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Protocol

Single cell qtSEQ: Cell-indexed quantitative and targeted RNA sequencing for sorted rare lymphocyte subpopulations

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https://doi.org/10.1016/j.xpro.2021.101064

SUMMARY

Adaptive T and B lymphocytes expand, respond, and persist across a multitude of separable cell differentiation states. Small compartments of these cells present defined cell surface phenotype, but express potentially divergent immune functions. Here, we use high resolution flow cytometry to provide direct access to rare lymphocyte subpopulations for evaluation of steady-state or reactive transcriptional programs. We sort and index single cells by phenotype in 384-well format for quantification of targeted gene amplification through RNA sequencing (single cell qtSEQ).

Before you begin

Single cell transcriptional analysis has emerged as a key methodology to interrogate the diverse molecular programs of adaptive immunity. However, achieving sensitivity at the single cell level remains elusive. Most single cell RNA sequencing techniques employ global 3′ transcriptomic approaches which are prone to dropout of biologically important signals and ill-suited to sequence plasma cell transcriptomes, which are highly enriched for immunoglobulin genes. Additionally, protocols that isolate single cells using droplet or bead-based methods forgo indexed phenotype data and have difficulty accessing significant numbers of rare immune subsets. To better address the technological limitations for the study of rare immune cell subsets, we developed single cell quantitative and targeted RNA-Seq (single cell qtSEQ).

Single cell qtSEQ relies on flow cytometry to index sort cell subsets of interest into 384-well plates for barcoding and cDNA library formation. The fluorescence-based cytometer must be equipped with an automatic cell dispensing unit, digital cell indexing and be calibrated to target 1 μL volumes for sorting. Single cell qtSEQ uses targeted and semi-nested PCR reactions to amplify 500 specific cDNA targets. While we have not verified the protocol beyond 500 it is likely additional targets can be added if proper consideration is taken into adjusting PCR conditions as described below. This strategy uses custom reaction mixes with increased selectivity and sensitivity to provide added depth for target mRNA species in each single cell. A multitude of primers must be designed and prepared beforehand and a guide for this process is included below. While the array of integrated analyses depicted in the graphical abstract is growing continually, we limit this protocol description to
the production of single cell libraries and deconvolution of digital information for the initial analytical presentation of the data.

**Design oligo-dT reverse transcription primers**

© Timing: 1 day for design plus ordering time

1. Single cell qtSEQ makes cDNA libraries from sorted cells by targeting polyA RNA sequences for reverse transcription. The oligo-dT reverse transcription primer (Figure 1) also contains a universal binding sequence (Univ1) (Illumina RA5) for amplification in the initial PCR step. Following the universal sequence (5’–3’) is a 6 basepair well barcode (BC) that labels each cDNA to identify the sorted well of origin. Single cell qtSEQ is a plate-based sequencing protocol optimized for 384-well sorting, the 384-well specific barcodes adapted from Soumillon et al. (2014) are listed in Table S1. Following the well barcode sequence is a randomized 10 basepair Unique Molecular Identifier (UMI) that will label each individual cDNA with a discrete sequence. This UMI sequence is used during in silico data processing to reduce PCR bias: each distinct UMI found among reads aligning to a singular gene is assumed to be derived from one original mRNA molecule in the original sample. Thus, amplified reads may be reduced into single molecule counts. Oligo-dT primers should be ordered at 10 mM suspended in 1X TE solution (10 mM Tris, 0.1 mM EDTA pH 8.0) in a 384-well plate format then distributed into working concentration (500 nM/well) 384-well plates. We recommend making smaller volumes per well (10 uL) to ensure stock plates
are not re-used too often which can increase the risk of primer contamination. Plates can be stored at −20°C for long term but should avoid excessive freeze thaw cycles. We found that at least 20 freeze-thaw cycles did not impact RT primer quality.

△ CRITICAL: Great care must be taken to ensure there is no oligo-dt primer contamination between wells. Any well cross over will eliminate the ability to accurately deconvolute cell barcodes in silico. As with all steps in this protocol, work in a laminar flow hood using sterile technique.

3’ based targeted primer design

⊙ Timing: 1–3 days per 100 unique gene specific primers plus ordering time

2. Single cell qtSEQ uses two nested PCR reactions to amplify the cDNA library therefore each targeted gene transcript will require an internal and external primer to be designed. These should target the 3’ end of the gene transcript such that the final amplicon length (including adapter sequences) will be ~500 bps as depicted in Figure 2. This is a workable size for the sequencing instrument used in this protocol which recommends an average library size insert of 550 bp with V2 chemistry (NextSeq 500, Illumina) although we have had success running libraries with longer (~800 bp) average inserts. Every internal 3’ targeted primer requires the addition of the RA3 universal adapter (Figure 1, Table S2). Primers should be ordered at 100 μM, then each primer carefully combined into an external primer mix working solution and an internal primer mix working solution. Add 1 μL from each primer into an external or internal PCR tube and calculate the working concentration of each primer in the mix (primer stock [c]/ # of primers added).

△ CRITICAL: The final concentration of each 3’ targeted primer in the primer mix working solution will depend on the number of primers used and should be taken into account for all downstream calculations.

3. We recommended designing the primers using NCBI Primer BLAST to ensure specificity. 3’ gene transcript information can be obtained from genome browsers (UCSC, or Ensemble). Ideal primers should meet the following criteria:
   a. Primer specificity is unique to a single gene transcript
   b. Primer length is between 20–30 bps
   c. Primers do not span intron-exon junctions
   d. Primers have consistent GC content (40%–60%) and melting temperature (~60°C)

Note: To reach the final amplicon length of ~500 bp, many targeted primer sequences will be located within the 3’UTR. While less annotated and more variable than the coding sequence there is still clear specificity from primers targeting the 3’UTR.
### Ordering universal primers

© Timing: 1 day plus ordering time

4. PCR amplification of selected cDNA targets is achieved using the 3’ targeted primers in a forward orientation, then an external (targeted PCR1) and internal (targeted PCR2) reverse primer sequence (labeled in this protocol as REV-PCR1-RA5 and REV-PCR2-RA5 respectively) binds to the RA5 universal sequence on successfully transcribed mRNA (Figure 1). The final round of PCR (adapter ligation PCR3) adds the Illumina flow cell adapters RP1 (P5 adapter) and RPIX (P7 adapter) to each amplicon (Illumina TruSEQ) (Figure 3). Several plates can be multiplexed into a single sequencing run by using different RPIX index sequences for each plate. While we provide four index sequences in this protocol additional RPIX primers can be ordered and plates multiplexed with due consideration of sequencing depth per plate.

**Note:** The application of all primers used throughout the protocol across PCR steps can be found in Figure 3.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Superscript II | Invitrogen | Cat#18064022 |
| RNaseOUT | Invitrogen | Cat#18427088 |
| ExoSAP-IT | Thermo Fisher Scientific | Cat#78205 |
| SPRIselect Beads | Beckman Coulter | Cat#823318 |
| Phusion | New England Biolabs | Cat#MO5315 |
| 1M Tris-HCl pH8 | Thermo Fisher Scientific | Cat#15568025 |
| RNase-H | Invitrogen | Cat#18021-071 |
| Bead Binding Buffer | Teknova | Cat#P4146 |
| **Critical commercial assays** | | |
| hsDNA Bioanalyzer kit | Agilent | Cat#5067-4626 |
| Qubit dsDNA high sensitivity kit | Invitrogen | Cat#Q33230 |
| NextSeq 500/550 Kit v2.5 | Illumina | Cat#20024907xs |
| **Oligonucleotides** | | |
| Primers used in this study | | |
| REV-PCR1-RA5: GAGTTCTACAGTCCGACGATC | N/A | N/A |
| REV-PCR2-RA5: CTACAGTCCGACGATC | N/A | N/A |
| Illumina P5: AATGATACGAGTGTTCCTCA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| TACACGTTGAGTTCTGCTACAGTCCGA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| Illumina P7 RP14: CAACGACAGAGCGGATACGAGATT | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| GACCACTGACTGGCTTGTGGCACCACCGAGATTCCA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| Illumina P7 RP16: CAACGACAGAGCGGATACGAGATGC | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| CAACTGGACTGGCTTGTGGCACCACCGAGATTCCA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| Illumina P7 RP17: CAACGACAGAGCGGATACGAGATGC | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| AGATCTGTGACTGGCTTGTGGCACCACCGAGATTCCA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| Illumina P7 RP112: CAACGACAGAGCGGATACGAGATGC | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| CTTGTAGTGACTGGCTTGTGGCACCACCGAGATTCCA | Illumina TruSeq Small RNA Guide | Document # 15042173 v01 |
| Reverse Transcription Primers: See Table S1 | This paper | N/A |
| 3’ Targeted External and Internal Primers: See Table S2 | This paper | N/A |
| **Other** | | |
| 384-well plates | Thermo Fisher Scientific | Cat#AB2384 |
| 96-well plates | Thermo Fisher Scientific | Cat#3482G |
| 1.5 mL DNA low bind tubes | Eppendorf | Cat#EP0030108051 |
| 200 μL PCR tubes | Eppendorf | Cat#EP0030124707 |
| 200 μL magnetic stand | PERMAGEN | Cat# MSRLV08 |
| 384-well metal cooler block | CORNING | Cat#432055 |
| 96-well metal cooler block | CORNING | Cat#432053 |
| Viaflow 12.5 μL, filtered, low retention sterile, 5 × 384 box tips | VIAFLOW | Cat#6455 |
| MicroAmp Clear Adhesive Films | Thermo Fisher Scientific | Cat#4306311 |
| **Software and algorithms** | | |
| Seurat (v3.2.3) | (Satija et al., 2015) | https://github.com/satijalab/seurat |
| Seurat (v3.2.9) | (Hafemeister and Satija, 2019) | https://github.com/ChristophH/seurat |
| FlowJo | FlowJo | https://www.flowjo.com/ |
| Bowtie2 (v2.2.9) | Langmead and Salzberg, 2012 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| Python (v2.7.11) | Python Software Foundation | http://www.python.org/ |
| R (v4.0.3) | The R Foundation for Statistical Computing | http://www.r-project.org/ |
| Rstudio (v1.3.1093) | RStudio | http://www.rstudio.com/ |
| HTSeq (v1.3.1093) | (Anders et al., 2015) | https://github.com/htseq/htseq/blob/release_0.11.1.1/python3/doc/index.rst |
| Bcl2fastq (v2.19.1.403) | Illumina | https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html |
MATERIALS AND EQUIPMENT

Flow cytometer and single cell sorting
In this protocol we use a BD FACS Aria III Cell Sorter capable of high parameter (~12 color) cytometry and single cell indexed sorting into 384-well plates. Any flow cytometer capable of index sorting into 384-well plates may be used.

Liquid handler
To effectively prepare plates for sorting, we used a VIAFLO liquid handler (Integra Biosciences) equipped with a 96-tip head. The plate preparation steps listed below are specifically designed to work with the 96-well head and two sample plates at once but could be simplified and adapted for a 384-well head liquid handler or additional sample plates.

NextGen sequencer
The library preparation steps listed in this protocol are designed for sequencing on a NextSeq500 (Illumina) with a v2.5 high output flow cell (150 cycles). Amplicon lengths, adapter sequences, and denaturing and dilutions steps can be adapted to accommodate alternate Illumina sequencers or flow cell versions.

STEP-BY-STEP METHOD DETAILS

Laminar flow hood setup

© Timing: 10 min

Ready the workspace to quickly and efficiently process four 384-well index sorted sample plates.

1. Prepare a laminar flow hood for single cell sequencing work
   a. Clean and sterilize the hood with 70% Isopropyl Alcohol or Ethanol
   b. Gather necessary materials in the hood:
      i. P2, P10, P20, P200, P1000 Pipettes
      ii. Filter tips for each pipette in use
      iii. 384-well plates (4)
      iv. 96-well plate
      v. MicroAmp Clear Adhesive Film plate covers
      vi. Ice trays/buckets
      vii. 200 μL magnetic stand
      viii. 1.5 mL Sterile Eppendorf Tubes and racks
      ix. PCR grade water
      x. Lab grade 200 proof Ethanol
      xi. 96- and 384-well plate cooling blocks
      xii. VIAFLO 96 head tips

   Note: While we found four plates (~1500 cells) to provide adequate cell numbers per experiment additional plates can be added as desired with requisite downstream calculations to the extra time and reagents required.

Flow cytometer setup

© Timing: 1–2 h dependent on instrument and user protocol

Prior to starting any library preparation steps such as generation of master mixes for RT, ensure the flow cytometer has completed daily maintenance and baseline validation, and that single cell sorting drop delays, targeting, and fidelity have been set. This will vary per user protocol and instrument, but an example of the steps required to properly target single cells is provided below.
2. Using a small aliquot of sample run ~10,000 events to ensure FL-antibody staining is adequate and sorting can go ahead.
3. To check alignment of single cells sorting into individual wells, place a film cover on top of a 384-well PCR plate. Sort 50 cells from your sample (can be unstained) into the four corner wells of each plate. Visually confirm that each droplet is centered perfectly over the wells.
4. Next remove the film from the 384-well plate and sort one cell into each corner well. Visually confirm the droplet has reached the bottom of each well with little splatter.

Reverse transcription master mix and plate preparation

**Timing: 1 h per 2 plates**

Once the flow cytometer is ready to sort, prepare two 384-well plates by adding 1 μL of reverse transcription (RT) solution into each well.

5. Remove the following components from –20°C storage to thaw on ice in the laminar flow hood:
   a. 384-well RT oligo-dt primer stock plate [500 nM/well]
   b. First strand synthesis buffer [5×]
   c. dNTPs [25 mM]
   d. DTT (Dithiothreitol) [0.1M]
   e. Phusion HF buffer [5×]
   f. REV-PCR1-RAS Primer [100 μM]
   g. External Primer Mix [200 nM/primer]
   h. Superscript II (leave in –20°C until ready to use)
   i. RNaseOUT (leave in –20°C until ready to use)

△ CRITICAL: During plate preparation keep all reagents and prepared plates on ice to ensure fidelity of the master mix.

**Note:** The limiting step of this protocol is length of time it takes to index sort cells of interest and the number of slots available in 384-well PCR machines for the reverse transcription reaction. It is CRITICAL: to ensure plates undergo RT as quickly as possible after sorting. Depending on the rarity of the cell population, sorting into a 384-well plate can take anywhere from 20 min to 1.5 h. We see improved cDNA formation when the length of time plates sat pre- and post-sort (even at 4°C) was minimized. As such, we designed the protocol to make reverse transcription master mix for only two plates at once. While those plates are being sorted additional plates can be prepared. Per experiment we typically prepared and sorted into 4 plates for about ~1500 cells.

6. Prepare the RT master mix in a 1.5 mL lo-bind Eppendorf tube on ice. This master mix does not contain any oligo-dT primer as these must be added to each individual well separately to maintain well-barcode specificity. To account for sample loss when working with small volumes and viscous solutions this master mix includes 25% excess to ensure each well of the 384-well plate receives the correct volume.

| Reagent                              | 1 cell* | 384 cells* | 768 cells* |
|--------------------------------------|---------|------------|------------|
| First Strand synthesis buffer [5×]   | 0.2 μL  | 96 μL      | 192 μL     |
| DTT [0.1 mM]                         | 0.03 μL | 14.4 μL    | 28.8 μL    |
| dNTPs [25 mM]                        | 0.012 μL| 5.76 μL    | 11.52 μL   |
| Superscript II                       | 0.03 μL | 14.4 μL    | 28.8 μL    |
| RNaseOUT                             | 0.03 μL | 14.4 μL    | 28.8 μL    |
| PCR water                            | 0.198 μL| 95.04 μL   | 190.1 μL   |
| Total                                | 0.5 μL  | 240 μL     | 480 μL     |

* Includes 25% excess volume
7. Using a P10 pipette, manually aliquot 5 μL of RT mastermix to each well of a 96-well plate on ice, and in a metal cooling block to ensure components are kept cold throughout processing. Cover plate with MicroAmp Clear Adhesive Film and spin down at 1000×g for 30 s ensuring all master mix is at the bottom of the wells without any bubbles.

8. Use the VIAFLO liquid handling instrument to dispense 0.5 μL of RT master mix into each well of the two 384-well plates keeping the plates on ice as much as possible.
   a. Set the VIAFLO liquid handling instrument to repeat dispense a volume of 0.5 μL for 8 repeats giving a total aspiration volume of 4.5 μL (Initial dispense step of 0.5 μL to clear air bubbles followed by 8 repeat dispenses of 0.5 μL to cover each quadrant of two 384-well plates).
   b. Place two 384-well plates each into a cooling block.
   c. Draw up 4.5 μL of master mix (leaving 0.5 μL in the well to ensure the full amount is aspirated) from the 96-well plates and dispense the initial clearance of 0.5 μL back into the well.
   d. Move the 384-well plates onto the VIAFLO platform and dispense 0.5 μL into each well of the two plates one quadrant at a time.
   e. Cover each 384-well plate with MicroAmp Clear Adhesive Film and spin down at 1000×g for 30 s to ensure all master mix is at the bottom of the wells without any bubbles.

**Note:** As the RT master mix is shared across wells, a single quadrant of a 384-tip box can be used to dispense master mix to all wells in both plates.

**Note:** To ensure tips are dispensing at the correct height, calibrate the z-height of the tips beforehand with the 384-well plates in the cooling blocks. Tips should be a few millimeters from touching the base of the wells.

⚠️ **CRITICAL:** Dispensing small volumes of viscous solutions requires careful use of the liquid handler. Once a volume is dispensed it is difficult to go back without compromising the remaining solution already in the tips by introducing air bubbles. Therefore, work under the assumption that a 96-well set of tips can only be used for a single distribution of the RT master mix.

9. Distribute 0.5 μL of the barcoded RT primer solution into each well of the 384-well samples plates
   a. Set the VIAFLO to repeat dispense 0.5 μL one time such that the total volume aspirated is 1 μL.
   b. Prepare the working space so that the 384-well RT oligo-dt primer stock plate and sample plates are in cooling blocks and easily accessible.
   c. Using a fresh set of VIAFLO tips aspirate 1 μL from quadrant 1 (Q1) of the 384-well RT oligo-dt primer stock plate. Dispense 0.5 μL into Q1 of the first sample plate then 0.5 μL into Q1 of the second sample plate. Eject sample tips; do not reuse tips as this will contaminate barcodes.
   d. Load a new set of tips and repeat process for Q2–4 changing tips in between each quadrant. See **Figure 4** schematic for graphical representation.
   e. Cover each 384-well plate with MicroAmp Clear Adhesive Film and spin at 1000×g for 30 s to ensure all of the master mix is at the bottom of the wells without any air bubbles.
   f. Store plates at 4°C until ready to sort. Ensure plates are tightly sealed to minimize evaporation from wells.

**Note:** Since oligo-dT primers are suspended in PCR-grade water, this solution is not viscous like the master mix, therefore excess solution is not required.

⚠️ **CRITICAL:** Very carefully ensure the correct quadrants from the RT primer stock plate are being added to the correct quadrants in the sample plates. Any form of barcode contamination could eliminate the bioinformatic capacity to link transcriptional data to a particular well.
Index sort single cells from targeted populations of interest.

10. Begin index sorting single cells into the prepared 384-well plates using a cooling block (if available) to keep the sample plates cold
   a. Ensure the “Index” sort option is selected to collect phenotypic data.
   b. Remove cover from sample plates and sort cells.
   c. After completion of cell sorting for a plate, cover the wells with MicroAmp clear adhesive film and spin down prior to running RT. If using a 2-block 384-well PCR instrument, store the first plate in a 4°C refrigerator until both plates are completed and ready for RT.

**Note:** While initial sample plates are being sorted, additional plates can be prepared for sorting.

**Note:** We do not recommend storing samples immediately after sorting. Users should proceed to cDNA library formation before pausing.
Note: We find that if the fraction of the cell type from the total spleen is ~0.01% it will take ~20 min to sort a plate. If the fraction of the total spleen is ~0.005% it will take closer to 1 h to sort a plate.

Reverse transcription

☀ Timing: 1 h total (5 min hands-on).

Make cDNA library from polyA mRNA of sorted cells.

Note: While sorting plates, start the 384-well PCR machine at a 42°C hold for hot start to RT.

11. Load sample plates into a 384-well PCR machine and run the reverse transcription program.

| Steps   | Temperature | Time   | Cycles |
|---------|-------------|--------|--------|
| RT      | 42°C        | 50 min | 1      |
| Denature| 80°C        | 10 min |        |
| Hold    | 4°C         | Forever|        |

Pause point: After RT is completed, library preparation can be halted for day 1 and continued another day. Store RT products at 4°C. While the cDNA-RNA hybrid product from RT is relatively stable, we advise completion of PCR 1 prior to stopping.

Pool sample plates, degrade excess primers and volume reduction

☀ Timing: 1.5 h

This section explains how to pool within sample plates across wells, treat with ExoSAP-IT to degrade excess RT primer and dNTPs, and reduce volume using SPRIselect beads.

12. After the conclusion of the RT reaction, spin down the plates and begin pooling steps (Figure 5).
   a. Using a P10 pipette, pool each well from a 384-well plate into a 1.5 mL low-bind Eppendorf tube. Alternately, a liquid handler or multi-channel pipette can be used for faster pooling.
   b. Repeat the pooling step for each plate using a separate tube for each plate.

Note: We recommend turning off the laminar flow in the hood for this pooling step as the air flow can quickly evaporate the reaction mix. Thus, aseptic technique is very important to avoid external contamination.

Note: While pooling each well by hand is more labor intensive, we found it resulted in the least sample loss. Any lost sample at this point pre-PCR amplification will result in reduced signal.

△ CRITICAL: It is essential that during this pooling step (and all subsequent steps) that each plate remains separate. Pooling across wells of a single plate is possible due to well barcodes; however, each plate uses the same 384 barcodes. Any pooling across plates will be impossible to separate bioinformatically so plates must remain separate until the plate identifier sequences are added in PCR3.

13. Once each plate has been pooled into its own 1.5 mL tube, excess RT primer and dNTPs are removed by treating with ExoSAP-IT. Excess RT primer could act as an unwanted primer during PCR1 and overwrite existing barcodes so it must be removed from the solution.
a. Record the volume recovered from each plate and then divide accordingly into 50 μL PCR tubes, labelling carefully to keep plates separate.

b. Add the ExoSAP-IT solution at a ratio of 2 μL to every 5 μL of pooled library according to manufacturer’s instructions.

c. Incubate PCR tubes at 37°C for 20 min then at 80°C for 15 min to inactivate ExoSAP-IT.

14. Recombine all PCR tubes from each plate back into a 1.5 mL Eppendorf tube for volume reduction using SPRIselct Beads. The ratio of SPRI beads in bead binding buffer versus the reaction volume can be used to size-select the fragments that will be bound by the beads versus left unbound as a measure of molecular crowding. Thus, for this initial bead cleaning step we used a 0.8 SPRI bead volume to 1.0 RT reaction volume ratio, which is meant to exclude molecules below 200 bp, which includes any oligo-dT primers not removed during the ExoSap-IT Express reaction. To avoid handling large volumes of beads, we combine only 100 μL of SPRIselct beads with an analog of the SPRIselct beads solution called Bead Binding Buffer (BBB, 2.5MNaCl, 20% vol/vol PEG; Teknova) to reach the 0.8 ratio.

a. Measure the total volume for each library after the ExoSAP-IT Express™ step. This volume is important for calculating the proper bead volume for SPRIselct bead clean and volume reduction.

b. Use that volume to calculate the required SPRIselct and BBB volumes which will likely differ between pooled sample plates.

| Component                  | Method                      | Example | Example volumes |
|----------------------------|-----------------------------|---------|-----------------|
| Pooled RT reaction         | Volume Calculate in step 14a| 500 μL  | 500 μL          |
| SPRI beads                 | 100 μL                      | 100 μL  |                 |
| Bead binding buffer        | (Volume × 0.8) – 100 μL     | (500 μL × 0.8) – 100 μL | 300 μL |

Note: SPRIselct beads can bind up to 7 μg of DNA per μL far beyond the amount of cDNA present in a pooled sample tube.
c. Combine 100 µL SPRSelect beads with calculated volume of BBB and vortex vigorously.
d. Add to correct pooled sample tube, vortex lightly, and let stand for 10 min to allow the DNA
to bind to the beads.
e. Set up a 200 µL magnetic stand for each plate to undergo volume reduction. Place a separate
200 µL tube onto each magnetic stand. Add 180 µL of the SPRSelect beads/BBB/RT reaction
mix to the tube. Allow the beads in the mix to pellet, about 2 min. Remove the supernatant
WITHOUT disrupting the bead pellet.
f. Repeat step 14e iteratively until the entire reaction mix has been bound in one pellet in a sin-
gle 200 µL tube.
g. Remove supernatant without disturbing the pellet and discard. Wash the pellet twice by
running 180 µL of 85% molecular-grade EtOH over it. Let sit for 30 s then remove the
EtOH without disturbing the pellet. A total of two washes, instead of just one, with 85%
EtOH is standard.

Note: Dilute 200 proof molecular-grade EtOH with PCR grade water to generate 85% EtOH.
h. Set the tubes aside to air dry for ~2 min or until the pellet glistens but not does not appear

Note: Over-drying of bead pellets can lead to sample loss as DNA does not elute effectively
once fully dried on the bead pellet.
i. Elute the DNA from the pellet by adding 35 µL pure PCR-grade water. Mix by pipetting and
let sit for 2 min. Return the sample to the magnetic stand to pellet the beads. Remove water
without disturbing the pellet. Discard tube and beads.

Note: Library quality control may be carried out at this point using qPCR assays. However, it
should be noted that removing sample for quantification before PCR will reduce library
diversity.

**PCR1: external nested amplification**

Δ **Timing: 1.5 h (15 min hands-on)**

PCR 1 is the first of 3 PCR steps necessary to selectively target and amplify targeted gene transcripts
of interest from the cDNA generated after RT. The first PCR utilizes the EXTERNAL primer set for
targeting of genes and the REV-PCR1-RA5 primer (Figure 3). An additional RnaseH step is added
before amplification to remove RNA from cDNA-RNA hybrids.

Δ CRITICAL: It is essential that the EXTERNAL primers are used for this first reaction. We
highly suggest color coding Internal and External primer library stocks to avoid using
the wrong set of primers.

15. Create the PCR 1 master mix and add to RT reaction eluted in water for a 60 µL total reaction
volume. Adjust volumes for the number of plates sorted. Volumes required for one reaction
(cDNA library from one sorted plate) listed below.

| Reagent                  | Amount for 1 reaction |
|--------------------------|-----------------------|
| RT reaction eluted in water | 35 µL                |
| RNase-H                  | 1.5 µL                |
| Phusion HF enzyme        | 1.2 µL                |

(Continued on next page)
16. Mix solutions thoroughly then perform PCR1 on each cDNA library.

| Steps            | Temperature | Time    | Cycles |
|------------------|-------------|---------|--------|
| RNase-H          | 37°C        | 20 min  | 1      |
| Initial denaturation | 98°C      | 30 s    | 1      |
| Denaturation     | 98°C        | 30 s    | 10 cycles |
| Annealing        | 60°C        | 3 min   |        |
| Extension        | 72°C        | 1 min   |        |
| Final extension  | 72°C        | 5 min   | 1      |
| Hold             | 4°C         | forever |        |

△ CRITICAL: The number of cycles used in PCR1 was empirically determined by titrating PCR cycles for the targeted lymphocyte cell subsets. Certain cell subsets may require more or less amplification at this stage to reach this endpoint. It is imperative to avoid overamplifying the library in PCR1 where gene transcript targets are not yet enriched; this can lead to mis-priming and mixing of cell barcodes and UMIs. On the other hand, enough library must be amplified to allow for accurate quantification. We recommend adding additional PCR cycles to either PCR2 or PCR3 rather than PCR1 if further amplification is required to reach the ideal final library concentration of 4–15 nM. qPCR assays may also be employed following PCR1 to determine a standard library concentration before proceeding.

¶ Pause point: After PCR1 is completed and cDNA targets amplified, libraries can be stored at 4°C and the protocol continued another day.

17. Bead clean PCR1 products using a 0.9× SPRIsel ect bead volume to 1.0× PCR 1 reaction volume ratio for the exclusion of DNA products below 150 basepairs, which includes all PCR1 primers.
   a. For a 60 μL PCR1 reaction, add 54 μL SPRIsel ect beads and mix thoroughly.
   b. Let beads bind for 5 min.
   c. Remove supernatant without disturbing the pellet and discard. Wash the pellet twice by running 180 μL of 85% molecular grade EtOH over it. (A total of two washes is beneficial) Let sit for 30 s then remove the EtOH without disturbing the pellet.
   d. Set the tubes aside to air dry for ~2 min or until the pellet glistens but does not appear cracked.
   e. Elute the DNA from the pellet by adding 16 μL pure PCR grade water. Mix by pipetting and let sit for 2 min. Return the sample to the magnetic stand to pellet the beads. Remove water without disturbing the pellet. Discard tube and beads.

Note: We recommend setting aside 6 μL of this reaction (store at 4°C) so that if the protocol fails downstream it will not result in the loss of an entire plate(s). This should also be done after PCR2. At this stage we find there is not enough product to test library quality using a Bioanalyzer trace. qPCR assays could be employed to indicate library quality but would not
give an indication of library diversity and are not much less labor or cost intensive as carrying on with the following PCR reactions.

**PCR2: internal nested amplification**

**Timing:** 1 h, hands on time 15 min

PCR2 enriches targeted amplicons from PCR1 with a second round of amplification. PCR2 utilizes the **INTERNAL** primer set for targeting genes and REV-PCR2-RA5 primer (Figure 3).

18. Create the PCR2 master mix and add at a 1:1 ratio to 10 µL of the each of the plate libraries from PCR1.

| Reagent                        | Amount for 1 reaction |
|-------------------------------|-----------------------|
| PCR1 reaction eluted in water | 10 µL                 |
| Phusion HF enzyme             | 0.4 µL                |
| Internal Primer Mix [200 nM stock] | 2.5 µL              |
| REV-PCR2-RA5 [20 µM stock]    | 2 µL                  |
| dNTPs [25 nM]                 | 0.28 µL               |
| Phusion HF buffer [5 x]       | 4 µL                  |
| Water                         | 0.82 µL               |

19. After mixing well perform PCR2 on each library.

| Steps            | Temperature | Time  | Cycles |
|------------------|-------------|-------|--------|
| Initial denaturation | 98°C       | 2 min | 1      |
| Denaturation      | 98°C        | 30 s  | 10 cycles |
| Annealing        | 60°C        | 3 min |        |
| Extension        | 72°C        | 1 min |        |
| Final extension  | 72°C        | 5 min | 1      |
| Hold             | 4°C         | forever |      |

20. Following PCR2 again carry out a 0.9× SPRselect bead clean to exclude DNA products below 150 basepairs and elute the library in a clean water solution.
   a. For a 20 µL PCR reaction add 18 µL of SPRselect beads.
   b. Follow the steps outlined in step 17b–e.

**PCR3: flow cell adapter linkage**

**Timing:** 45 min

PCR3 adds the Illumina P5 and P7 adapters to the targeted amplicons with a few cycles of PCR.

21. Create the PCR3 master mix and add at a 1:1 ratio to 10 µL of each of the plate libraries from PCR1. Make the master mix without the index primers (RPI-X library barcode) which need to be added separately to each library.

| Reagent                        | Amount for 1 reaction |
|-------------------------------|-----------------------|
| PCR2 reaction eluted in water | 10 µL                 |
| RP1 Primer [10 µM stock]      | 2 µL                  |

(Continued on next page)
△ CRITICAL: Each library will require a separate RPI(X) primer. Thus, if creating a mastermix for multiple libraries, do not include the RPI primer, they must be added separately to each library. Each RPI primer is identical except for a 6 bp plate identifier sequence, called a plate index, which is used to separate each library computationally if multiplexing is used. These library identification sequences are based on the 48 Illumina TruSeq Small RNA Index sequences and can be found in the Illumina TruSeq Library Prep Pooling Guide.

22. Mix well and perform PCR3 on each library.

| Steps              | Temperature | Time  | Cycles |
|--------------------|-------------|-------|--------|
| Initial Denaturation | 98°C        | 2 min | 1      |
| Denaturation       | 98°C        | 15 s  | 8 cycles |
| Annealing          | 60°C        | 30 s  |        |
| Extension          | 72°C        | 30 s  |        |
| Final extension    | 72°C        | 5 min | 1      |
| Hold               | 4°C         | forever |       |

23. Following PCR3 the average library size will be larger and primer dimers may form at ~120 bp. We use a lower ratio of 0.75 x SPRIselect Beads to ensure the exclusion of DNA products below ~200 bp.
   a. For a 20 µL PCR3 reaction add 15 µL SPRIselect Beads.
   b. Perform the remaining bead clean steps as previously detailed in step 17b–d.
   c. Elute in 18 µL or PCR water. Store at 4°C until ready to quantify and sequence.

Note: This elution volume was selected so that the libraries we created would be dilute enough to run on a high sensitivity (hsDNA) chip on an Agilent Bioanalyzer without the need for additional dilution while being detectable using a Qubit dsDNA High Sensitivity kit (Invitrogen, Cat# Q32854). You may find your libraries need to be more dilute or more concentrated at this step for ideal quantification. We found best results for the BA trace if samples are loaded in a range of 2–6 ng/µL.

△ CRITICAL: It is essential that in the final elution step absolutely no beads are aspirated since the elute will be used for quantification and sequencing. Retention of paramagnetic beads in your eluted library will disrupt proper size quantification using a hsDNA chip on an Agilent Bioanalyzer and will necessitate a repeat with a new quantification chip.

DNA library quantification and sequencing

○ Timing: 1 h

Quantify targeted amplified libraries from each plate to determine required dilution for sequencing.
24. Quantify the libraries using Qubit dsDNA HS Assay Kit (Invitrogen, Cat# Q32854) following the manufacturer’s instructions using 1–2 µL of PCR 3 DNA library.

25. Check library size distributions and specific target amplification on a microcapillary array, such as an Agilent Bioanalyzer (High Sensitivity DNA Kit, Agilent, Cat# 5067-4626), TapeStation (High Sensitivity D1000 Screen Tape and Re-agents, Agilent, Cat# 5067-5583, and Cat# 5067-5584) or Fragment Analyzer (High Sensitivity Small Fragment Kit (1–1,500 bp), Advanced Analytical, Cat# DNF-477-0500)

**Note:** For this protocol we used an Agilent Bioanalyzer for peak size determination. Figure 6 shows a sample plot of a library within the correct range of 300–1000 bps. Also indicated are primer dimer peaks and PCR bubbles that indicate a need for reconditioning cycles of PCR to remedy.

26. Combining the library peak size with the ng/µL concentration from the Qubit, calculate the nanomolar concentration of DNA in each library using the following conversion formula:

\[
\text{Nanomolar value} = \left( \frac{\text{concentration in ng/µL}}{660 \times \text{Avg basepair size}} \right) \times 10^4
\]

27. Sequence the libraries with an Illumina NextGen Sequencer.
   a. Dilute and denature libraries to flow cell loading concentrations according to manufacturer’s instructions (Illumina Denature and Dilute Libraries Guide)
   b. This protocol used a NextSeq500 with a paired-end read setting (75 bp) in the following sequencing configuration:

   - Read 1: 19 bp (reads 6 bp well barcode, 10 bp UMI, 3 poly T)
   - Read 2: 67 bp (reads gene specific sequence)
   - Index 1: 6 bp (reads 6 bp RPI-X plate identifier index sequence)
   - Index 2: 0 bp (not used in this protocol)

28. Bioinformatically process the targeted sequencing data generated by qtSEQ library prep to count matrixes for analysis (Figure 7). Add in the indexed protein information from the flow data by matching well origin to get the protein and gene count matrix.

**Note:** To assign identity/align fastq files, we used a custom genome consisting of the targeted amplicon sequences identified during primer design. This method gives very similar results to whole genome alignment at a fraction of the computational cost.

**EXPECTED OUTCOMES**

Following the library preparation steps you should generate targeted amplified libraries with a final concentration of ~4–15 nM. Library size should range from 300–1000 bp with most amplicons clustered around 500 bp without evidence of primer dimer or PCR bubble (Figure 6). If using a NextSeq500, a good sequencing run should have most bases in read 3 with a Q30>85%. However, as we are only seeking to count molecules with this protocol (rather than look for variants) some mismatches in the gene sequence (but not the barcode or UMI) can be tolerated and we have generated usable data with lower Q30 scores. We find the median number of reads/cell to be in the range 10^5 although this will vary by cell type. A depth of even 10^4 will often be enough to detect most genes due to the targeted nature of the protocol. There remains a strong linear correlation to UMI count and read depth. For cell quality control we typically employ
a cutoff of >85 UMI counts and >25 features in addition to the expression of a few key marker genes specific to the sorted cell subset (e.g., \textit{Cd3d} expression in T cells) to determine if a well would be used in downstream analysis. These parameters will vary depending on the cell subset being investigated.
LIMITATIONS

qtSEQ was designed to provide hypothesis driven transcriptional information from immune cell populations that are not well represented in randomly seeded droplet based scRNA-seq protocols. While this protocol uses a plate-based sorting method, which lowers throughput (hundreds of cells compared to thousands for droplet-based approaches), it does provide more control over which cells are allocated for sequencing and offers indexed protein data.

qtSEQ uses a 3’ targeted method to amplify 500 genes of interest via a series of nested PCR reactions. While this increases the sensitivity of gene transcript detection and ensures access to key immune cell genes, the targeted approach has some drawbacks. Our gene panel was handpicked from the literature to focus on genes that impact immune cell contact, secretory molecules, and transcriptional programming, however, this list is far from exhaustive. Therefore, it is possible to unknowingly exclude relevant genes from analysis. Additionally, some single cell transcriptional analysis tools designed for global transcriptional analysis must be used with caution (such as GSEA) and others (such as scRNA velocity) cannot be used at all.

Overall qtSEQ is better suited for specific hypothesis driven analysis of phenotypically defined immune cells rather than exploratory analysis or subset discovery.

TROUBLESHOOTING

Problem 1
No library is detected during DNA library quantification after PCR3 (step 24–25).

Potential solution
Ensure your fluorescent-activated cell sorting alignment is correct and that droplets are hitting the bottom of each 384 well (step 10). The protocol can be adapted into a 96-well format if the cell sorter used is not easily adaptable to 384 wells. Additionally, over-drying of AMPureXP beads can result in library loss as DNA does not properly elute off dried beads (step 14, 17, 20, 23). Ensure that bead pellets are eluted before they completely dry out and start cracking.

Ensure that all primers used in PCR1 and PCR2 are producing product using bulk RNA methodologies prior to application to single cell library preparation. Our approach to testing primer quality was to bulk sort 50 cells per well from various cell types of interest spanning the expected expression of our targeted genes. Then using the final sequencing read out to judge primer quality. Additional wells can be sorted as 10 cells 3 cells and 1 cell to determine the single cell sensitivity of a particular assay. Inconsistent cDNA amplification by targeted primers will result in failure to produce a viable library.
Failure of any PCR step can result in no end product. Utilize the saved aliquots from each PCR step to redo PCR1–3 ensuring the correct primers and reagents are used. Quantification of library quality after each step may be employed to help with troubleshooting and determining the optimal number of PCR cycles. The library quality of the unamplified cDNA, PCR1 and PCR2 product is not detectable using a trace or Qubit. It would be possible to use qPCR assays (several qPCR based NGS quantity assays are commercially available) to give an indication of library quantity after each PCR stage of the protocol. It should be noted that before PCR1 amplification any library lost to use for sample quantification will be a permanent reduction in the total library diversity.

Problem 2
There is a visible PCR bubble on bioanalyzer plot (step 24–25 demonstrated in Figure 6).

Potential solution
If there is a large PCR “product” above 2000 bp it is likely the result of a PCR “bubble” forming, which is an artifact that occurs when PCR3 has run out of primer. Denatured sequences will anneal to each other at adapter sequences but not the gene specific sequence between, generating a larger dsDNA product that runs slowly on a gel, giving the appearance of a larger basepair size. A reconditioning PCR step will usually resolve the issue. Repeat 1 cycle of PCR3 on the PCR3 product including the initial denaturation step (step 22).

Running out of primer in PCR3 could also be an indication of overamplification. If there is enough library to consume all available PCR3 primer, consider reducing the number of PCR3 cycles, or using less PCR2 library volume in the PCR3 reaction (step 21).

PCR3 eluate retaining AMpureXP beads can clog the microfluidic channels in the dsDNA chip. This results in slower movement of DNA bands or sometimes halting entirely. Place the PCR3 product back onto a magnetic stand for 5 min and carefully remove the eluate leaving behind any remaining beads (step 23).

Problem 3
There is a dominance of a 30–200 bp peak on the bioanalyzer plot (step 24–25 demonstrated in Figure 6).

Potential solution
The peaks at the lowest range of the bioanalyzer plot consist of primer dimer which can bind to the flow cell and lower the quality of the sequencing run. Significant presence of primer dimer is likely an indication of a low template to primer ratio to start the PCR3 reaction which would favour the formation of primer dimer instead of amplicons. Consider increasing the number of cycles in PCR2 (step 19) by 2–3 to raise the baseline template concentration being added to PCR3.

Problem 4
There is a low percentage of clusters passing filter during sequencing (step 27).

Potential solution
A low percentage of passing clusters can indicate too much library was added to the flow cell causing clusters to overlap. Consider loading less library on a new chip. Take into consideration the length of the amplicons for bridge amplification. We found that using a NextSeq500 with Illumina v2.5 flow cells, amplicons around 500 bp in length were best suited to loading 100 μL of library opposed to the Illumina Denature Guide suggesting 117 μL of library. Alternatively, poor-quality library could have been loaded onto the flow cell. If there are major peaks of primer dimer when visually quantifying peaks, these could be binding the flow cell and generating clusters that lack any actual data. Ensure that PCR dimer peaks are removed using AMpureXP bead cleaning prior to sequencing.
Problem 5
After bioinformatic processing you have significant detection of gene counts in the empty control wells (step 28).

Potential solution
We recommend including 3–4 no sort or negative control wells per plate (with different plates having different no sort wells) (step 10). If after bioinformatic processing of sequencing data the control wells have significant gene counts, it likely indicates contamination of well barcodes occurred during the RT master mix addition (step 9). Dispose of all reagents (even those not used in the RT master mix), wipe down all equipment used with ethanol and start over again ensuring high standards of single cell aseptic technique.

An additional cause of this problem may be overamplification of the targeted libraries especially during PCR1 when the targeted gene transcripts are not yet enriched. If the PCR reaction has a primer to amplicon ratio that is too low, then amplicons may start annealing to each other and over-writing barcode sequences. Expression of one or two highly expressed gene transcripts in all wells is a particular indication of this effect. Redo the experiment with a significantly lower number of PCR1 cycles.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to Michael McHeyzer-Williams: mcheyzer@scripps.edu

Materials availability
Primers used for library formation and targeted gene-specific primers sequences are available in Tables S1 and S2.

Data and code availability
The code used to process qtSEQ sequencing results is not included in this article as it is adapted from the method employed by Hashimshony et al., 2016. Scripts specific to qtSEQ are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101064.

ACKNOWLEDGMENTS
Graphical abstract was created using BioRender.com. This work was supported by the US National Institutes of Health (AI047231, AI040215 and AI071182) and Bill & Melinda Gates Foundation (BMGF OPP1154835) to M.G.M.-W.

AUTHOR CONTRIBUTIONS
A.G.S., B.W.H., L.M.W., and M.M.W. devised and developed the method used to establish the protocol; C.R.D., K.B.M., A.M.R., and K.D. contributed to methodology refinement, protocol update, and utility.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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