals with the names of your arrays. Fill each conical with 35–40 mL of Wash Buffer (*See* **Note 47**).
4. Remove the hybridization buffer from one well of the 4-well dish (*See* **Note 48**). Optionally, replace with 1–2 mL of wash buffer (*See* **Note 49**).
5. Take one array out at a time. Use wafer forceps to collect the array from its position out in the 4-well dish.
6. While the array is still in the dish, use pointed forceps to gently remove the membrane and discard (*See* **Note 50**).
7. Place the array at a 45-degree angle above a clean prelabeled falcon tube. About one-quarter of the array should be in the falcon tube.
8. Use a p1000 pipette to carefully yet vigorously squirt 1000 uL of wash buffer directly onto the loaded surface of the array.
9. With a clean glass slide, scrape the array from its top to its middle 3–5 times. In between scrapes dunk the array in a clean wash buffer and, while it is at the bottom of the tube, use clean wafer forceps to partially lift and lower the array (displacing about 1 cm) 3–5 times, before pulling it back up to its original position (Fig. 32).
10. Flip the array in the tube (the side that was inside of the tube becomes the side on top) while maintaining that 45-degree angle.
11. Repeat **step 8**.
12. Wash again with 1 mL wash buffer and a P1000 pipet, this time aiming at the part of the array inside of the Falcon tube. This step washes beads left on the edge (Fig. 33).
13. Return the array to its position in the 4-well dish.
14. Repeat for each array **steps 5–13** for each array.
15. Inspect the arrays under a microscope. For any arrays with more than 10% of beads remaining, repeat **steps 5–13** on the already scraped arrays, and inspect again (*See* **Note 51**).
16. Once all arrays are complete, spin the 50 mL conical for 5 min at 1000G.While this spin is occurring, label microcentrifuge tubes with the array names.
17. Aspirate all but 500 uL from the conicals, be very careful not to disturb the bead pellet.
18. Using a 1 mL pipette, resuspend the bead in the 500 uL of wash buffer and transfer to the prelabeled microcentrifuge tubes.

Fig. 32 With a clean glass slide, scrape the array from its top to its middle 3-5 times

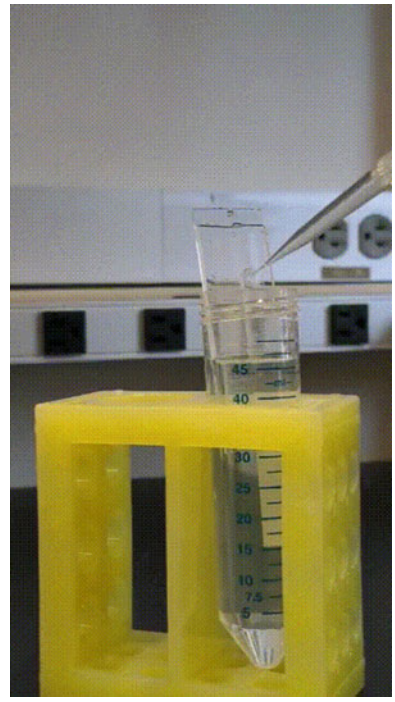


Fig. 33 Wash again with 1mL wash buffer and a P1000 pipet, this time aiming at the part of the array inside of the Falcon tube

19. Rinse the 50 mL conicals with 500 uL of fresh wash buffer—add this wash to the same 1.5 mL microcentrifuge tube. Repeat for each array. Proceed immediately to reverse transcription.

3.7 Reverse Transcription

1. During hybridization, prepare the following Maxima RT Mastermix with 1.1× overage (volumes below are for 1 array). Add reagents in the order listed, store the mastermix on ice.

   Maxima RT Mastermix

   40 μL RNAse-free H20

   40 μL Maxima 5× RT Buffer

   80 μL 30% PEG 8000

   20 μL 10 mM dNTPs

   5 μL SuperaseIN RNAse Inhibitor

   5 μL 100 uM Template Switch Oligo

2. Also prepare 1× Maxima RT Buffer, by diluting 5× Maxima RT buffer in RNAse-free H20. Prepare 250 μL 1× Maxima RT Buffer per array, again using 1.1× overage.
3. Centrifuge the 1.5 mL microcentrifuge tubes with collected beads at 800G for 1 min.
4. Aspirate supernatant and resuspend beads in 250 μL of 1× Maxima RT Buffer. Flick the bottom of the tube to resuspend the beads (*See* **Note 52**).
5. Centrifuge the 1.5 mL centrifuges tubes containing the beads collected from the arrays in the previous step at 800G for 1 min.
6. Centrifuge the tubes with collected beads at 800G for 1 min.
7. Aspirate supernatant (*See* **Note 53**) and resuspend beads in 200 μL of RT Mastermix. Flick to mix.
8. Incubate the tubes at room temperature for 30 min with end-over-end rotation on HulaMixer or equivalent device.
9. Incubate the tubes at 52 C for 90 min with end-over-end rotation (*See* **Note 54**).
10. Wash beads 1× with 1 mL of TE-TW, 1× with 1 mL of TE-SDS and 2× with 1 mL of TE-TW (*See* **Note 55**).
11. This step is a stopping point: After the final TE-TW wash, beads can be resuspended in TE-TW and stored at 4 C for up to 2 weeks.

3.8 Exonuclease I Treatment

1. For each sample, prepare the following Exonuclease I Mix with a 1.2× overage:

   Exonuclease I Mix

   20 μL 10× Exo I Buffer

170 μL RNAse Free H20

10 μL Exo I Enzyme

2. Centrifuge beads (stored in TE-TW) at 800G for 1 min.
3. Aspirate TE-TW storage solution and resuspend in 500 μL of 10 mM Tris–HCl pH 8.0.
4. Centrifuge beads again at 800G for 1 min.
5. Aspirate Tris–HCl and resuspend beads in 200 μL Exonuclease I Mix.
6. Incubate beads in tubes at 37 C for 50 min with end-over-end rotation.
7. Wash beads 1× with 500 μL of TE-SDS, 2× with 500 μL TE-TW.
8. Beads can be stored at 4 C in TE-TW for up to 2 weeks.

3.9 Second Strand Synthesis

1. For each sample, prepare the following Second Strand Synthesis Mastermix with 20% additional total volume (1.2× overage):

   Second Strand Synthesis Mastermix

   40 μL Maxima 5× RT Buffer

   80 μL 30% PEG 8k

   20 μL 10 mM dNTPS

   2 μL 1 mM dN-SMRT oligo

   5 μL Klenow Enzyme (add the Klenow enzyme immediately before adding the beads)

   53 μL H20
2. Centrifuge samples at 800 G for 1 min at room temperature.
3. While samples are spinning, prepare 550 μL of 0.1 N NaOH from a >1 N stock solution for each sample.
4. Aspirate supernatant and resuspend beads in 500 μL 0.1 N NaOH.
5. Rotate the tube for 5 min (end-over-end) at room temperature
6. Centrifuge for 1 min at 800 g. Aspirate supernatant.
7. Wash samples 1× with 500 μL of TE-TW (*See* **Note 56**) and 1× with 500 μL of TE.
8. Resuspend beads in 200 μL Second Strand Synthesis Mastermix and incubate at 37 C for 1 hour with end-over-end rotation.
9. Wash beads 2× with 500 μL TE-TW and 1× with 500 μL TE.
10. Proceed directly with WTA.

3.10 Whole Transcriptome Amplification (WTA)

1. Wash beads once with 500 μL ultrapure water, pellet beads. After spin down, the bead pellet is easily disturbed—remove the supernatant as much as you can without disturbing the bead pellet—usually this condition means leaving about 20 μL of ultrapure water—and resuspend in 220 μL of water.
2. Prepare the following PCR Mastermix with 1.2× overage. Volumes are per reaction. It is standard protocol to perform 24 reactions per array

   WTA Mastermix

   25 μL 2× Kapa HiFi Hotstart Readymix

   24.6 μL H20

   0.4 μL 100 uM SMART PCR Primer
3. Pellet beads, remove supernatant, and resuspend in 50 μL of PCR Mastermix for every 2000 beads or 1200 μL for 24 reactions.
4. To a 96-well plate, aliquot 50 μL of beads and PCR Mastermix to each well, resuspending periodically to prevent beads from settling.
5. Add 10 uL of water to the bottom of the tube you emptied in the previous step, and use it to wash the bottom of the tube.
6. Distribute the water you used to wash the bottom of the tube dropwise across the 24 reactions for that array, focusing on wells that might have received fewer wells in **step 6** due to beads settling.
7. Use the following cycling conditions to perform whole-transcriptome amplification (*See* **Note 57**) (Table 1).

Table 1
Cycling conditions to perform whole-transcriptome amplification

	Temperature	Time
	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 3 min
Final extension	72 C	5 min
	4 C	Infinite hold

3.11 Purification of WTA Products

1. Retrieve Ampure XP beads from 4 C and allow equilibrate to RT for 20 min (*See* **Note 58**).
2. Pool 8 wells of PCR reactions in a 1.5 mL microcentrifuge tube (*See* **Note 59**).
3. Thoroughly mix Ampure XP beads with the product at a 0.6× volumetric ratio (Ampure XP: PCR products) (*See* **Note 60**).
4. Incubate for 3 min at RT before placing on a magnet. Incubate for another 3 min.
5. During incubations, prepare 5 mL of 80% ethanol from a stock solution RNA-clean 200 proof ethanol.
6. Wash beads 3× with freshly made 80% ethanol.
7. After the third wash, allow the beads to dry for 5 min on the magnet.
8. After 5 min, while samples remain on the magnet, use a P200 pipette to remove any liquid that has pooled at the bottom of the microcentrifuge tubes.
9. Incubate on the magnet for another 5 min.
10. Once the beads appear matte in color, but before they develop significant cracks, remove the beads from the magnet.
11. Elute the dried beads in 100 μL RNAse-free water.
12. Incubate for 1 min, then place samples on the magnet.
13. Incubate for 1 min on the magnet, then transfer the 100 μL to new microcentrifuge tubes.
14. Repeat **steps 3–7** with the 100 μL product, combine the beads with the product at 0.8× volumetric ratio.
15. After the third wash, allow the beads to dry for 10 min on the magnet.
16. After 5 min, use a P200 pipette to remove any liquid that has pooled at the bottom of the microcentrifuge tubes.
17. Once the beads appear matte in color, remove the beads from the magnet.
18. Elute the beads in 16 μL RNAse-free water.
19. Incubate for 1 min, then place samples on the magnet.
20. Incubate for 1 min on the magnet, then transfer 15 μL to new Lobind microcentrifuge tubes.
21. It is helpful to analyze the distribution of fragment lengths in the purified WTA product by running a High Sensitivity D5000 Screen Tape by electrophoresis such as by agarose gel or on an Agilent 4200 Tapestation. The distribution of products should extend between 400 bp and 1500 bp with a peak size greater than 900 bp. Distribution of product will vary by sample type. Additionally, Seq-Well S^3 cDNA libraries will

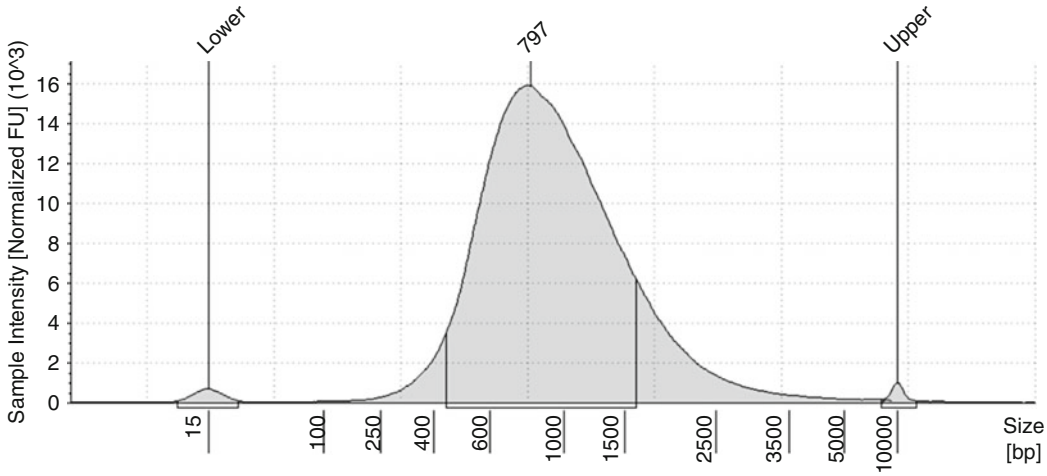


Fig. 34 An ideal WTA product distribution is a smooth bell curve with a peak between 700 and 2000 bp

appear shorter than full-length cDNA libraries as the dN-SMRT primer allows for recovery of transcripts that are not fully extended by the RT Polymerase and thus do not possess a TSO (Fig. 34).

22. Proceed to library preparation or store the WTA product at −20 C.

3.12 Nextera Library Preparation

1. Ensure that your thermocycler has been set to run tagmentation (**step 5**) and Nextera PCR (**step 11**) protocols.
2. For each sample, combine 800 pg of purified cDNA with water in a total volume of 5 μL (*See* **Note 61**).
3. To a new 96-well plate, add 10 μL of Nextera TD Buffer followed by 5 μL of ATM Buffer (*See* **Note 62**). Finally, add 5 μL of diluted PCR product.
4. Mix by pipetting up and down about 5 times. Seal the plate and spin the plate down for 1 min at 800G.
5. Incubate at 55 C for 5 min.
6. Once the thermocycler has finished its program, immediately remove the plate and place it in a bucket of wet ice, with 1" of ice on top of it.
7. Wait for 2 min, or until water condenses onto the plate seal over the wells.
8. Spin down for 30 s at 800G.
9. Immediately unseal and add 5 μL of Neutralization Buffer (NT). Mix by pipetting 10–15 times, try to pipette smoothly to minimize the production of bubbles. Spin samples briefly after mixing.
10. Incubate at room temperature for 5 min.

Table 2
Cycling conditions

	Temperature	Time
	72 C	5 min
	95 C	30 s
12 cycles	95 C 55 C 72 C	10 s 30 s 30 s
Final extension	72 C	5 min
	4 C	Infinite hold

11. Add to each well, in the order listed:
    8 μL RNAse-free H20
    1 μL 10 uM New-P5-SMART PCR hybrid oligo
    1 μL 10 uM Nextera N700× oligo
    15 μL Nextera PCR Mix
12. Seal the plate and centrifuge for 1 min at 800G.
13. Carry out PCR using the following cycling conditions (Table 2):
14. Store tagmentation products at −20 C or proceed to purification steps.
15. To each sample add Ampure XP Beads at 0.6× volumetric ratio.
16. Let samples incubate for 3 min off the magnet.
17. Transfer to a microcentrifuge tube magnet and incubate for another 3 min.
18. Perform three washes with freshly made 80% ethanol.
19. After the third wash, allow the beads to dry for 10 min on the magnet. Cover the open microcentrifuge tubes with the top of a pipette tip box to prevent dust from falling into the tubes while they are incubating.
20. After 5 min, use a P20 pipette to remove any liquid that has pooled at the bottom of the microcentrifuge tubes.
21. Once the beads appear matte in color, but ideally before they develop significant cracks, move the tubes off the magnet to elute.
22. Elute the dried beads in 101 μL RNAse-free water, pipette up and down to mix well, until the solution is uniform in color. Incubate for 1 min, then return the plastic rack to the magnet.

23. Incubate for 1 min on the magnet, then transfer the 100 μL to new microcentrifuge tubes.
24. Repeat **steps 3–7** with the 100 μL product, combine Ampure XP beads with the product at 0.8× volumetric ratio.
25. After the third wash, allow the beads to dry for 5–10 min on the magnet. Watch the beads closely, as overdrying beads can impair DNA recovery. Cover the open microcentrifuge tubes with the top of a pipette tip box to prevent dust from falling into the tubes while they are incubating.
26. After 5 min, use a P20 pipette to remove any liquid that has pooled at the bottom of the microcentrifuge tubes.
27. Once the beads appear matte in color, but ideally before they develop significant cracks, remove from the magnet and add 16 μL of RNAse-free water. Pipette up and down to mix well, until the solution is uniform in color.
28. Incubate for 1 min, then return to the magnet.
29. Incubate for 1 min on the magnet, then transfer 15 μL to new microcentrifuge tubes

3.13 Quality Control and Library Prep

1. Analyze the distribution of fragment lengths in the purified product by running a High Sensitivity D5000 Screen Tape on an Agilent 4200 Tapestation. The distribution should be fairly smooth with an average size of 600–750 bp. We have successfully sequenced tagmentation products with average fragment lengths of 350 bp to 700 bp (Fig. 35).

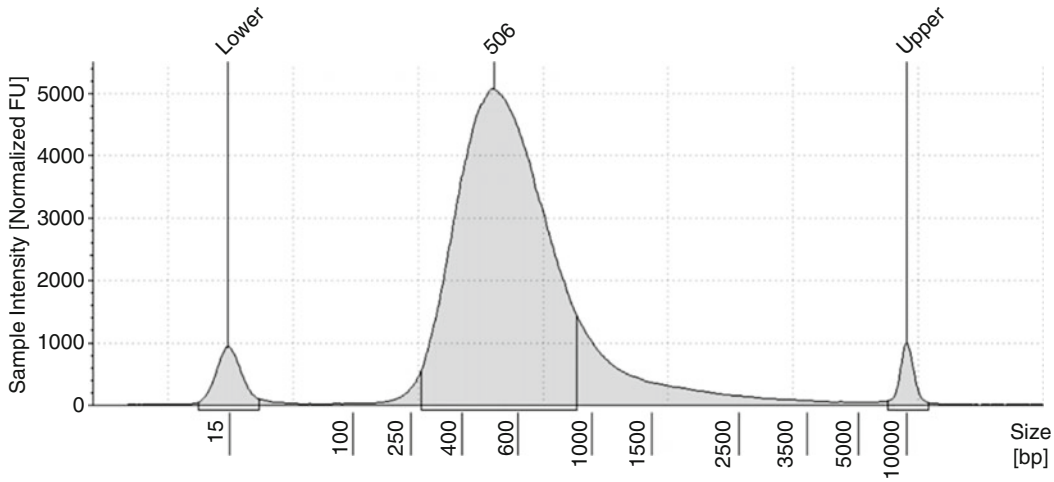


Fig. 35 An ideal NTA product distribution is a smooth bell curve with a peak between 350 and 700 bp

2. Determine the concentration of sequencing libraries using the high-sensitivity dsDNA kit for Qubit Fluorometer.
3. Library can be stored at −20 C until sequencing (*See* **Note 63**). For best results, prepare the 5 μL 4 nM dilution on the day of sequencing (*See* **Note 64**).

3.14 Sequencing

1. Make a 5 μL library pool, diluted to 4 nM.
2. Prepare a fresh aliquot of 0.2 N NaOH by combining 180 μL of ultrapure water and 20 μL of 2 N NaOH in a 1.5 mL Lo-Bind microcentrifuge tube.
3. Program a timer for 5 min. Add 5 μL of the freshly diluted 0.2 N NaOH to 5 μL of the 4 nM library.
4. Pipette to mix, spin briefly, then incubate at RT for 5 min.
5. After 5 min (*See* **Note 65**), add 5 μL of Tris–HCl pH 7.5. Pipette up and down to mix.
6. Add 985 μL freshly defrosted HT1 Buffer to the same tube. This is a 1 mL 20 pM library (solution 1).
7. In a new tube, add 165 μL of Solution 1 to 1.5 mL HT1 buffer for a final library concentration of 2.2 pM (Solution 2).
8. Add 6 μL of Custom Read 1 Primer to 1994 μL of HT1 to make 2 mL of 0.3 μM Custom Read 1 Primer.
9. Load 1.5 mL of Solution 2 to reservoir #10 on the Illumina reagent cartridge, labeled "Load Library Here."
10. Load 2 mL the Custom Read 1 Primer solution prepared in **step 8** to the NextSeq reagent cartridge reservoir # (look up).
11. Follow Illumina's guide for loading a NextSeq500/550 High Output sequencing kit and starting the sequencing run. Seq-Well requires paired end sequencing with the following read structure: Read 1: 20 bp, Read 2: 50 bp, Index 1:8 bp.

4 Preprocessing of Sequencing Data

4.1 Alignment and Preprocessing

This section outlines the general steps from sequencer to digital gene expression matrix (DGE) cell-gene-count matrix and provides a high-level overview of possible methods for downstream analysis of data generated with Seq-Well S^3(Fig. 36).

Upon the completion of a sequencing run, one should first review the run's performance metrics to check for errors and to determine if the run performed within the manufacturer's expectations [15]. Low Cluster Density or low Clusters Passing Filter, for instance, could imply low sample purity, an instrument error, or inaccurate measurements of sample concentrations. Please refer to the manufacturer's guide to troubleshoot such issues [15]. It is still advised to proceed with demultiplexing as the output files may help to identify the source of most issues.

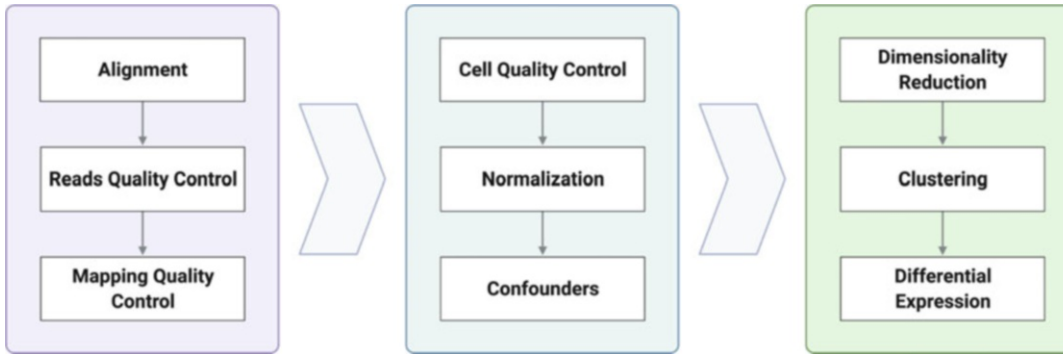


Fig. 36 Flowchart of scRNA-seq analysis with Seq-Well S^3 [14]

Demuliplexing refers to the process of assigning sequencing reads to their sample of origin. For each sequencing run, sequencing reads are first converted from the standard machine output files (in bcl type format) to FASTQ format. Demultiplexing sequencing reads from Illumina machines is performed using the free software bcl2fastq [16]. Briefly, bcl2fastq demultiplexes samples according to sample-specific indices introduced in the post-tagmentation PCR. A helpful representation of the library structure is provided by the Teichmann Group [17]. A user-generated spreadsheet is provided as input to bcl2fastq listing the index-sample pairings. The summary file generated by bcl2fastq lists the number of reads sequenced and the Most Common Unknown Index Sequences which can help troubleshoot issues relating to low quality libraries [18]. Demultiplexed FASTQs are then aligned to the corresponding reference genome using an implementation of the Drop-seq Pipeline maintained by the Broad Institute [19]. The key output file is a DGE cell-gene-count matrix that groups a 12 bp cell barcode to 8 bp unique molecular identifier (UMI) contained in each sequencing fragment.

4.2 Cell Quality Control

Determining the degree of sequencing saturation with a saturation curve, shown below, is a useful initial measure of quality. Sequencing to a depth of 10–50 k reads per cell increases the likelihood of capturing lowly expressed genes and thus reduces the chances of false-dropouts. Tuning the cells used as input to Seq-Well S^3, as well as understanding the sequencing depth required for the experiment, is critical when determining whether or not to resequence a sample [20] (Fig. 37).

At the single-cell level, several tools exist to analyze single-cell RNA-seq data such as Seurat and Scanpy. These platforms can be used to filter data based on quality control metrics, visualize common features, transform and normalize data, and provide an integration point for novel methods of analyses developed by third parties. The modes of analysis will vary based on experimental

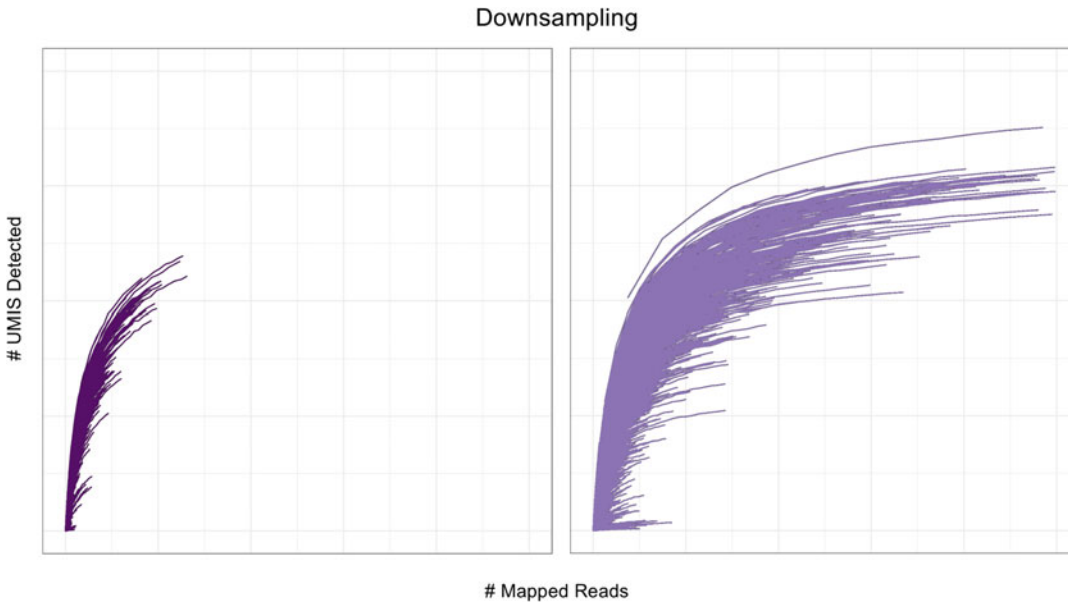


Fig. 37 Estimating Sequence Saturation: In the image to the right, we see that the number of UMIs detected per cell begins to plateau. This is representative of a sample that is adequately sequencing. Further sequencing will not recover a substantial amount of additional UMIs from cells of such a sample. By comparison, the image to the left is representative of a sample that is under-sequenced

design, and it is important to review current literature where this is reviewed comprehensively [21].

A general first step is quality control. Beginning with the DGE matrices generated in **step 4**A, low-quality cells are identified and filtered out from downstream analysis. Second strand synthesis in Seq-Well S^3 increases the expected average number of genes recovered per cell. Visualizing the distribution of genes per cell may be helpful in determining a threshold. The goal is to retain high-quality cells. An initial threshold can be set above the first quartile and adjusted based on experiment needs. It is helpful to remember that different cells with varying transcriptional activity, as well as samples with different levels of integrity, will affect the genes captured per cell. Low quality cells are initially discarded on the basis of gene detection (e.g., <500 genes per cell) and transcript detection (e.g., <700 UMIs per cell). Different thresholds can be tested to see what is lost at a given cut-off, and inform threshold selection (Fig. 38).

In a similar fashion, genes that are not present in more than a handful of cells, due to low expression or difficulties in capturing a gene, are filtered out of the datasets as they do not necessarily serve as reliable or statistically significant carriers of information for differential gene expression analysis. The remaining genes are carried through analysis for further examination. If a rare cell population is expected, the metric can be more permissive and adjusted as

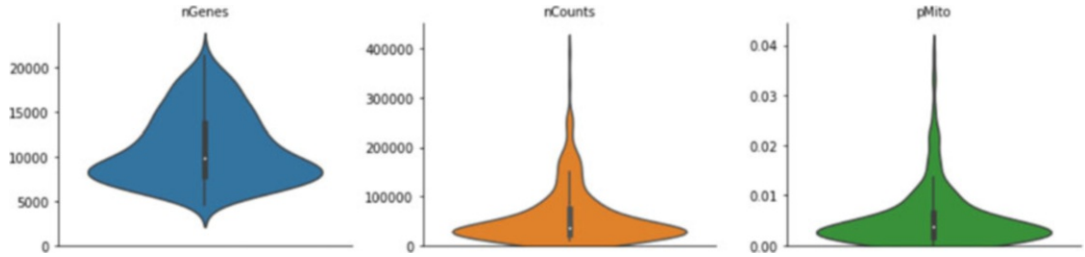


Fig. 38 Violin plots used to visualize QC metrics used to filter low-quality cells. These plots show data from one array

more samples are processed and integrated into the dataset. If certain genes continue to be expressed in a handful of cells and these cells do not belong to a distinct cell type, as discussed below, removing these genes from the dataset is advised. Oftentimes, cells with greater than 10% of reads mapping to mitochondrial genes are also discarded as a high mitochondrial percentage of total transcripts is an indicator of cell death and/or stress. Note that while high mitochondrial mapping percentages can sometimes be due to sample degradation (e.g., during handling of the cell), they can sometimes represent key biology (e.g., very active respiration). It is worth noting that Seq-Well S^3 has been shown to preserve cellular integrity and biological state. Therefore, with new datasets or for samples where cell death or high-mitochondrial activity is expected (in neurons, for example), these cells may be retained in the dataset through clustering, or examined separately.

4.3 Doublets and Ambient RNA Detection and Removal

Occasionally, more than one cell is captured in a single picowell of the Seq-Well array. As the transcripts from these cells will be captured on the same bead, they will be assigned to the same cell barcode, despite originating from separate cells. While rare, this effect can mask true transcriptional heterogeneity in a sample and skew any methods to normalize the data. Thankfully, streamlined protocols exist that are designed to identify doublets within a dataset with a variety of strategies, assumptions, and sample specificities [23]. No method is perfect and it is recommended to review the most up-to-date literature.

Ambient mRNA contamination is another possible source of background noise that may be encountered and removed using computational methods [24]. These methods typically seek to define the nonspecific binding of free-floating RNA to beads loaded on an array. Unlike doublets, ambient RNA does not typically represent a significant portion of the transcripts captured on any particular bead. It is most typically observed when processing primary tissue samples with large amounts of debris and/or low cell-viability. As the background noise is thought to be relatively uniform across the sample, it may not, in some instances,

significantly affect downstream analyses that seek to contrast cells. It is always a good idea to check and remove if necessary. Note that ambient RNA is array-specific, and thus methods for removing ambient RNA should be run separately for each Seq-Well S^3 array.

5 Computational Analysis

Once low-quality cells have been filtered from a sample, various computational and statistical tools can be applied to characterize and interpret transcriptional heterogeneity and the underlying cellular processes that inform it. The following sections provide a general approach for interpreting the high-dimensional data generated from Seq-Well S^3. Various methods exist that attempt to resolve what is meaningful variation, find structure within it, and then link gene expression covariation to putative biological drivers (e.g., cell type/differences in activation); these methods are covered extensively elsewhere [21].

5.1 Variable Genes

A critical step in the analysis is the process of choosing variable genes. It is an iterative process. Beginning with higher degrees of variation, such as treatment groups, tissue types, and cell types, the goal is to identify expression profiles that distinguish subsets of the data.

The list of genes will change based on the question that is being asked. As an example, a gene that is expressed in T cells but is not expressed highly in epithelial cells may be highly variable (ranging from 0 in some cells to high expression in others) and of much interest at the first step of analysis when the goal may be to subset T cells from epithelial cells. When identifying heterogeneity between T cells, however, this same gene may not be as informative if it is similarly expressed across these cells.

5.2 Dimensionality Reduction

To identify orthogonal axes of variation in the dataset, a Principal Component Analysis may be performed on the highly variable genes. Ranked in order from small to large, the principal components represent orthogonal linear combinations of variable genes that reflect decreasing degrees of variation.

Critically, not all principal components represent a high degree of variance (or biologically relevant variation) and therefore should not be included in dimensionality reduction. Selecting the number of principal components to include is an iterative process. Visualizations of the percentage variance explained by each principal component may be used to identify the set of principal components that represent the majority of variance present in a sample; this may also be calculated statistically [22] (Fig. 39).

Other methods such as Independent Component Analysis and consensus non-negative matrix factorization (cNMF) exist are reviewed elsewhere [25, 28] (Figs. 40 and 41).

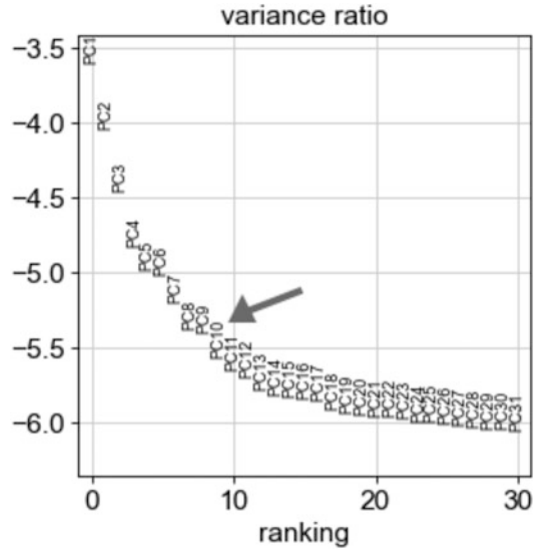


Fig. 39 Plot of ranked principal components based on percent of variance.[10] Arrow points to the elbow

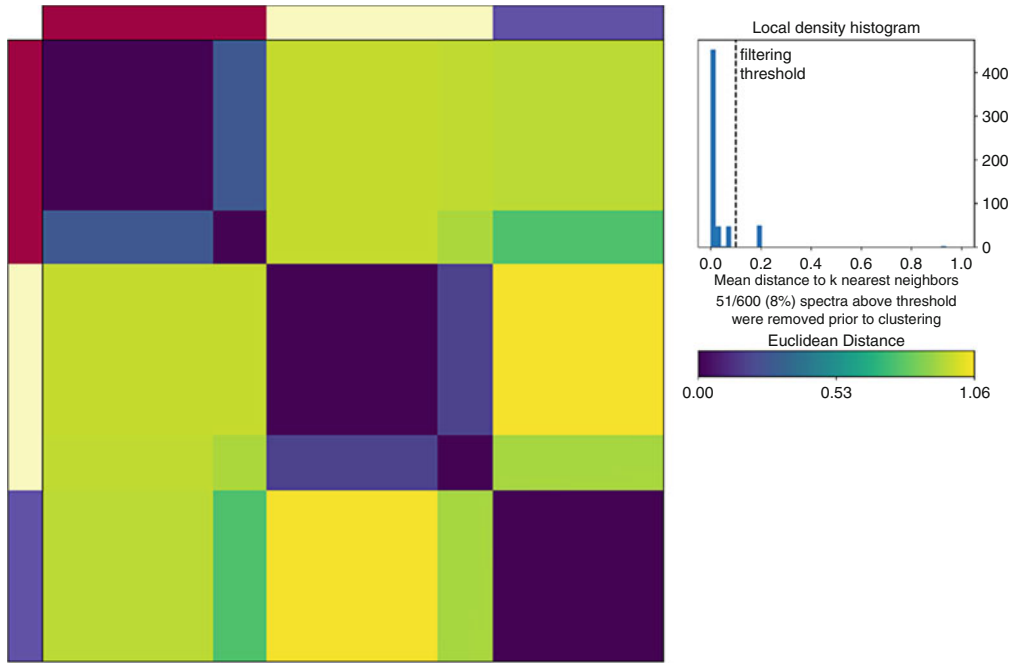


Fig. 40 Plot demonstrating the use of cNMF to identify gene programs

A common approach used to visualize dimensionally reduced data is UMAP (Uniform Approximation and Projection) [27]. Simply, UMAP works by constructing a high-dimensional representation of the dataset and then a low-dimensional analogue that attempts to minimize differences between the two models.

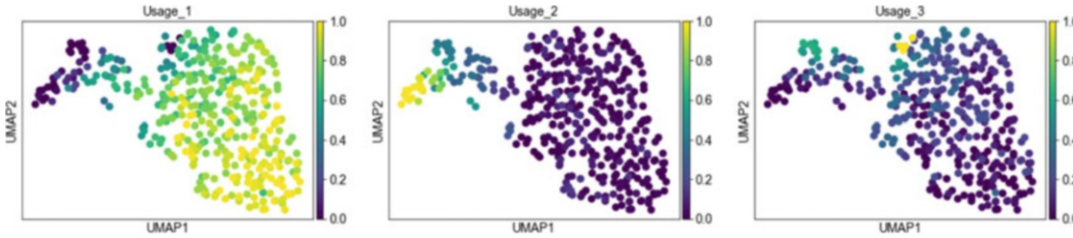


Fig. 41 Gene program usage plotted on UMAP of gene expression profiles

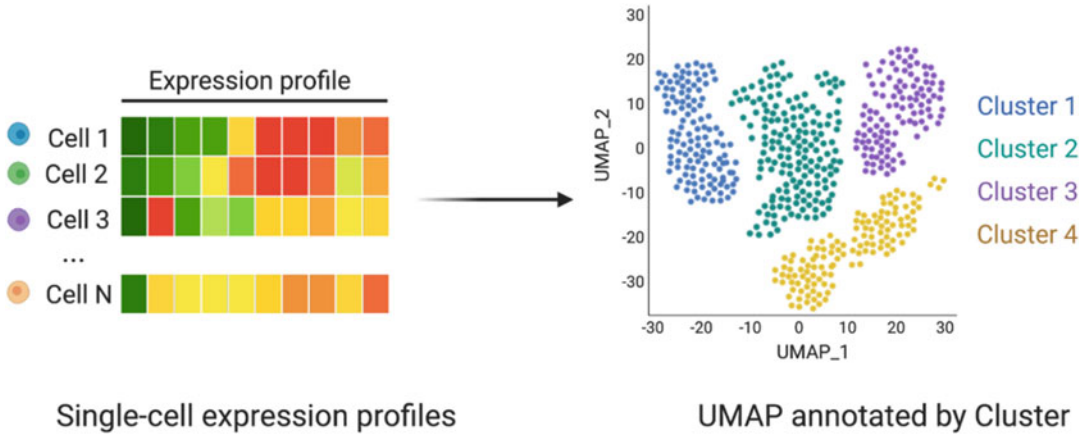


Fig. 42 Visualizing a dataset after dimensionality reduction and clustering

Ultimately, all dimensionality reduction methods serve to improve interpretability of the high-dimensional datasets created with methods like Seq-Well S^3. Identifying the appropriate balance between interpretability and information loss will be largely dataset-dependent (Fig. 42).

5.3 Clustering

Grouping cells based on the similarity of their expression profiles allows one to identify cell types and states, as well as infer differences between groups. This is typically done by clustering or graph-based learning [26]. Unsupervised clustering is a common approach that includes methods such as hierarchical clustering and k-means clustering. These methods compute a distance metric between cells based on a low-dimensional representation of the dataset, such as PCA or UMAP. Cells are then iteratively grouped into clusters based on these distances.

Similarly, graph-based learning methods aim to identify similar groups of cells [21]. Specifically, graph-based learning treats each cell as a node in a graph, with edges connecting each node to its k nearest neighbors. Based on the connectivities, the graph and thus cells are partitioned into clusters. The most widely used graph-based method is the Leiden algorithm which groups cells with high connectivity, compared to the level of connectivity observed throughout the dataset.

One important parameter that is shared across most clustering and graph-based learning approaches is a resolution parameter [26]. Selecting this parameter is important as it establishes the basis for downstream analyses which compares the distribution of gene expression in one cluster or group versus another. An initial strategy could involve tuning this parameter until the dataset is partitioned in a manner that closely resembles the groups observed by a dimensionality reduction method of choice, UMAP for example; we typically select our resolution parameter by maximizing the average silhouette score across all clusters [27, 29]. The resolution can be increased or decreased depending on the level of heterogeneity that is expected from the sample.

6 Notes

1. You will want ~25 mL of bead loading buffer (BLB) for each array. It is important that you do not add the sodium carbonate directly to the BSA, as this will denature 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 uM filter, and should be kept at 4 C. If the BSA solution begins to turn amber or yellow, it should be discarded.
2. The guanidine thiocyanate will take ~2 hours to go into solution, prepare this solution in advance.
3. Prelysis buffer is photosensitive. Wrap the buffer's container with aluminum foil to protect it. Wrapped prelysis buffer can be stored in a glass bottle at room temperature for approximately 6 months.
4. Prepare complete lysis buffer fresh, just prior to use.
5. Prepare 50 mL of wash buffer per array.
6. Unused beads should be stored at 4 C in TE-TW buffer in 1.5 mL microcentrifuge tubes. When they arrive from the manufacturer, beads should be processed according to the manufacturer's package insert, namely, washed 2× with 30 mL 200 Proof Ethanol for Molecular Biology, 2× with 30 mL TE-TW, passed through a 100 uM filter, and resuspended in 50 mL TE-TW.
7. Currently, Chemgenes is the only supplier manufacturing these beads.
8. Functionalized arrays can be made using the protocol in Hughes et al. 2020 [2] or purchased from the Nanowell Cytometry division of the Biomicro Core at the Koch Institute for Integrative Cancer Research at MIT, Cambridge, Massachusetts, contact: biomicro@mit.edu.

9. There are two ways to transport arrays. The preferred method is to place arrays in 50 mL conical tubes, filled with array quenching buffer or aspartic acid solution. Two arrays can be transported in each conical; arrays should be arranged back-to-back with glass slides touching. Alternatively, the arrays can be dried and transported in a histology slide box. To dry the arrays, remove them from the storage buffer and (being careful not to touch the surface of the array) use a paper towel to wick off excess liquid from the slide. Cover the arrays with the top of a pipette tip box to prevent dust from falling onto them as they dry. Let the arrays dry overnight at room temperature. Dried arrays can be stored at room temperature for 2 weeks. Rehydrate the arrays by placing them in a 4-well dish with either array quenching buffer or aspartic acid solution. Place the arrays under vacuum until (by microscope inspection) there are no air bubbles remaining in the wells of the rehydrated arrays. If no vacuum chamber is available, the arrays can be left to soak overnight and should be ready to use the following day.
10. You will need one hybridization clamp for each array that you plan to process in parallel. Prepare the hybridization clamps by cleaning them with 70% ethanol. You may also find it helpful to apply a small piece of lab tape to the screws—this mark will enable you to keep track of sample identities during membrane sealing.
11. If the plasma cleaner has multiple shelves, place the membranes on the bottom shelf. This placement will reduce the risk that the treated membranes will fly off the slides when the vacuum is released.
12. If your plasma is not bright pink, adjust the bleed valve until the desired color is observed. If you suspect a vacuum has not formed, power off the plasma cleaner—but not the pump—and pull gently on the door to see if a seal has been formed.
13. This amount of time required for membrane functionalization will vary based on the size of the chamber, the power of the instrument, and the settings of the needle valve. When beginning to use a new plasma cleaner, you will need to calibrate to find the correct time for membrane treatment, typically between 3 and 8 min. Anytime you reassemble a plasma cleaner, you will connect NPT tapered threads (3-way valve). For best results, wrap the thread in PFTE (Teflon tape) to ensure a good seal.
14. The sound of the air being released at this low level is very faint, it may be beneficial to put your ear near to the valve as you slowly turn the valve (with appropriate personal protective equipment, like eyeshields), so that you can detect the exact moment when air starts to be released.

15. If you open the door to find that the membranes are slightly folded over, but still on their original slides, slowly flip the membranes back using tweezers. If the membranes have blown off the slides entirely, it is impossible to know which side of the membrane has been exposed to the plasma, and these membranes should be discarded. The plasma-treatment procedure should be repeated with a new set of membranes.
16. Ensure the *entire* membrane is hydrated, usually 5 mL is sufficient. Continue adding PBS over the top of the membrane until it is fully submerged. Avoid touching the top of the membrane with the pipette tip.
17. If any of the arrays have folded over completely and you are unsure which side was UP during the plasma treatment, discard the membrane. This folding can also happen when membranes are being transferred, and it may be helpful to prepare extra membranes.
18. If bubbles remain after the initial solvation step, rock aggressively along the x-axis (i.e., slide the dish across the table) to remove them.
19. Looking down on the 4-well dish, inspect the solvated membranes and discard any which are bright white and opaque. Next, position yourself so that you can view the membranes from the side of the 4-well dish. Membranes which are floating completely on top of the PBS after solvating for 2 min should be discarded.
20. After solvation and before bead loading, use a microscope to inspect wells. If this inspection reveals air bubbles in the wells of the arrays, place arrays 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 bubbles from the wells of the array.
21. If a tabletop centrifuge is unavailable, spin beads for 1 min at 800–1000G.
22. In order to remove all the BLB, you may find it helpful to pipette directly off the top of the array. Be sure to remove excess BLB under the array, as this will allow the array to shift and slide, making bead loading more difficult.
23. This placement is very important, if the long sides of the array are touching the wells of the dish, the loaded beads will slide off the array before they are able to fall into wells.
24. A completely dry array can be identified by its surface finish. Arrays with a small amount of liquid on top of them will remain shiny, while those that have dried out completely will appear matte.

25. During this step, do not lift the pipette tip more than 0.5 cm above the surface of the array, as the force of the beads falling from a greater height will result in a pattern of uneven bead loading.
26. Place a black background behind the 4-well dish to better visualize the bead coverage of the array while loading.
27. If you do get bubbles on the surface of the array, use a fresh pipette tip to carefully pop them. Alternatively, use a pipettor to pipette air out of the bubbles.
28. Manually agitate the arrays by pushing the entire 4-well dish slowly across the benchtop in the X and Y direction. Do not pick up the dish during this step, as tilting the 4-well dish relative to the ground will result in the beads sliding off the array.
29. If the array starts to dry out and less than 25% of the surface of the array looks dry (matte finish), carefully add more BLB to the top of the array dropwise. If the array starts to dry out and more than 25% of the array is dry, add 5 mL of BLB to the well and place the array under vacuum for 5 min. You will likely have to add more beads to this array.
30. If the beads have fallen off—usually this is caused by the long side of the array touching the side of the 4-well dish—use a pipette tip to re-center the array, pipette up beads from the side/bottom of the well, and redeposit the beads on the top of the array. Be careful not to introduce bubbles to the surface of the array during this step. If you do see bubbles in your pipette tip, you may find it useful to transfer the beads you have aspirated into a clean microcentrifuge tube, and spin them down on a tabletop centrifuge to remove bubbles.
31. The edges of the array will not generally have full bead occupancy, this observation is not reason for concern.
32. Loaded arrays can be stored for up to 12 hours at 4 °C. If you are preparing the arrays more than 12 hours in advance, place the loaded array in array quenching buffer and store at 4 °C.
33. For every step of the protocol where beads meet TE-TW, the beads take longer to pellet when they are spun. When pipetting to remove supernatant during these steps, watch closely to ensure that you are not also removing beads. We recommend that you collect the supernatant two steps—spin down for 1 min, remove the first 200 uL of supernatant, spin for another minute, then aspirate the remainder of the supernatant.
34. Ensure that tubes containing beads are stored upright. If tubes are knocked over and beads are incompletely submerged, they may dry out, rendering them unusable.

35. While automated cell counters are time-efficient, we find that the most replicable results are achieved with manual cell counting. We typically mix 20 μL of rigorously resuspended cell suspension with 20 μL of 0.4% Trypan Blue, and load 10 μL into each chamber of a Neubauer Improved disposable hemocytometer. If cells are plentiful, we count samples in triplicate. If counting on a flow cytometer, be sure to include both a live stain (like Calcein) and a dead stain (such as an amine dye).
36. Do not use RPMI with any additives.
37. It is very important that this solution contains FBS, because FBS allows the cells to fall into the wells instead of just sticking to the surface of the array. The FBS minimizes nonspecific interactions at the surface of the array, thereby preventing cells from sticking to the surface of the array.
38. Cells can also be loaded in DMEM + 5% FBS or PBS + 0.05% BSA.
39. The fan in biosafety cabinets tends to quickly dry arrays out. If safety protocols allow, place the arrays on the benchtop for 5 min or turn off the fan in the biosafety cabinet (only if feasible with your biosafety protocol requirements).
40. Once the cells are loaded, do not put the arrays under vacuum.
41. If the array is touching the side and the liquid spills off, collect cells, re-center, and pipette cells directly onto the top of the array again.
42. If the array begins to dry, use P200 pipette to add a little bit (~50 μL) of RPMI + 10% FBS.
43. It is *critical* that nothing is added to this PBS.
44. Sometimes the array will stick to the top piece of the clamp. In this case, carefully separate the array sandwich from the top piece. Hold the array sandwich from the sides to prevent forces being applied unequally to the two sides. *Do not pry the array off the metal*, as this action can reverse membrane sealing.
45. During this transfer, be careful to maintain the orientation that the slide sandwich had when it entered the clamp—with the slide attached to the array down.
46. The hybridization buffer may contain trace amounts of guanidine thiocyanate and should not be mixed with bleach. Aspirate the hybridization buffer into the same container you used to collect lysis buffer in **Step 5**.
47. The amount of wash buffer you used in this step should be sufficient to fill the 50 mL conical to the top of the array.
48. The hybridization buffer may contain trace amounts of guanidine thiocyanate and should be collected in the lysis waste container.

49. If you are only processing a few arrays and working quickly, you can elect to not replace the aspirated hybridization buffer with a wash buffer. This exchange will prevent the beads that float from being lost, and may make the arrays easier to image under the microscope. Once the arrays have dried out completely, it will be very difficult to remove beads from them.
50. You may find it helpful to make a note of how much resistance you felt when removing the membrane—was it stuck onto the array, or did it slide right off? Membrane sealing is one of the primary points of failure for this protocol.
51. This step is optional. Once air enters the microwells, it can be very difficult to identify beads in the microwell array and distinguish them from empty wells. We recommend this step if previous experimental runs have had low yields of cDNA, or when samples are irreplaceable.
52. Pipetting up and down at this step will cause beads to get stuck in the pipette tip, leading to a loss of product.
53. At this point, the bead pellet will be easily disturbed, and you may not be able to completely aspirate the supernatant. Remove the supernatant as completely as possible. For best results, do not leave more than 10 μL of 1× RT buffer covering the beads.
54. Alternatively, you can let the RT continue overnight at 52 C and wash the beads the following morning.
55. We start with a single TE-TW wash because salts in the RT buffer can cause SDS to precipitate, making it difficult to remove.
56. For every step of the protocol where beads meet TE-TW, we recommend collecting the supernatant in two steps—spin down for 1 min, remove the first 200 uL of supernatant, spin for another minute, then aspirate the remainder of the supernatant.
57. The optimal number of PCR cycles depends on the cell type used. In general, 13 cycles are optimal for cell lines or larger cells (e.g., macrophages) while 16 cycles are optimal for primary cells.
58. To quickly gauge if the Ampure SPRI beads are ready to use, quickly invert the bottle. If you observe no beads stuck to the bottom, the beads are ready to use. If you see a significant number of beads stuck to the bottom, incubate another 10 min at RT.
59. It is especially important to use Lo-Bind 1.5 mL centrifuge tubes for the following steps, using non-Lo-Bind tubes will result in the loss of a significant amount of WTA product.
60. For example, if you have 200 μL of PCR product add 120 μL of SPRI beads for a 0.6× volumetric ratio.

61. Dilute your PCR product in a separate tube/plate so that you can transfer 5 μL of this dilution to the tagmentation reaction.
62. Change pipette tips as you add the ATM buffer to prevent the ATM stock from being contaminated with TD buffer.
63. Final libraries are compatible for all Illumina sequencing machines that allow for a custom Read 1 primer. It is standard protocol to allot 200 million reads per sample (per Seq-Well Array). The following protocol is prepared for sequencing using a High-Output 75 Cycle v2.5 Kit for the NextSeq 500/550 platform. This kit produces 500 million reads and thus provides sufficient sequencing depth for 2 arrays per sequencing run.
64. Use the average length from the tape station and the average concentration from the Qubit fluorometer (take measurements in triplicate) to calculate the required dilution.
65. The timing of this step is important, an overly denatured library may significantly reduce the quality of your sequence data. If you accidentally allow the denaturing to proceed for longer than 10 min, make a fresh 4 nM library and repeat the denaturation.

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