Takara Bio USA, Inc.

Shasta™ Total RNA-Seq Kit - 2 Chip User Manual for the Shasta Single Cell System

Cat. No. 640288
for Shasta CELLSTUDIO™ v1.0.1 Software
(052925)

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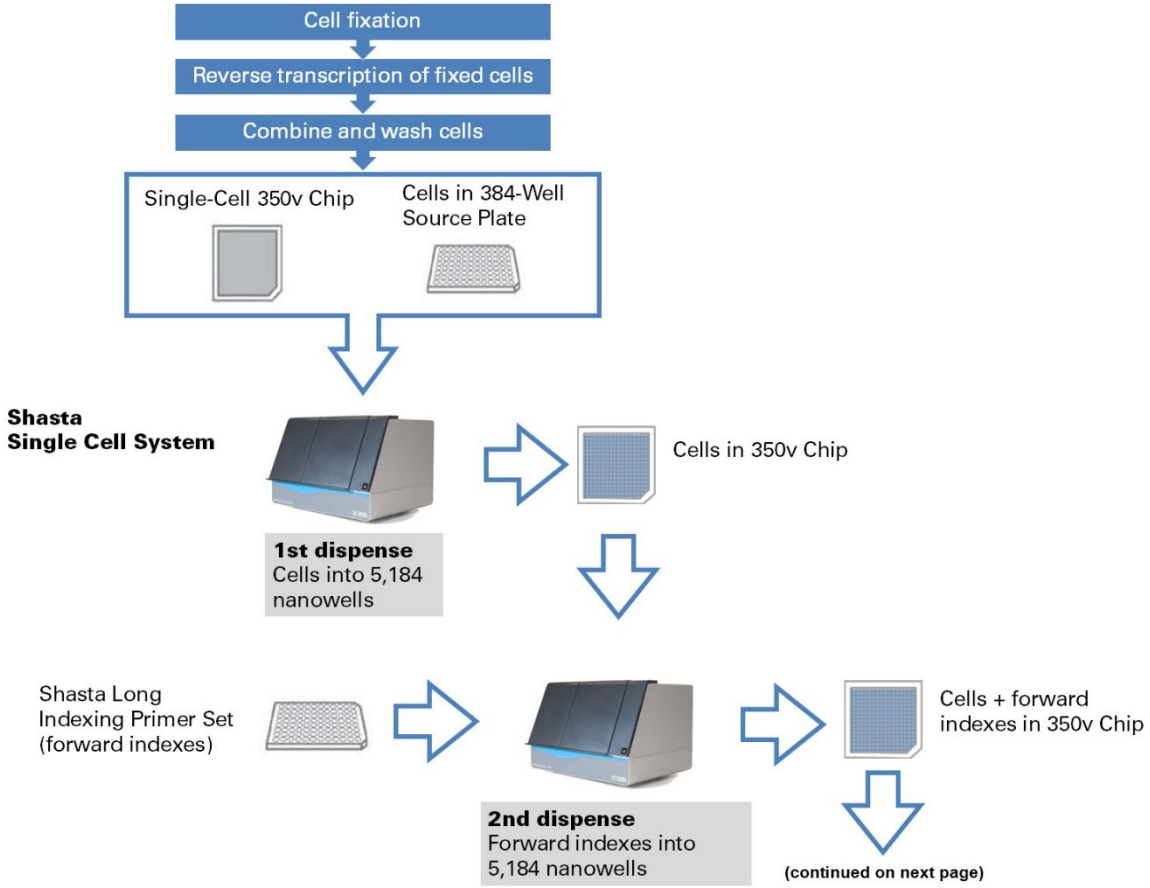
I. Introduction

High-throughput full gene-body analysis of single cells

The **Shasta Total RNA-Seq Kit - 2 Chip** (Cat. No. 640288) protocol enables full-gene body transcriptome analysis of up to 100,000 single cells using the Shasta Single Cell System (Cat. No. 640282). By leveraging the capabilities of the Shasta system along with SMART® (**S**witching **M**echanism **a**t 5′ end of **R**NA **T**emplate) technology, this protocol provides an efficient, cost-effective solution for high-throughput full-gene body transcriptome analysis of up to 100,000 single cells per experiment.

The workflow (Figure 1, below) begins with cell fixation. The fixed individual cells then function as the container for the random priming-based *in situ* reverse transcription, upon which cDNA is synthesized and a sample-specific barcode is added. Up to 96 sample-specific barcodes can be used in a single experiment. The cells are then combined, washed, and dispensed into the 5,184 nanowells of the Single-Cell 350v Chip using the Shasta system and Shasta CELLSTUDIO Software.

After cell dispense, on-chip PCR is carried out by the addition of 72 different i5 indexing primers (by row), 72 different i7 indexing primers (by column), and PCR master mix using the Shasta system. This on-chip PCR lyses cells and incorporates i5 and i7 indexes into the final library construct. The resulting libraries are extracted from the chip and purified. Ribosomal cDNA (cDNA fragments originating from rRNA molecules) are then removed using ZapR® technology, which features R-Probes targeting ribosomal RNA and mitochondrial rRNA sequences. The cleaned-up cDNA libraries are then amplified and purified again. After validation steps, the libraries are ready for sequencing on Illumina® platforms.



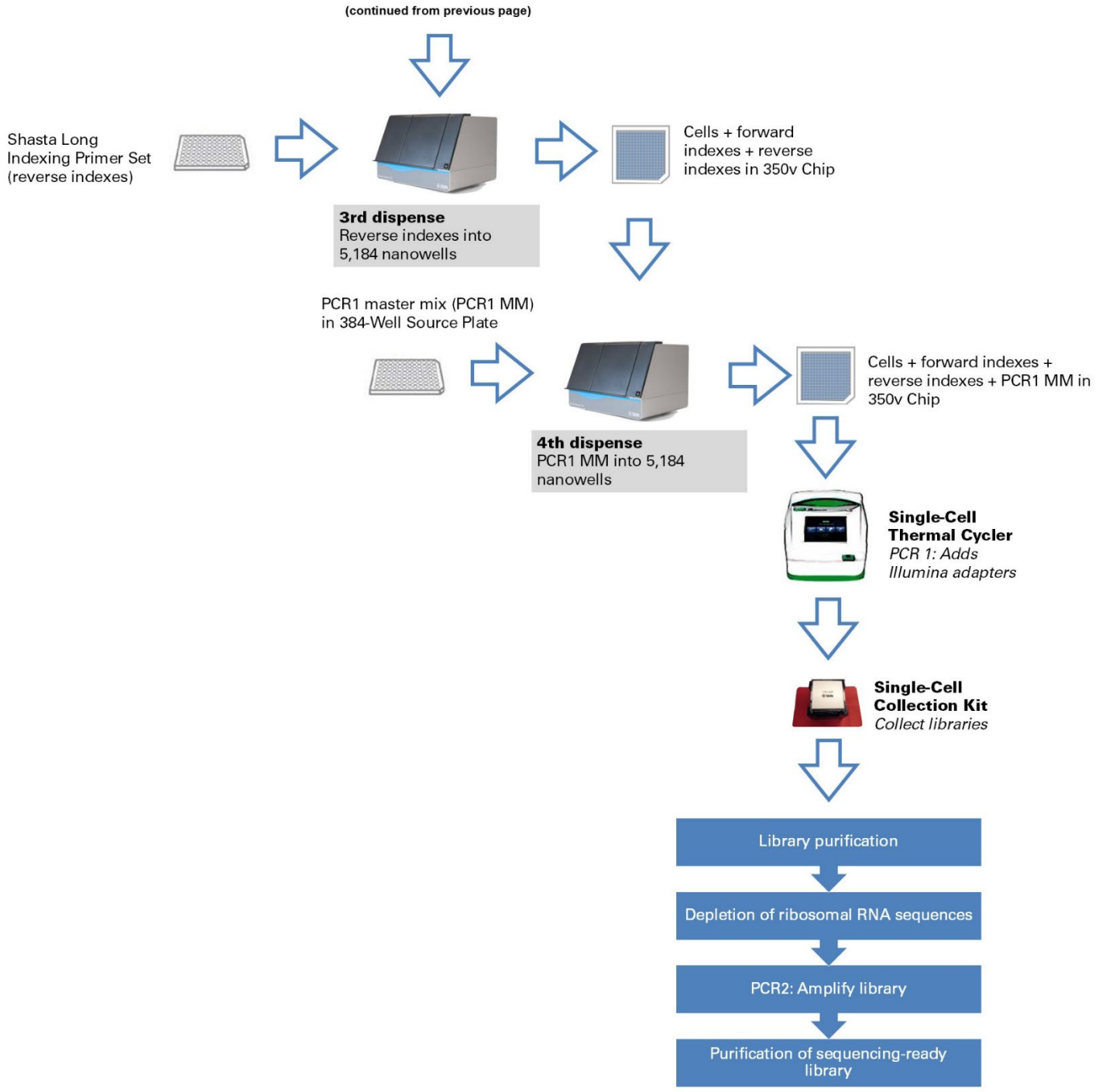


Figure 1. Complete Shasta Total RNA-Seq Kit workflow.

Figure 2 on the next page shows an illustrated breakdown of the protocols that comprise the workflow as presented in this user manual.

Data analysis should be performed using Cogent™ NGS Analysis Pipeline (CogentAP), available for free from our website. For more information on demultiplexing and data analysis, refer to Section VI.

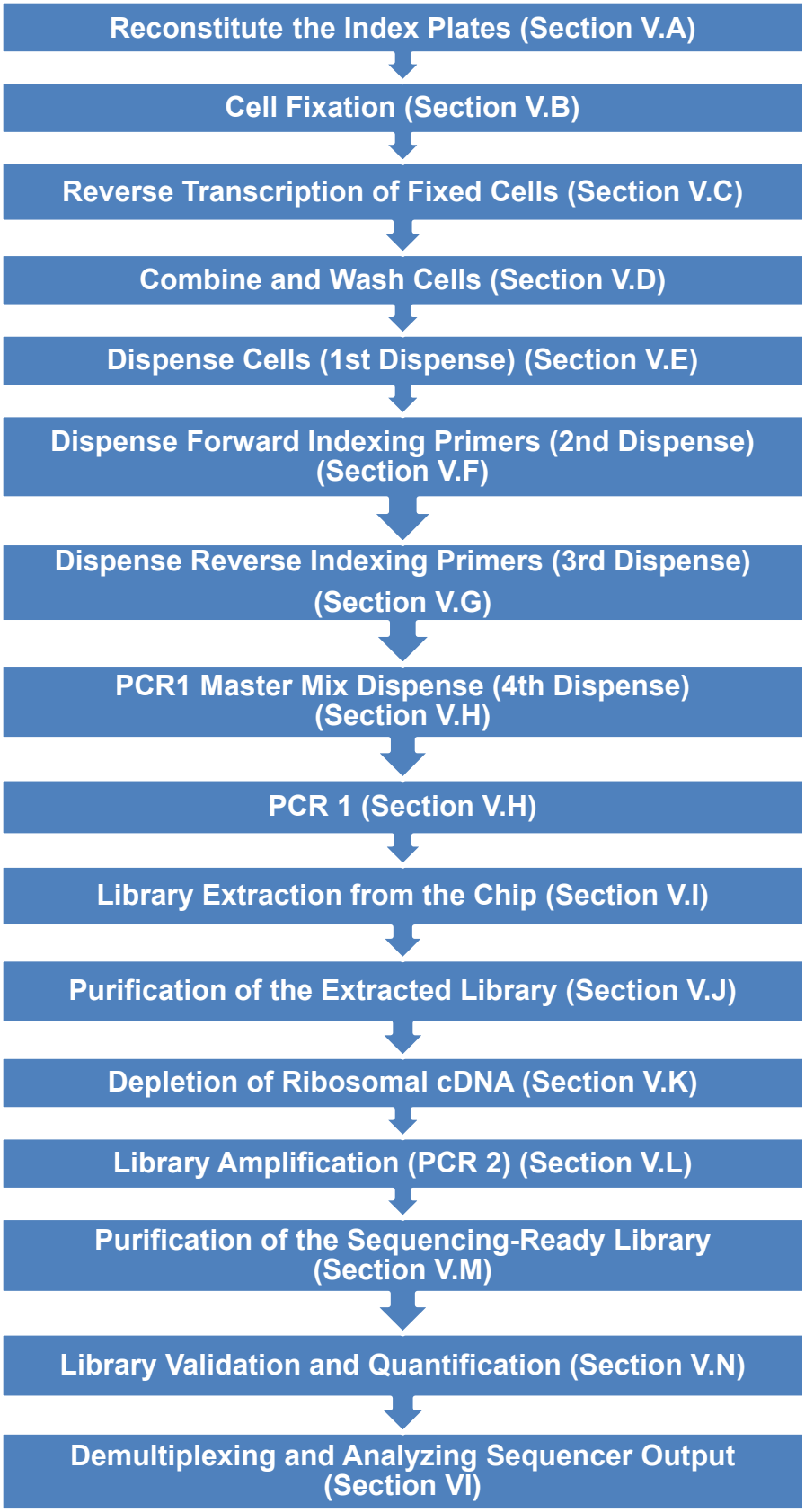


Figure 2. Protocols in the Shasta Total RNA-Seq workflow.

II. List of Components

The Shasta Total RNA-Seq Kit workflow requires use of a Shasta Single Cell System (Takara Bio, Cat. No. 640282) and the Shasta Total RNA-Seq Kit - 2 Chip (Cat. No. 640288).

Table 1. Shasta Total RNA-Seq Kit - 2 Chip components.

Shasta Total RNA-Seq Kit - 2 Chip	**640288 (2 chip)***
Shasta Total RNA-Seq Reagents (Cat. No. 640289)†	**1**
Package 1 (Store at –70°C)	
R-Probes	2 x 7.5 µl
Package 2 (Store at –20°C)	
Second Diluent (100X)	2 x 250 µl
BSA (1%)	2 x 250 µl
RNase Inhibitor (40 U/µl)	2 x 125 µl
Fixation Solution	2 x 1 ml
Quenching Solution	2 x 12.5 ml
Shasta Total RNA Reverse Transcriptase (200 U/µl)	2 x 60 µl
Permeabilization Buffer	2 x 25 µl
RT Buffer (5X)	2 x 500 µl
Fragmentation Buffer (5X)	2 x 500 µl
ZapR Buffer (10X)	2 x 15 µl
ZapR Enzyme	2 x 15 µl
SeqAmp™ DNA Polymerase	2 x 65 µl
SeqAmp CB PCR Buffer (2X)	2 x 10 ml
PCR2 Primers	2 x 10 µl
Nuclease-Free Water	2 x 10 ml
Package 3 (Store at room temperature)	
TSO-index plate (0.05 nmol /well) ‡	2
Shasta Long Indexing Primer Set - A (Cat. No. 640283) (Store at room temperature)	**2**
Shasta Long Indexing Primer Set - A (0.05 nmol /well) ‡§	2 x 1
Plate Sealing Film	2 x 1
Single-Cell 350v Chip (Cat. No. 640019) (Store at room temperature)	**2**
Single-Cell 350v Chip, 5,184 Wells, 2.2 mm (350 nl)	2 x 1
Single-Cell Collection Kit (Cat. No. 640212) (Store at room temperature)	**2**
Single-Cell Collection Fixture	2 x 1
Collection Tube (2.0 ml)	2 x 2
Collection Film	2 x 1
Single-Cell Loading Kit (Cat. No. 640206) (Store at room temperature)	**2**
Chip Freezing Film	2 x 1
RC Film	2 x 6
Blotting Paper	2 x 7

*Enough reagents are included for two runs of the protocol described in this document.

†Not sold separately.

‡Index sequences are listed in Appendix D.

§Each well of the Shasta Long Indexing Index Primer Set - A contains a specific forward or reverse primer.

III. Additional Materials Required (Not Provided)

Required general lab supplies

- Personal protective equipment (PPE): powder-free gloves, safety glasses, lab coat, sleeve protectors, etc.
- Three (3) 384-well plates and seals are needed **per chip**: Nanodispenser 384-Well Source Plate and Seals (Takara Bio, 20/pack, Cat. No. 640018; 120/pack, Cat. No. 640037)
- Nanodispenser Chip Holder (Cat. No. 640008); two chip holders are included with the Shasta system, additional chip holders can be ordered separately
- 384-Well Plate Seal Applicator, included with the Shasta system
- Film Sealing Roller for PCR Plates ("film sealing roller") (Bio-Rad, Cat. No. MSR0001)
- Microseal 'B' PCR Plate Sealing Film (Bio-Rad, Cat. No. MSB1001)
- PCR thermal cycler compatible with 0.2 ml tubes
- Minicentrifuges for 1.5 ml tubes and 0.2 ml tubes or strips
- 384-well plate orbital shaker with 3 mm mixing orbit
- Vortex mixer
- Centrifuges, rotors, and adapters. Recommended:
  - Eppendorf 5810R with Microplate Buckets (VWR, Cat. No. 53513-874), ≥2,600*g*, room temperature and 4°C operation
  - Kubota 3740 with rotor SF-240 for cell preparation
- Nuclease-free, non-stick 0.2 ml PCR tubes
- Nuclease-free LoBind 1.5 ml microcentrifuge tubes (Eppendorf)
- Conical tubes, 50 ml and 15 ml sizes
- 25 ml reagent reservoirs (Thermo Scientific, Cat. No. 8093-11)
- MacroTube 5ml Non-Sterile Snap-Cap Centrifuge Tube (MTC Bio, SKU C2500)
- Single-channel pipettes: 2 µl, 10 µl, 20 µl, 200 µl, and 1,000 µl
- Multi-channel pipettes: 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Wide-bore pipette tips: 200 µl and 1,000 µl
- Serological pipets and controller
- Nuclease-decontamination solution
- Exhaust hood system with UV

For cell processing (Section V.B)

- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4; Thermo Fisher Scientific, Cat. No. 10010-023)
- Appropriate cell culture medium*
- TrypLE Express (Thermo Fisher Scientific, Cat. No. 12604-021)*

*Required only if performing cell culture, frozen cell recovery, or dissociation of adherent cells.

For cell counting (Section V.D)

- Recommended: Countess 3 FL Automated Cell Counter (ThermoFisher, Cat. No. AMQAF2000), Moxi Z Mini Automated Cell Counter Kit, U.S. Version (ORFLO, Cat. No. MXZ001) with Moxi Z Cell Count Cassettes, Type M (25 pack; ORFLO, Cat. No. MXC001), or Moxi Z Cell Count Cassettes, Type S (25 pack; ORFLO, Cat. No. MXC002)

NOTES:
- Refer to the cell counter's user guide for guidance.
- Alternatively, you may use a hemocytometer or any preferred cell counter with demonstrated, accurate cell counting.

For library purification (Section V.K and Section V.N)

- NucleoMag NGS Clean-up and Size Select is preferred (Takara Bio; 5 ml size: Cat. No. 744970.5; 50 ml size: Cat. No. 744970.50; 500 ml size: Cat. No. 744970.500). The AMPure XP PCR purification kit (Beckman Coulter; 5 ml size: Cat. No. A63880; 60 ml size: Cat. No. A63881) is an appropriate substitute.

NOTES:
- The kit has been specifically validated with the beads listed above. Substitutions may lead to unexpected results.
- Beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Using individual tubes for each experiment allows tubes to be equilibrated quickly to room temperature (~30 min) and avoids bead contamination.

- 100% ethanol (molecular biology grade)
- Magnetic separation device for small volumes—used to purify amplified libraries.
  - For 8-tube strips: SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
  - Magnetic Separator compatible with 1.5 ml tubes

For library validation and quantification (Section V.O)

- Agilent 2100 Bioanalyzer instrument or similar
- Agilent High Sensitivity DNA Kit (110 samples; Agilent Technologies, Part No. 5067-4626)
- Qubit 2.0 Fluorometer (Thermo Fisher Scientific) or similar
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854)
- Library Quantification Kit (Takara Bio, Cat No. 638324) and DNA Standards for Library Quantification (Takara Bio, Cat No. 638325)

IV. General Considerations

A. Sample Considerations

- For best results, start with a sample that has >2 x 10^6 cells.
- For samples with lower cell numbers, please see Section V.B.3 for details on determining the cell numbers needed for this protocol.

B. Protocol Best Practices

- The kit contains two sets of reagents and was designed for performing two tests (one chip each). Use one set of reagents and consumables each time and dispose of leftovers. Do not reuse the leftovers.
- Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease decontamination solution, water, and ethanol. Change gloves routinely.
- Minimize the exposure time of unsealed Single-Cell 350v Chips, reservoirs, reagents, and other consumables to the open air. Wearing laboratory sleeve protectors may reduce the likelihood of introducing contaminants from exposed hands and arms.
- The assay is very sensitive to variations in pipette volume. Please make sure that all pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outsides of the tips when dispensing liquids.
- All lab supplies related to cDNA synthesis need to be stored in a closed, DNA-free cabinet. Ideally, reagents for cDNA synthesis should be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Use nuclease-free, molecular biology- or PCR-grade reagents to set up all molecular biology reactions.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- DO NOT change the amount or concentration of any of the components in the reactions; they have been carefully optimized for the Shasta Total RNA-Seq workflow.
- Because of the large volume or viscosity of mixtures subject to purification using NucleoMag NGS Beads or AMPure beads, each round of purification requires a very strong magnet. Place the samples on the magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant.
- UV-treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use.

C. Safety

Refer to safety guidelines in the user manuals for all equipment used in this protocol.

WARNING: Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease decontamination solution, water, and ethanol. Change gloves routinely.

WARNING: Use of equipment and reagents for cell preparation and isolation with the Shasta Single Cell System may cause exposure to toxic or biohazardous chemicals, thereby presenting a hazard. Always wear appropriate personal protective equipment (PPE), which should, at minimum, include gloves, eye protection, and a lab coat, when handling equipment and reagents and operating instruments.

Note and heed all warning labels on the instruments used in this protocol.

D. Shasta System Application Notes

Refer to the Shasta Single Cell System User Manual for full details. Included below are general reminders.

- All dispensing steps in the Shasta Single Cell System stage module should be performed with a 384-Well Source Plate oriented with the A1 well positioned at the top-right corner of the 384-well plate nest (Figure 3). The source plate must be fully seated. This may be accomplished by pushing the source plate down after it has been placed on the plate nest.
- All dispensing steps in the Shasta Single Cell System stage module should be performed with the Single-Cell 350v Chip oriented with the chamfered (beveled) corner positioned towards the bottom-right corner of the chip nest (Figure 3).

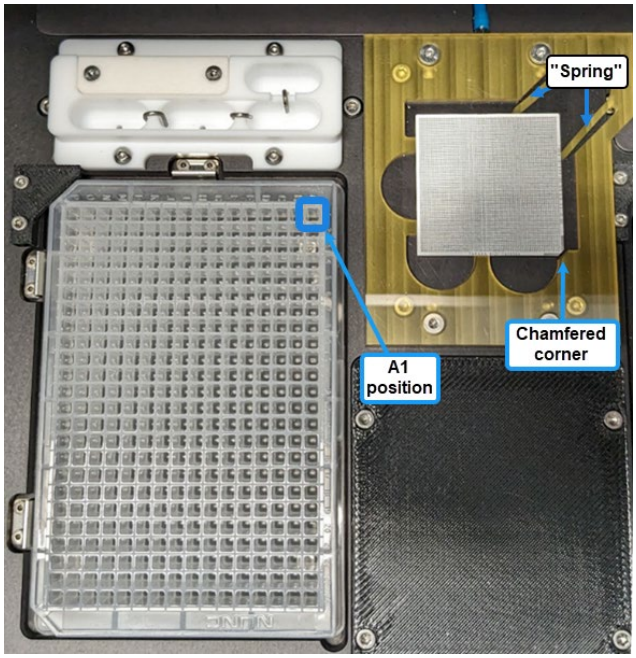


Figure 3. Shasta Single Cell System stage module. **(Left)** 384-Well Source Plate nest, with the "A1" well of the plate highlighted. **(Right)** Single-Cell chip nest.

E. Single-Cell 350v Chip

Each Single-Cell 350v Chip is engraved with a unique number (Figure 4). You can use this number to link your chip images and other experimental record files.

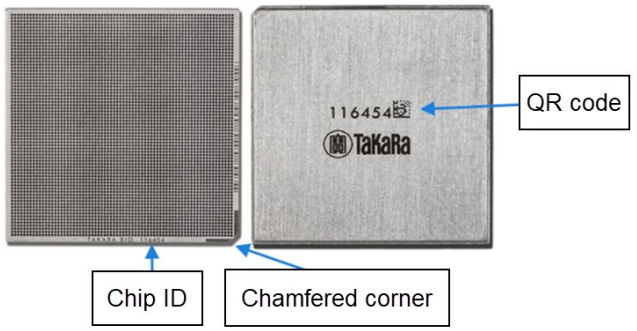


Figure 4. Single-Cell 350v Chip features. **(Left)** Top view of the Single-Cell 350v Chip. Note the chamfered (beveled) corner at the bottom right. The "TaKaRa" logo and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. **(Right)** Bottom view of the Single-Cell 350v Chip. The chip ID is also engraved on the reverse side of the chip with a corresponding QR code that can be scanned by a barcode reader, allowing for the chip ID to be easily entered into the software.

F. Software

The instructions in this manual are written for use with Shasta CELLSTUDIO v1.0 and CellSelect v1.0 Software. Please refer to the Shasta Single Cell System User Manual and the Shasta CellSelect Software User Manual for more detailed information.

V. Procedure

For all on-chip protocols (Section V.F–V.I), the post-dispense instructions documented in this manual are also displayed for your convenience within CELLSTUDIO software.

It is recommended that you perform the workflow at least once with the manual, but the embedded instructions can be used on subsequent runs.

For more information about using the Shasta system, refer to the Shasta Single Cell System User Manual.

A. Protocol: Reconstitute the Index Plates

These steps should be done prior to cell preparation so that there is sufficient time for the oligos to fully reconstitute before use. Do NOT vortex the plate at any step.

NOTES:

- If dispensing Index 1 and Index 2 on the second day, the Shasta Long Indexing Primer Set - A can be reconstituted on Day 2, before index dispensing.
- If the Shasta Long Indexing Primer Set - A plate is reconstituted on Day 1, it can be stored at 4°C overnight and used on Day 2.
- Barcode sequences for both plates are listed in Appendix D.

1. Centrifuge the TSO-index plate (0.05 nmol/well) plate at 3,000*g* at room temperature for 10 min.
2. Add 7 µl of Nuclease-Free Water to each well of the TSO-index plate using a multichannel pipette to pierce the sealing foil.

   IMPORTANT: Do not peel off the foil seal.

3. Reseal the plate with an adhesive sealing film on top of the pierced sealing foil and centrifuge at 1,000*g* at room temperature for 1 min.
4. Shake the plate at 700 rpm at room temperature for 10 min and then leave on ice until use.
5. Centrifuge the Shasta Long Indexing Primer Set - A plate at 3,000*g* at room temperature for 10 min.
6. In a 15 ml conical tube, prepare the rehydration buffer using the following recipe.

Rehydration buffer:

5,000 µl	(5 ml)	Nuclease-Free Water
4,000 µl	(4 ml)	SeqAmp CB PCR Buffer (2X)
9,000 µl	**(9 ml)**	**Total volume per plate**

7. Vortex the rehydration buffer then transfer to a reagent reservoir.
8. Use a multichannel pipette to add 50 µl of rehydration buffer into each well as shown in the 384-well plate layout in Figure 5 (72 pink wells + 72 blue wells = 144 total wells).

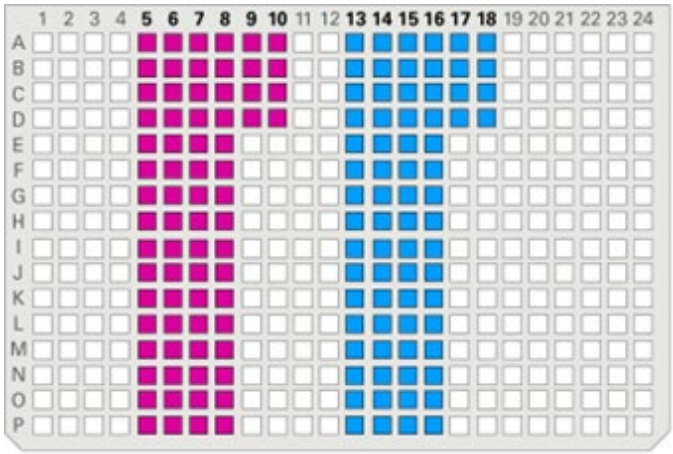


Figure 5. Rehydration buffer-dispense pattern for the Shasta Long Indexing Primer Set - A plate.

9. Seal the plate with sealing film and centrifuge the plate at 1,000*g* at room temperature for 1 min.
10. Shake at 700 rpm at room temperature for 10 min and leave on ice until use.

B. Protocol: Cell Fixation

In this protocol, cells are fixed for the in situ reverse transcription (RT) reaction. Prepare single-cell suspensions and fix samples following Procedure 1 (Section V.B.3). Preparation of single-cell suspensions may vary depending on the sample types and needs of the user.

Here are some examples:

- If starting from a single-cell suspension sample, such as K-562 cells, use Procedure 1 (Section V.B.3)
- If starting from an adherent culture, such as 3T3 cells, use Procedure 2 (Section V.B.4) to prepare single-cell suspensions, and then follow Procedure 1 for cell fixation.
- If starting from frozen cells in cryovials, such as frozen PBMCs, use Procedure 3 (Section V.B.5) to process cells and prepare single-cell suspensions, and then follow Procedure 1 for cell fixation.

1. Cell Handling notes

- This protocol requires several milliliters of healthy cell suspensions with a cell density ≥0.7 x 10^6 cells/ml.
- Keep cells at 37°C with 5% CO_2 in a cell culture incubator when not performing manipulations.
- Perform all wash steps in an exhaust UV hood. Avoid exposing cell cultures to ambient air to reduce the likelihood of contamination.
- Treat cells gently; do not vortex. Minimize bubble formation and frothing.
- Wear nitrile or powder-free gloves to reduce imaging artifacts.
- Centrifugation speed and time may need to be modified for different cell types.

2. Before You Start

- Set the chip centrifuge to 4°C.
- Chill PBS on ice.
- Thaw RNase Inhibitor (40 U/µl), and Fixation Solution on ice. Thaw the Quenching Solution at room temperature. Once it is thawed, put it on ice.

3. Procedure 1: Fix Cells in Suspension

1. Transfer ~3 ml of suspension cells (≥ 2 x 10^6 cells) to a fresh 5 ml centrifuge tube.

   NOTE: 2 x 10^6 cells are recommended. When handling more than 1 sample, the required cell number can be lower for each sample. The goal is to make sure the whole RT plate has 0.5–1 x 10^6 cells so that sufficient cells can be recovered after RT for downstream cell dispensing. If one sample has a lower cell number, more cells from other samples will be needed to make sure the whole RT plate has 0.5–1 x 10^6 cells.

2. Centrifuge at 200*g* at 4°C for 5 min.

   NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. Examples:

   – Regular to large cells, such as K-562 cells: 150–200*g* for 5 min.
   – PBMCs or smaller cells: 300–500*g* for 5 min.

3. Carefully remove 2.9 ml of the supernatant then tap the tube bottom to completely resuspend the pellet.
4. Add 5 ml of cold PBS to wash the cells. Filter the cells with a 40 µm cell strainer if there are big cell clumps.
5. Centrifuge again at 200*g* at 4°C for 5 min.
6. Carefully remove all the supernatant without touching the pellet.
7. Tap the tube bottom to completely loosen the pellet then add 200 µl of cold PBS to cells.

   NOTE: Check if the cells are clean. If the sample has many clumps or cell debris, repeat Steps 4–7.

8. Transfer 150 µl of the resuspended cells to a clean 5 ml tube. Add 50 µl of cold Fixation Solution to the cell solution. Gently pipette up and down 3–4 times with a wide-bore tip. Leave on ice for 15 min.
9. Add 1 ml of cold Quenching Solution to the cell solution. Mix by inverting the tube 2–3 times.
10. Centrifuge at 300*g* at 4°C for 5 min.

NOTE: 1.5X centrifugation speed is used on fixed cells. Examples:

- Regular to large cells, such as K-562 cells: 300g for 5 min.
- PBMCs or similarly sized cells: 450–750*g* for 3 min.

11. Carefully remove 1.1 ml of supernatant. Tap the tube bottom to completely resuspend the pellet.
12. Add 0.5 ml of cold PBS to the cells.

NOTE: If handling one sample, a minimum of 0.5 ml is required for resuspending the cells. When handling multiple samples, the minimum resuspension volume for each sample will be reduced based on the sample number. For example, when handling 2 samples, the minimum resuspension volume for each sample will be 250 µl. When handling 10 samples, the minimum resuspension volume for each sample will be 50 µl. This minimum resuspension volume does not include the volume for cell counting. Please always include extra volumes for cell counting depending on the cell counter requirements.

13. Count cells to make sure that the concentration is 1.5–2.5 x 10^6 cells/ml in 500 µl of solution.

NOTE: This cell concentration will allow the whole RT plate to have 0.5–1 x 10^6 cells. The goal is to recover sufficient cells after RT for the downstream cell dispensing step. When handling multiple samples, if the cell concentration of some samples is lower than 1.5 x 10^6 cells/ml after fixation, more cells will be needed from other samples to ensure the total cell number of the RT plate is 0.5–1 x 10^6 cells.

If the cell concentration is higher than 2.5 x 10^6 cells/ml, please use PBS to dilute the cells to the above concentration range. Please use the Shasta Total RNA-Seq Experiment Planner for guidance.

14. Proceed immediately to the next protocol (Section V.C, "Protocol: Reverse Transcription of Fixed Cells"). Keep the prepared cell suspension on ice.

4. Procedure 2: Preparing Adherent Cell Types from a 75 cm Culture Flask*

*Adjust volumes accordingly for different-sized flasks.

1. Carefully remove culture media from a 75 cm flask containing adherent cells using a serological pipette.
2. Add 10 ml of 1X PBS prewarmed to 37°C by dispensing the PBS on the side walls of the flask. DO NOT pour PBS directly onto cells.
3. Wash the cells by tilting the flask gently. DO NOT mix by pipetting.
4. Remove the PBS from the cells using a serological pipette.
5. Add 3 ml of TrypLE Express prewarmed to 37°C to the flask to dissociate the cells.

IMPORTANT: The efficiency of cell dissociation from the flask surface may vary with cell type. Monitor the process visually using a microscope.

6. When cell dissociation is sufficient, neutralize the trypsinization reaction by gently adding 7 ml of complete cell media for your sample (containing 10% serum), prewarmed to 37°C. DO NOT vortex or overagitate cells.
7. Follow "Procedure 1: Fix Cells in Suspension" (Section V.B.3, above) starting from Step 1.

5. Procedure 3: Preparing frozen cells from cryovials (e.g., PBMCs)

1. Remove cryovials (e.g., PBMCs) from storage and immediately thaw at 37°C in a bead or water bath for 2–3 min until most cells are thawed, with only small ice crystals remaining.
2. Slowly transfer 1 ml of thawed cells to a 50 ml conical tube using a wide-bore pipette tip.
3. Rinse the cryovial with 1 ml of prewarmed cell-culture media (depending on cell types) and slowly add the warm media dropwise (1 drop per 5 sec) to the 50 ml conical tube while gently shaking the tube by hand.
4. Sequentially add prewarmed cell-culture media to the tube in the following steps at a rate of 1 ml/3–5 sec.

   Gently swirl the tube after each volume increment to mix and pause ~1 min before proceeding to the next addition.

   a. 2 ml
   b. 4 ml
   c. 8 ml
   d. 16 ml
5. Centrifuge the cells at 300*g* at 4°C for 10 min. Remove the supernatant completely and then tap the tube bottom to loosen the cell pellet.

   NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. Examples:
   - Regular to large cells, such as K-562 cells: 150–200*g* for 10 min.
   - PBMCs or smaller cells: 300–500*g* for 10 min.
6. Add 4 ml of cold PBS and transfer the cells to a new 5 ml centrifuge tube. Centrifuge at 300*g* at 4°C for 5 min.
7. Repeat step 6 for a second wash. Filter cells with a 40 µl cell strainer if there are clumps in the cell suspension.
8. Follow "Procedure 1: Fix Cells in Suspension" (Section V.B.3, above) starting from Step 1.

C. Protocol: Reverse Transcription of Fixed Cells

In this protocol, a reverse transcription (RT) reaction takes place inside the fixed cells, and the first barcode will be incorporated into the resulting product.

1. Required Components

- From the Shasta Total RNA-Seq Kit - 2 Chip:
  - Shasta Total RNA-Seq Reagents

  - 5X Fragmentation Buffer, RNase Inhibitor (40 U/µl), Nuclease-Free Water, Permeabilization Buffer, 5X RT Buffer, and Shasta Total RNA Reverse Transcriptase (200 U/µl)
- Reconstituted TSO plate (from Section V.A)
- One (1) 96-well plate and two (2) 96-well plate seals
- Multichannel pipette
- 8-tube strips

2. Before You Start

- Thaw 5X Fragmentation Buffer, Nuclease-Free Water, Permeabilization Buffer, 5X RT Buffer, and Shasta Total RNA Reverse Transcriptase (200 U/µl) on ice. Once thawed, keep them on ice for the remainder of the protocol.
- Preheat the thermal cycler with the following program settings for Step 5: cycler temperature at 85°C, lid temperature at 95°C, and volume at 9 µl.
- Centrifuge the reconstituted TSO plate (from Section V.A) at 1,000*g* at 4°C for 1 min.

3. Procedure

IMPORTANT: Cells will settle over time. Steps 1–4 need to be done quickly.

1. Samples are barcoded and, therefore, differentiated by the TSO index during the RT reaction. Calculate how many wells on a 96-well plate are needed for each sample based on the sample number.

   Example:

   If you're running an experiment with six samples and decide to distribute each sample to an equal number of wells on the 96-well plate, each sample would be distributed to 96 / 6 = 16 wells on the 96-well plate. Depending on the experiment design, you can choose to distribute their samples into an equal or non-equal number of wells (Figure 6).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
B	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
D	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
E	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
F	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
G	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
H	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6

Figure 6. Sample distribution in the 96-well plate using 6 samples and an equal number of sample wells.

2. In a 1.5 ml tube, mix all components in the order listed to prepare the fragmentation mix based on the well number required for each sample as calculated in Step 1.

Fragmentation mix for one well on the 96-well plate*:

5.0 µl	5X Fragmentation Buffer
5.0 µl	Fixed cells (From Section V.B)
0.6 µl	RNase Inhibitor (40 U/µl)
10.6 µl	**Total volume per well**

*This formula includes +25% for overage.

Use a 1.5 ml wide-bore pipette tip to pipette the fragmentation mix up and down 4–5 times to mix. Do NOT vortex.

NOTE: Do not let the fragmentation mix sit too long, as cells will settle.

Example:

In an experiment with six samples, create six fragmentation mixes (one for each sample) using the following calculation:

5 µl/well x 16 wells =	80.0 µl	5X Fragmentation Buffer
5 µl/well x 16 wells =	80.0 µl	Fixed cells (From Section V.B)
0.6 µl/well x 16 wells =	9.6 µl	RNase Inhibitor (40 U/µl)
16 sample wells =	**169.6 µl**	**Total volume per sample**

3. Split the fragmentation mix evenly into each tube of an 8-tube strip.

4. Using a multichannel pipette, transfer 8.5 µl of the fragmentation mix into the bottom of each well of a fresh 96-well plate to create the fragmentation cell plate (Figure 7).

NOTE: When using the multichannel pipette to transfer the fragmentation mix to the plate, insert the tips to the bottom of the 8-tube strip and pipette gently up and down 3 times to mix before transferring.

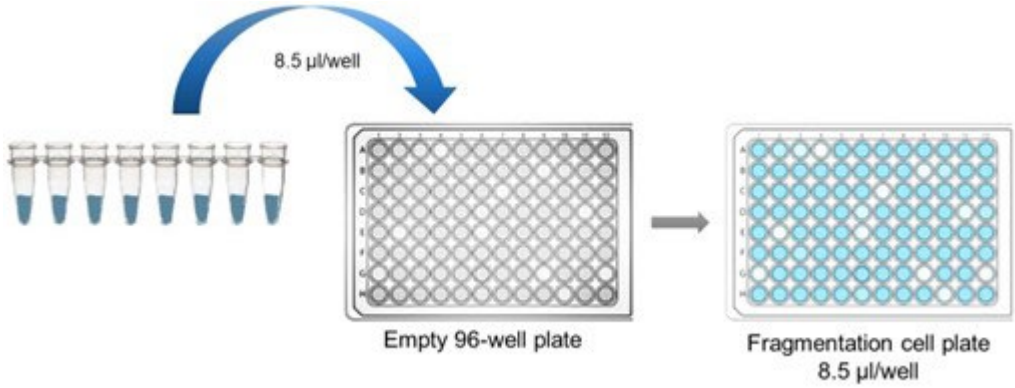


Figure 7. Creating the fragmentation cell plate. The workflow illustrates Steps 2–3.

5. Seal the plate with a sealing film and put in the preheated thermocycler at 85°C for 6 min. At the end of the 6 min, immediately put the plate on ice.
6. Preheat the thermocycler according to the RT program in Step 13: cycler temperature at 10°C, lid temperature at 55°C, and volume at 20 µl.

7. In a 1.5 ml tube, mix all components in the order listed to prepare the RT mix.

RT mix:

207.0 µl	Nuclease-Free Water
23.0 µl	Permeabilization Buffer
460.0 µl	5X RT Buffer
57.5 µl	RNase Inhibitor (40 U/µl)
60 µl	Shasta Total RNA Reverse Transcriptase (200 U/µl)
807.5 µl	**Total volume per plate**

Vortex to mix well and centrifuge briefly.

8. Split the RT mix evenly into each tube of an 8-tube strip. Each tube will contain 97 µl of RT mix.

NOTE: The solution is viscous and has detergent. Pipette slowly. Do not pipette up and down repeatedly because it will generate bubbles.

9. Using a multichannel pipette, add 7 µl RT mix into each well of the **TSO plate** to create the RT-TSO plate (Figure 8). There is no need to pipette up and down to mix. Centrifuge the plate at 1,000*g* for 1 min at 4°C.

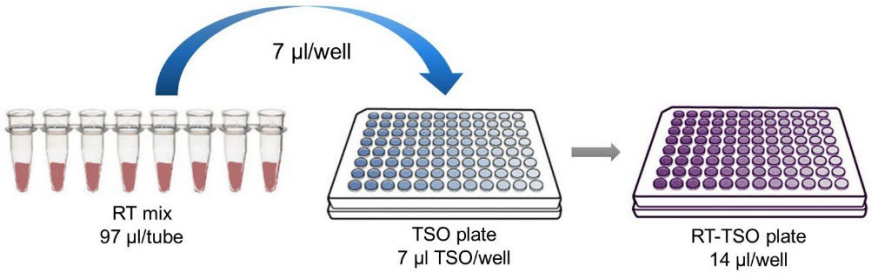


Figure 8. Creating the RT-TSO plate. The workflow illustrates Steps 8–9.

10. Transfer 11.5 µl RT mix from the RT-TSO plate (from Step 8) into the fragmented cell plate (from Step 3) as shown in Figure 9 to create the plate that will be used in the RT reaction. Pipette up and down twice to mix well.

IMPORTANT: Make sure to add the RT mix+TSO contents to the bottom of the wells containing fragmented cells. **Do not switch the order**.

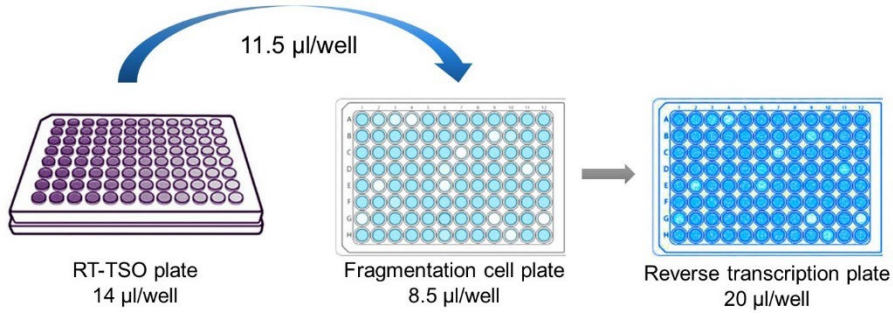


Figure 9. Creating the final plate used in the reverse transcription reaction.

11. Seal the fragmented cell (reverse transcription) plate with the sealing film. Do not vortex.
12. Centrifuge the plate at 100*g* at 4°C for exactly 5 sec. Do not centrifuge at a higher speed or for a longer time because this will pellet the cells.

13. Place the plate into the preheated thermal cycler. Run the RT program (lid temperature: 55°C) below.

10°C	10 min
15°C	10 min
20°C	10 min
25°C	10 min
30°C	10 min
35°C	10 min
42°C	60 min
4°C	forever

IMPORTANT: This is NOT a safe stopping point. Immediately proceed to the next protocol, Section V.D.

D. Protocol: Combine and Wash Cells

In this protocol, cells containing RT products from Section V.C will be washed to remove excess primers.

1. Required Components

- From the Shasta Total RNA-Seq Kit 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - Second Diluent (100X) and BSA (1%)
- PBS
- Reagent reservoir
- 5 ml centrifuge tube
- 12-channel multichannel pipette

2. Before You Start

- Thaw Second Diluent (100X) and BSA (1%) on ice. Once thawed, keep on ice for the remainder of the protocol

3. Procedure

1. From the RT products plate, use a 12-channel multichannel pipette set to 20 µl to gently pipette the well contents (containing cells) up and down 3–5 times to mix. Transfer the contents of Rows B, Row C, and Row D to Row A (Figure 10, Panel A).
2. Transfer each of the 12 wells in Row A into a single 5 ml centrifuge tube (Figure 10, Panel B).

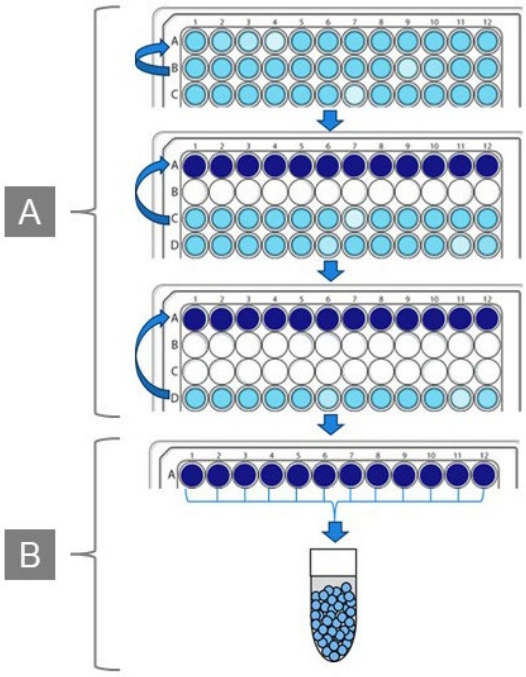


Figure 10. Combining RT products from Rows A–D of the plate into a 5 ml centrifuge tube. **Panel A**. Moving the contents of Rows B, C, and D on the plate to pool in the Row A wells. **Panel B**. All wells of Row A are further pooled into the 5 ml centrifuge tube.

3. From the same RT products plate, transfer the contents of Row G, Row F, and Row E into Row H (Figure 11, Panel A).
4. Using a 200 µl single-channel pipette set to 180 µl, transfer each of the 12 wells in Row H into the same 5 ml centrifuge tube as Step 2 (Figure 11, Panel B).

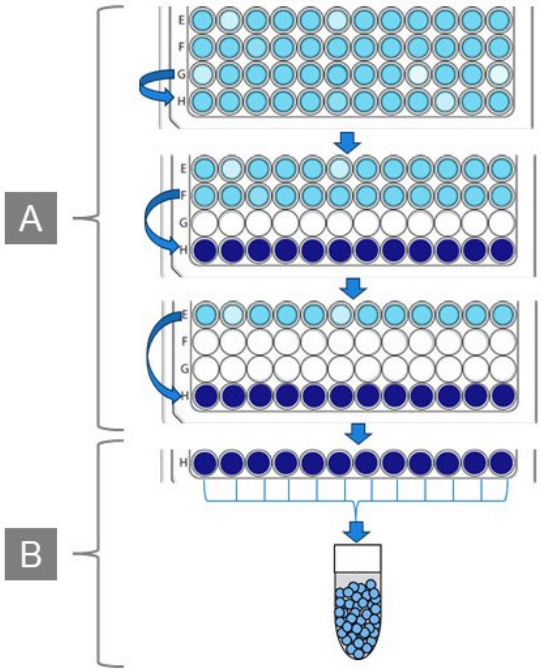


Figure 11. Combining RT products from Rows E–H of the plate into a 5 ml centrifuge tube. **Panel A**. Moving the contents of Rows G, F, and E on the plate to pool in the Row H wells. **Panel B**. All wells of Row H are further pooled into the 5 ml centrifuge tube.

5. In a 15 ml conical tube, mix all components in the order listed to prepare a wash buffer.

   Wash buffer:

5,880 µl	PBS
60 µl	Second Diluent (100X)
60 µl	BSA (1%)
6,000 µl	**Total volume (6 ml) per plate**

   Pour the wash buffer into the reagent reservoir.

6. Use a multichannel pipette to add 30 µl of wash buffer to each well of the RT products plate.
7. Using a 12-channel multichannel pipette set to 30 µl, repeat Steps 1–4 to wash each well of the plate. Transfer all content in Row A and Row H into the same 5 ml tube as in Steps 2 and 4.

   IMPORTANT: Do not discard the leftover wash buffer.

8. Invert the 5 ml tube five times. Centrifuge the tube at 1,000*g* at 4°C for 10 min.
9. Gently remove the tube from the centrifuge and pipette out 4.4–4.9 ml of supernatant, leaving about 100 µl of solution, taking care not to disturb the invisible pellet. Gently tap the bottom of the tube to resuspend the cells.
10. Add 1 ml of wash buffer prepared in Step 5 to the cells. Pipette the cells up and down with a wide-bore tip at least five times to resuspend the cells.

    IMPORTANT: If you do not have enough remaining wash buffer, prepare a new tube of wash buffer following the recipe in Step 5.

11. Check the cell morphology and count cells with a hemocytometer. Dilute the cells with leftover wash buffer into a desired concentration for cell dispensing. Refer to the table below and Appendix A, Section A, "Final Cell Dispense Concentration" for additional guidance.

Table 2. Cell dilution guidance for cell dispensing.

Cell concentration for dispensing (cells/ml)	Final volume	Cell count per nanowell	Expected cell number output in final data per chip
20,000–50,000	1 ml	1–2	5,000–10,000
50,000–80,000	1 ml	2–4	10,000–20,000
80,000–200,000	1 ml	4–10	20,000–50,000
200,000–400,000	1 ml	10–20	50,000–100,000

NOT A SAFE STOPPING POINT: Immediately proceed to Section V.E to initiate the experiment on the Shasta system.

E. Protocol: Initiate a Total RNA-seq Experiment on the Shasta System

1. Follow the procedure in the Shasta Single Cell System User Manual, Section VII, "Protocol: Prepare the Shasta System".
2. When you reach Section VII.D, "Initiate or Resume an Experiment", follow the directions to start a new experiment.
   a. From the *Home* screen, select 'Total RNA-Seq' under "Start new experiment" or [More applications].

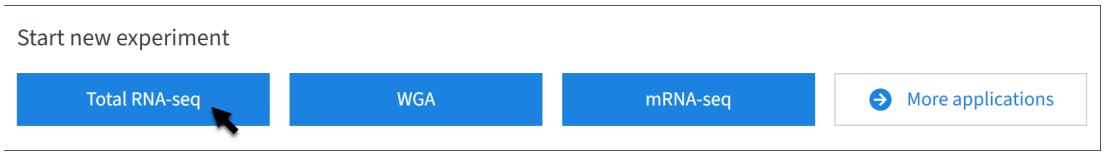


Figure 12. Selecting the Total RNA-Seq application in CELLSTUDIO software.

   b. Fill out the required and (if desired) optional fields in the *Experiment setup* screen. For the "Barcode" drop-down, select 'Long SetA'.

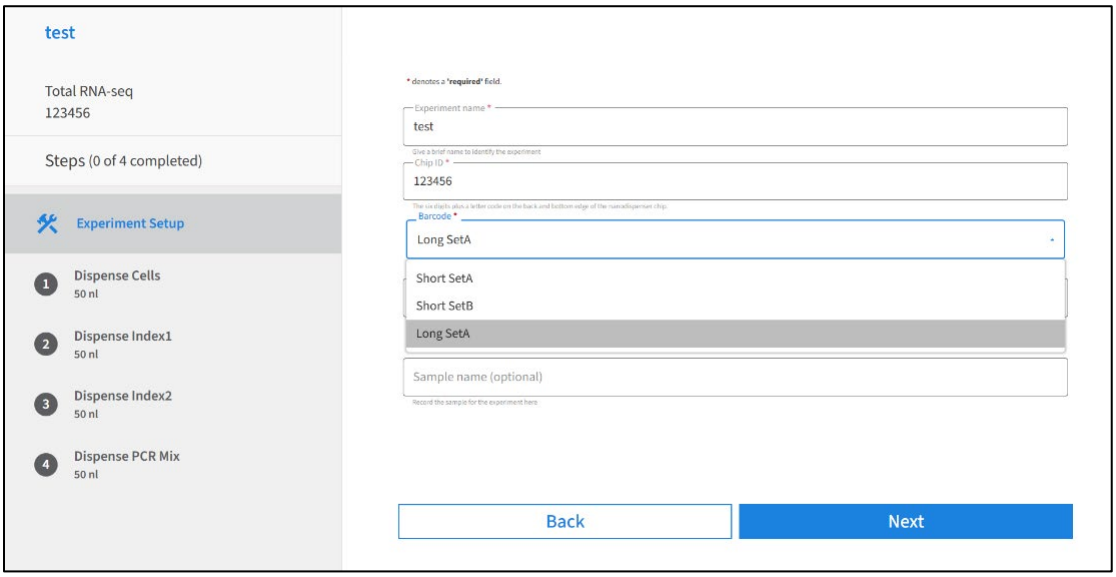


Figure 13. Selecting 'Long SetA' (Shasta Long Indexing Primer Set - A) in *Experiment setup*.

   c. When all the desired fields are populated, click [Next]. This will advance you to the "Dispense Cells (50 nl)" step.

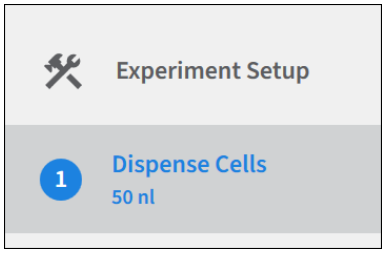


Figure 14. Use CELLSTUDIO software to dispense the cells.

F. Protocol: Dispense Cells on the Shasta System (1st Dispense)

In this protocol, the washed cells are dispensed into the Single-Cell 350v Chip.

1. Required Components

- From the Shasta Total RNA-Seq Kit - 2 Chip:
  - Single-Cell Loading Kit
    - Chip Freezing Film
- From the Nanodispenser 384-Well Source Plate and Seal: three (3) 384-Well Source Plates and Plate Seals
- A Plate Seal Applicator and film-sealing roller
- A razor blade
- Pre-chilled Nanodispenser Chip Holder(s)

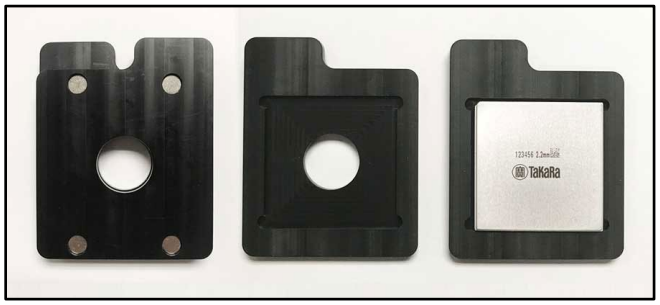


Figure 15. Nanodispenser Chip Holder.

- Nanodispenser Chip Centrifuge Spinner and Chip Balance (included with the Shasta system)

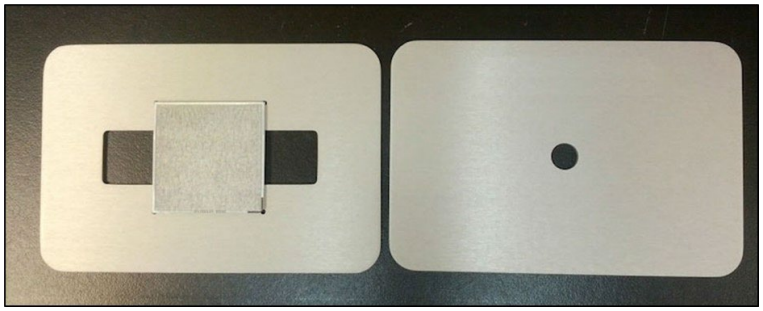


Figure 16. Nanodispenser Chip Centrifuge Spinner (left, with chip loaded) and Chip Balance (right).

2. Procedure

NOTE: More details about the dispense procedure can be found in the Shasta Single Cell System User Manual, Section IX.

1. Follow the prompts in CELLSTUDIO software to load the Single-Cell Chip and an **empty** 384-Well Source Plate. Then click "Dispense Cells".

2. The instrument will start preparation. After a couple of minutes, the software will display a

prompt to remix or change reagents (Figure 17). This is Pause 1. When that occurs, follow the steps below to load cells.

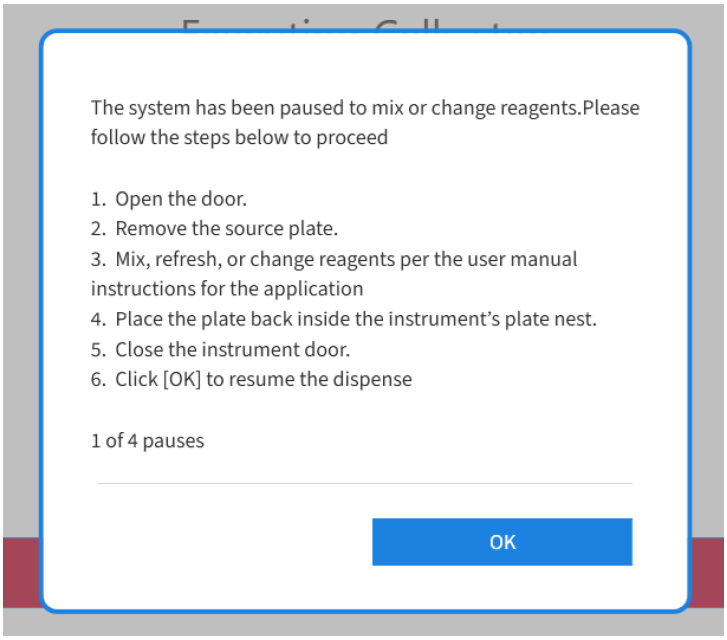


Figure 17. Example dialog window when the dispense is paused, prompting to remix the source plate.

a. Open the door of the instrument.

b. Take out the empty 384-Well Source Plate from the plate nest.

c. Use a regular pipette with a 200 µl tip to slowly and carefully load 30 µl of cell solution into the bottom of wells A1, A2, B1, B2, C1, C2, D1, and D2 of the 384-Well Source Plate (Figure 18). Make sure no bubbles are trapped at the bottom of the wells. Wide-bore tips are NOT recommended here since they are more likely to trap bubbles when loading cells to the source wells. Keep the original cell solution tube on ice. Make sure to invert and mix the cell solution each time prior to loading to the source plates.

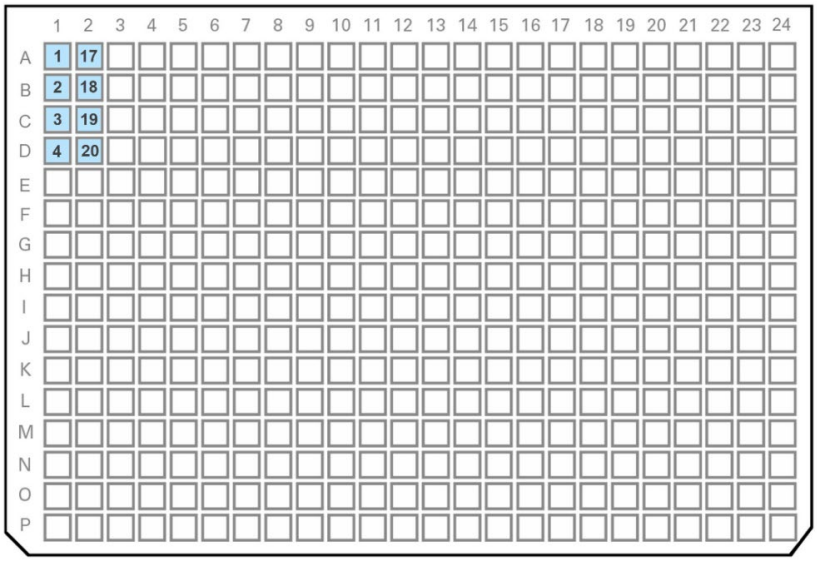


Figure 18. 384-Well Source Plate map for cell dispense. Wells A1–D2 (labeled 1–4, 17–20) are filled for the cell dispense.

d. Load the 384-Well Source Plate with cells in the Shasta Single Cell System plate nest. Do not let the plate sit for a longer than 1 minute. Proceed to the next step right away.

e. In the software, click [OK]. Cell dispensing will resume.

3. There will be 3 more pauses (total: 4 pauses) and you will repeat Step 2 three more times. Every time, discard the previously used source plate. Invert the tubes containing cells to mix them well and load 30 µl of the cell solution into the bottom of wells A1, A2, B1, B2, C1, C2, D1, and D2 of a **fresh** 384-Well Source Plate.

4. After the dispense is complete, blot and seal the chip using the RC Film from the Single-Cell Loading Kit. Centrifuge the chip at 3,000*g* at 4ºC for 5 min with full acceleration and full brake.

NOTE: Do not proceed if there are big droplets on the chip surface after dispensing (Shasta Single Cell System User Manual, Figure 61). This indicates a problem with the instrument that needs to be checked.

5. Discard the final 384-Well Source Plate.

SAFE STOPPING POINT: The chip can be stored in the Nanodispenser Chip Holder at –20°C overnight.

G. Protocol: Dispense Forward Indexing Primers (2nd Dispense)

In this protocol, 72 forward indexing primers are dispensed from the Shasta Long Indexing Primer Set - A plate (prepared in Section V.A).

1. Required Components

- From the Shasta Total RNA-Seq Kit - 2 Chip:
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- Shasta Long Indexing Primer Set - A plate (prepared in Section V.A)
- A razor blade
- Film sealing roller

2. Before You Start

- If this step is being done on a different day than the cell dispense, prepare the Shasta system with the procedure in the Shasta Single Cell System User Manual, Section VII, "Protocol: Prepare the Shasta System" and the previous experiment needs to be resumed. The Chip ID is required for resuming the experiment. More details can be found in the Shasta Single Cell System User Manual, Section VII.D.

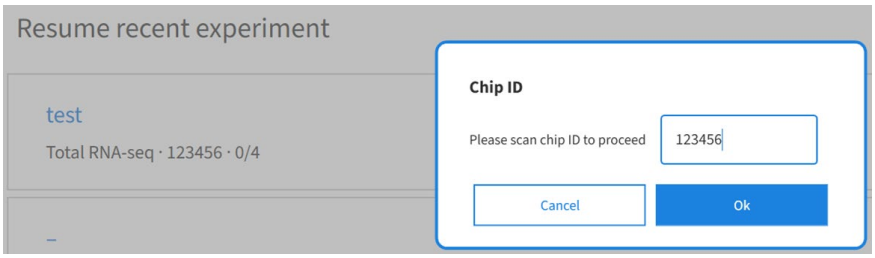


Figure 19. Resuming a previous experiment started on the Shasta system. The chip ID can either be scanned in with the barcode reader or manually typed into the input box.

- If the chip was stored frozen at –20°C after the cell dispense step (Section V.F), take the frozen chip out of the chip holder and thaw the chip facing nanowells up on the benchtop at room temperature for at least 10 min.
- Centrifuge the thawed chip at 3,000*g* at 4°C for 3 min prior to use.
- Set the centrifuge(s) used for spinning the Single-Cell 350v Chip and 384-Well Source Plate to 4°C.

3. Procedure

NOTE: More details about the dispense procedure can be found in the Shasta Single Cell System User Manual, Section IX.

1. Centrifuge the reconstituted Shasta Long Indexing Primer Set - A plate at 1,000*g* at 4°C for 1 min before use.
2. Using the razor blade, cut the index plate seal into two halves along Columns 11 and 12 (Figure 20, next page).

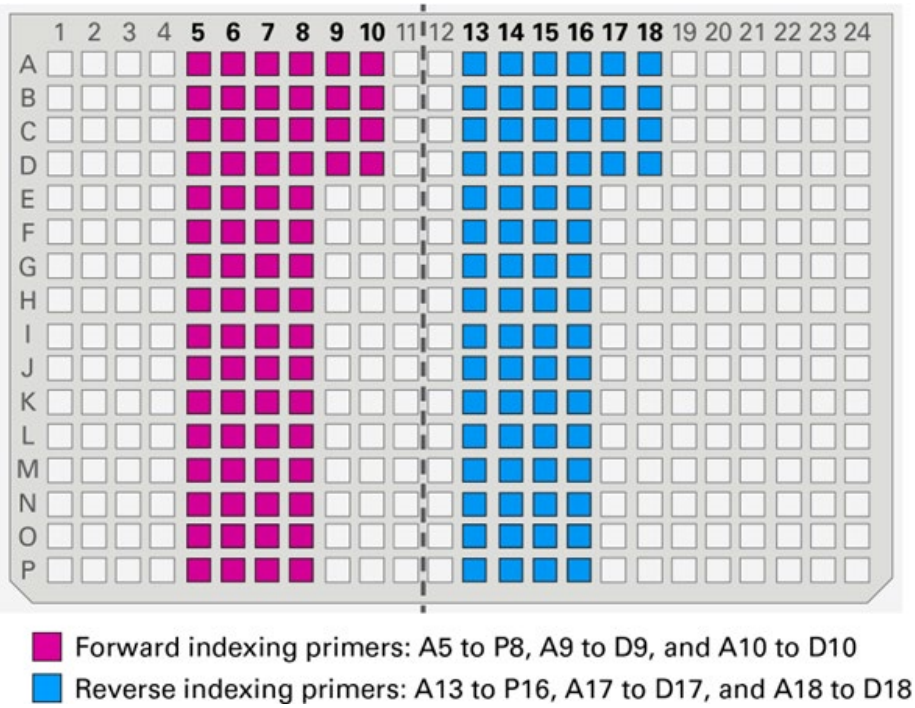


Figure 20. Plate map of the indexing primers, indicating the cut line. The indexes (shown in pink and blue) were reconstituted in Section V.A. The razor cut placement is shown by the black dotted line.

3. Remove the plate seal on the left-hand side to expose forward indexing primers (Columns 5–10, Figure 21).

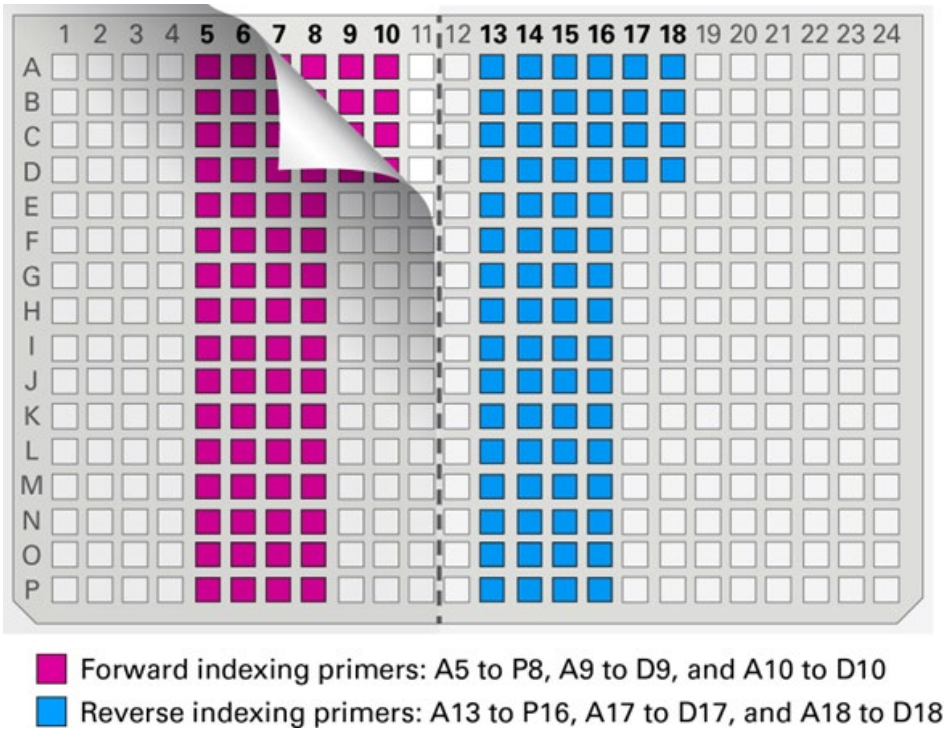
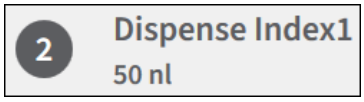


Figure 21. Peeling the plate seal from the forward indexing primers. The forward indexing primers are shown in pink. The plate seal should remain on the right-half of the plate, covering the reverse indexing primers (blue).

4. Follow the prompts in CELLSTUDIO software to load the Single-Cell Chip with cells and the reconstituted Shasta Long Indexing Primer Set - A and remove seals. After that, click "Dispense Index1".



5. After the dispensing is complete, Blot, Seal, and Centrifuge the Chip. You will be using the RC Film from the Single-Cell Loading Kit to seal the chip and centrifuge the chip at 3,000g at 4ºC for 5 min with full acceleration and full brake.

   NOTE: Do not proceed if there are big droplets on the chip surface after dispensing (Shasta Single Cell System User Manual, Figure 61). This indicates a problem with the instrument that needs to be checked.

6. Centrifuge the chip: 3,000*g* at 4ºC for 5 min with full acceleration and full brake.

   IMPORTANT: Do NOT discard the index source plate as it will be used in the next protocol step. It is recommended to leave the index source plate in the plate nest during Steps 5 & 6.

NOT A SAFE STOPPING POINT: Immediately proceed to the next step (Section V.H, "Protocol: Dispense Reverse Indexing Primers (3rd Dispense)").

H. Protocol: Dispense Reverse Indexing Primers (3rd Dispense)

In this protocol, 72 reverse indexing primers are dispensed from the Shasta Long Indexing Primer Set - A plate used in the previous protocol.

1. Before You Start

- Set the centrifuge used for spinning the Single-Cell 350v Chip to 4°C.

2. Procedure

1. Remove the rest of the seal from the Indexing Primer Plate to expose the reverse indexing primers (Columns 13–18, Figure 22).

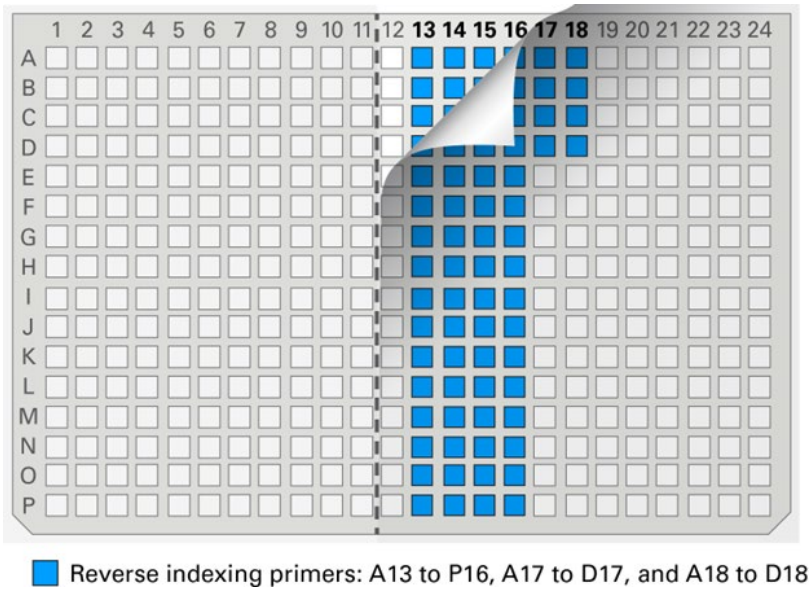
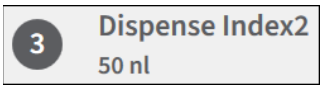


Figure 22. Peeling the plate seal from the reverse indexing primers. Remove the remaining seal from the right-side of the plate.

IMPORTANT: If bubbles were noted in the Shasta Long Indexing Primer Set - A plate wells for the reverse primers during the previous protocol (Section V.G) and the plate was not centrifuged to remove them, remove the bubbles gently by pipette now.

2. Follow the prompts in CELLSTUDIO software to load the Single-Cell Chip with cells and the reconstituted Shasta Long Indexing Primer Set - A and remove seals. After that, click "Dispense Index2".



3. After the dispensing is complete, blot and seal the chip using the RC Film from the Single-Cell Loading Kit. Centrifuge the chip at 3,000*g* at 4ºC for 5 min with full acceleration and full brake.

NOTE: Do not proceed if there are big droplets on the chip surface after dispensing (Shasta Single Cell System User Manual, Figure 61). This indicates a problem with the instrument that needs to be checked.

4. During centrifugation, prepare the Single-Cell Thermal Cycler (see "Before You Start" in the next section).

NOT A SAFE STOPPING POINT: Immediately proceed to the next step (Section V.I, "Protocol: PCR1 Master Mix Dispense (4th Dispense) and PCR 1").

I. Protocol: PCR1 Master Mix Dispense (4th Dispense) and PCR 1

In this protocol, the PCR reaction takes place inside nanowells of the chip and the second barcode is incorporated into the PCR product.

1. Required Components

- From the Shasta Total RNA-Seq Kit - 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - Nuclease-Free Water, SeqAmp CB PCR Buffer (2X), SeqAmp DNA Polymerase
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- Plate Seal Applicator and film sealing roller

2. Before You Start

- Thaw the Nuclease-Free Water, SeqAmp CB PCR Buffer (2X), and SeqAmp DNA Polymerase. Keep them on ice.
- Preprogram the Single-Cell Thermal Cycler with the PCR 1 program (Steps 5 and 6) before the experiment. Run and immediately hold the program before the reaction.

3. Procedure

NOTE: More details about the dispense procedure can be found in the Shasta Single Cell System User Manual, Section IX.

1. In a clean 1.5 ml tube, mix all components in the order listed to prepare the PCR1 Master Mix.

   PCR1 Master Mix:

285.6 µl	Nuclease-Free Water
340.0 µl	SeqAmp CB PCR Buffer (2X)
54.4 µl	SeqAmp DNA Polymerase
680.0 µl	**Total volume per chip**

   Vortex the tube to mix well and spin briefly.

2. Pipette 80 µl of the PCR1 Master Mix per well to the 384-Well Source Plate from Section V.H in wells A3, A4, B3, B4, C3, C4, D3 and D4 (Figure 23). Directly add to the bottom of the wells without trapping bubbles at the bottom or leaving the liquid on the well walls.

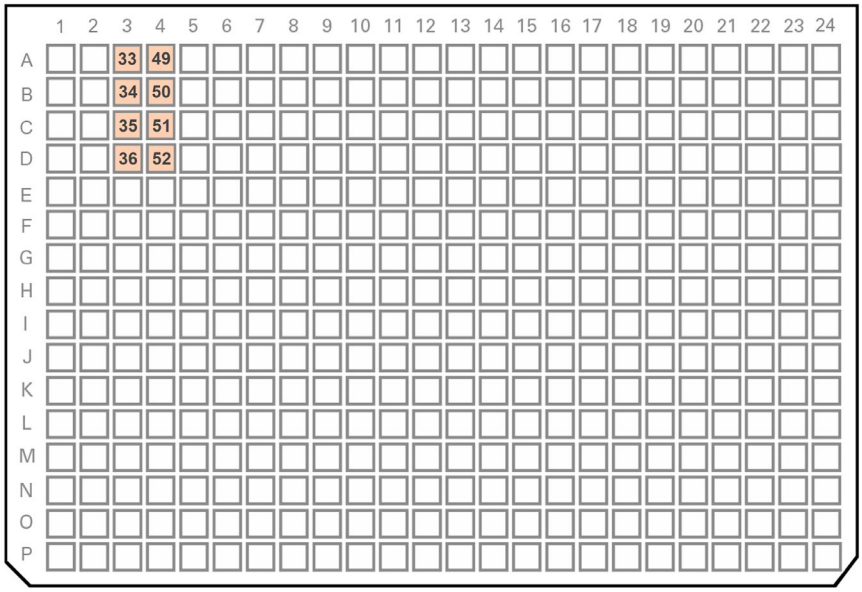
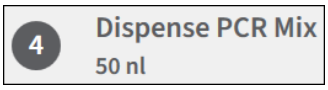


Figure 23. 384-Well Source Plate map for PCR1 Master Mix dispense. Wells A3–D4 (labeled 33–36, 49–52) are filled for the "Dispense PCR" step (Step 3).

3. Follow the prompts in CELLSTUDIO software to load the Single-Cell Chip (Section V.I) and the 384-Well Source Plate containing the PCR1 Master Mix. Then click "Dispense PCR Mix".



4. After the dispense is complete, blot and seal the chip using the RC Film from the Single-Cell Loading Kit to seal the chip. Centrifuge the chip at 3,000*g* at 4ºC for 5 min with full acceleration and full brake.

NOTE: Do not proceed if there are big droplets on the chip surface after dispensing (Shasta Single Cell System User Manual, Figure 61). This indicates a problem with the instrument that needs to be checked.

5. Discard the 384-Well Source Plate.
6. After centrifugation finishes, place the chip into the thermal cycler. Make sure the thermal cycler lid temperature is at 72°C, the block temperature is at 96.5°C, and the volume is 0.
7. Once the chip is loaded into the thermal cycler, resume the PCR 1 program:

96.5°C	3 min
5 cycles:	
100.0°C	10 sec
54.4°C	5 sec
59.6°C	10 sec
72.2°C	9 sec
67.9°C	21 sec
67.9°C	2 min
4°C	forever

SAFE STOPPING POINT: The chip can be left in the thermal cycler at 4°C overnight.

J. Protocol: Library Extraction from the Chip

This protocol extracts the PCR1 product from the Single-Cell 350v Chip.

Refer to the Shasta Single Cell System User Manual, Section XIII, " Extracting Library from the Chip " for the procedure. The collected volume should be no less than 830 µl.

- Transfer 250 µl into a fresh 1.5 ml tube for the downstream protocol and store the rest at –20°C as a backup.
- If <20,000 cells were dispensed into the Single-Cell 350v Chip in Section V.F (cell dispense protocol), transfer 500 µl of the PCR1 product into a fresh 1.5 ml tube and adjust the amount of NucleoMag beads to 350 µl in Section V.K.3, Step 1. Store the rest of the PCR 1 product at –20°C for use as a backup.

SAFE STOPPING POINT: The PCR 1 product can be frozen at –20°C.

K. Protocol: Purification of the Extracted Library

In this protocol, the extracted library is purified by immobilization on NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol, and the libraries are eluted with Nuclease-Free Water.

1. Required Components

- From the Shasta Total RNA-Seq Kit 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - Nuclease-Free Water
- Prealiquoted tube of NucleoMag NGS Clean-up and Size Select (see note in Section III)
- Molecular grade anhydrous ethanol

2. Before You Start

- Equilibrate a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.
- Prepare fresh 80% (v/v) ethanol just prior to cleanup. You will need 2 ml.

3. Procedure

1. If the library has been kept frozen after extraction from the chip in Section V.J, thaw the library on ice. If this is directly after extraction, proceed to Step 2.

2. Add 175 µl of NucleoMag beads to the 250 µl of the extracted PCR 1 product. Vortex the tube briefly 2–3 sec to mix well.

   NOTES:
   - The beads are viscous; pipette the entire volume and push it out slowly.
   - The bead:sample ratio is 0.7. Using higher concentrations of NucleoMag beads is not recommended because it will introduce small peaks, such as primer dimers, into the library.
   - If 500 µl of the extracted PCR 1 product is used, add 350 µl of the NucleoMag beads.

3. Incubate the tube at room temperature for 8 min to let the DNA bind to the beads.
4. Briefly spin the samples to collect the liquid from the side of the tube. Place the tube in the 1.5 ml tube magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant; any bead carryover will decrease the efficiency of size selection.

   NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Use the supernatant to resuspend them and pipette them onto the magnet with the rest of the beads.

5. Keep the tube on the magnetic separation device. Remove the supernatant with a pipette and discard it.
6. Add 1 ml of freshly made 80% ethanol to the tube without disturbing the beads, to wash away contaminants. Wait for 30 sec and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
7. Repeat the ethanol wash (Step 5) once more.
8. Briefly spin the tube (~2,000*g*) to collect the remaining liquid at the bottom.
9. Place the tube on the magnetic separation device for 30 sec then remove the remaining supernatant with a pipette and discard it.
10. Let the tube rest open on the magnetic separation device at room temperature for ~1–2 min until the pellet appears dry and is no longer shiny.

    NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads, and recovery may be reduced.

11. Once the bead pellet has dried, remove the tube from the magnetic separation device and add 64 µl of Nuclease-Free Water to cover the pellet. Vortex the tube to ensure complete bead dispersion and briefly spin down.

    NOTE: Be sure that the beads are completely resuspended. The beads can sometimes stick to the sides of the well or tube.

12. Incubate the tube at room temperature for 5 min to rehydrate.
13. Mix thoroughly by vortexing for 3–5 sec and spin briefly.
14. Transfer all liquid to a new 0.2 ml PCR tube. Place the tube on the magnetic separation for PCR strips for 2 min or longer until the solution is completely clear.

    NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Pipette these nonpelleted beads (without disrupting the existing pellet) up and down to resuspend them with the supernatant and then pipette them towards the magnet where the rest of the beads have already pelleted. Continue the incubation until there are no beads left in the supernatant.

15. Transfer 62 µl clear supernatant containing the purified library to a fresh 0.2 ml PCR tube.

SAFE STOPPING POINT: The purified library can be frozen at –20°C indefinitely.

L. Protocol: Depletion of Ribosomal cDNA

In this protocol, library fragments originating from rRNA and mitochondrial rRNA are cut by the ZapR Enzyme in the presence of R-Probes (mammalian-specific). These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA sequences and human mitochondrial m16S and m12S rRNA sequences. Non-human mitochondrial rRNA sequences will not be removed.

1. Required Components

- From the Shasta Total RNA-Seq Kit 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - R-Probes, ZapR Buffer, and ZapR Enzyme

2. Before You Start

- Thaw ZapR Buffer, ZapR Enzymes, and R-Probes on ice. Once thawed, keep them on ice for the remainder of the protocol.
- Preheat the thermal cycler at 72°C, the lid at 85°C, and volume at 7 µl before the experiment.

3. Procedure

1. Transfer 7 µl thawed R-Probes into a 0.2 ml PCR tube.
2. Heat R-Probes in the pre-heated thermal cycler at 72°C for 2 min then put on ice right away for 1 min to cool, but no longer than 10 min.
3. Program the thermal cycler as in Step 7 below with lid temperature 85°C and volume 22 µl. Run and immediately hold the program before the reaction.
4. In a clean 0.2 ml PCR tube, mix all components in the order listed to prepare the ZapR mix.

   ZapR mix:

9.8 µl	ZapR Buffer
4.2 µl	ZapR Enzyme
6.8 µl	Preheated R-Probes (from Step 2)
8.4 µl	Nuclease-Free Water
29.2 µl	**Total volume per chip**

   Gently vortex to combine.

5. Add 6.5 µl ZapR mix into four clean 0.2 ml PCR tubes.
6. Add 15.5 µl of the purified library from the previous step (Section V.K) into each tube to combine with ZapR mix. Close the caps of the tubes. Gently vortex the tubes to mix and briefly centrifuge to bring all liquid to the bottom of the tubes.
7. Place the reaction tubes in the thermal cycler and run the program below:

37°C	60 min
72°C	10 min
4°C	forever

NOT A SAFE STOPPING POINT: Immediately proceed to the next step (Section V.M, "Protocol: Library Amplification (PCR 2)") to perform the second PCR.

M. Protocol: Library Amplification (PCR 2)

In this protocol, PCR amplifies and yields the final sequencing-ready library.

1. Required Components

- From the Shasta Total RNA-Seq Kit 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - Nuclease-Free Water, SeqAmp DNA Polymerase, SeqAmp CB PCR Buffer, and PCR2 Primers

2. Before You Start

- Thaw Nuclease-Free Water, SeqAmp DNA Polymerase, SeqAmp CB PCR Buffer, and PCR2 Primers on ice. Once thawed, keep them on ice for the remainder of the protocol.
- Program the thermal cycler as in Step 4 below with the lid at 105°C and volume at 100 µl before the experiment. Run and immediately hold the program before the reaction.

3. Procedure

1. In a 1.5 ml tube, mix all components in the order listed to prepare the PCR 2 master mix:

PCR2 master mix:

225 µl	SeqAmp CB PCR Buffer (2X)
108 µl	Nuclease-Free Water
9 µl	PCR2 Primers
9 µl	SeqAmp DNA Polymerase
351 µl	**Total volume per chip**

Vortex and spin it down briefly.

2. Transfer 80 µl into each of the four tubes containing the resulting products from the previous protocol (Section V.L).
3. Vortex to mix and briefly spin down the tubes.

4. Load the tubes into the thermal cycler and resume the PCR 2 program.

94°C	1 min
15-25 cycles*:	
98°C	15 sec
55°C	15 sec
68°C	30 sec
68°C	2 min
4°C	hold

* Review Appendix B for additional PCR Cycling guidelines.

5. Combine the four tubes of PCR 2 product into one 1.5 ml tube.

SAFE STOPPING POINT: The PCR 2 product can be frozen at –20°C indefinitely.

N. Protocol: Purification of the Sequencing-Ready Library

In this protocol, the PCR2 product is purified by immobilization on NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol, and the libraries are eluted with Nuclease-Free Water.

1. Required Components

- From the Shasta Total RNA-Seq Kit 2 Chip:
  - Shasta Total RNA-Seq Reagents
    - Nuclease-Free Water
- Prealiquoted tube of NucleoMag NGS Clean-up and Size Select (see note in Section III)
- Molecular grade anhydrous ethanol

2. Before You Start

- Bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min. Mix well by vortexing. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube.
- Prepare fresh 80% (v/v) ethanol just prior to cleanup. You will need 2 ml.

3. Procedure

1. Add 280 µl of NucleoMag beads to the 1.5 ml tube containing PCR 2 products.

NOTES:
- The beads are viscous; pipette the entire volume and push it out slowly.
- The bead:sample ratio is 0.7.
- Using higher concentrations of NucleoMag beads is not recommended because it will introduce small peaks, such as primer dimers, into the library.

Gently vortex the tube for 3 sec to mix well.

2. Incubate the tube at room temperature for 8 min to let the DNA bind to the beads.

3. Briefly spin the samples to collect the liquid from the side of the tube. Place the tube in the 1.5 ml tube magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant; any bead carryover will decrease the efficiency of size selection.

   NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Use the supernatant to resuspend them and pipette them onto the magnet with the rest of the beads.

4. Keep the tube on the magnetic separation device. Remove the supernatant with a pipette and discard it.
5. Add 1 ml of freshly made 80% ethanol to the tube without disturbing the beads to wash away contaminants. Wait for 30 sec and use a pipette to carefully remove the supernatant containing contaminants. The DNA will remain bound to the beads during the washing process.
6. Repeat the ethanol wash (Step 5) once more.
7. Briefly spin the tube (~2,000*g*) to collect the remaining liquid at the bottom.
8. Place the tube on the magnetic separation device for 30 sec then remove the remaining supernatant with a pipette and discard it.
9. Let the tube rest open on the magnetic separation device at room temperature for ~1–2 min until the pellet appears dry and is no longer shiny.

   NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

   https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

   If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads, and recovery may be reduced.

10. Once the bead pellet has dried, remove the tube from the magnetic separation device and add 100 µl of Nuclease-Free Water to cover the pellet. Vortex the tube to ensure complete bead dispersion and briefly spin down.

    NOTE: Be sure that the beads are completely resuspended. The beads can sometimes stick to the sides of the well or tube.

11. Incubate the tube at room temperature for 5 min to rehydrate.
12. Mix thoroughly by pipetting up and down 10 times to elute the DNA from the beads. Place the tube back on the magnetic separation device for 3 min or longer until the solution is completely clear.

    NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Pipette these nonpelleted beads (without disrupting the existing pellet) up and down to resuspend them with the supernatant and then pipette them towards the magnet where the rest of the beads have already pelleted. Continue the incubation until there are no beads left in the supernatant.

13. Transfer 95 µl of supernatant to a clean new tube. Keep the tube with the rest of the elutes on the magnetic separation device for the library quantification (Section V.O).

SAFE STOPPING POINT: The purified library can be frozen at –20°C indefinitely.

O. Protocol: Library Validation and Quantification

To determine whether library production and purification were successful:

- Measure the concentration of the final libraries by Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854),
- Dilute and analyze on an Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626), and
- Quantify by qPCR using Takara Bio's NGS Library Quantification Kit (Cat. No. 638324).

Please refer to the corresponding reagent and instrument user manuals for detailed instructions.

1. Before You Start

- Bring the Agilent High Sensitivity DNA Kit to room temperature for ~30 min prior to use.

2. Procedure

1. Keep the tube containing the final library on the magnetic separator (Section V.N). Using 1 µl of the library, measure the concentration using the Qubit fluorometer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851). Refer to the Qubit dsDNA HS assay kit user manual for sample prep instructions.
2. Based on the Qubit measurement, dilute the library with Nuclease-Free Water to 2–3 ng/µl. Label the tube "D1". We recommend making at least 20 µl of "D1" as this library will be used for downstream Bioanalyzer, qPCR, and Illumina sequencing.

   Save the original, undiluted library at –20°C.
3. Use 1 µl of the "D1" library to load the Bioanalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit for validation. See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
4. Use the Bioanalyzer results to determine library quality and average size for qPCR quantification. See Figure 24 for an example of a typical Bioanalyzer profile for a library generated by this protocol.

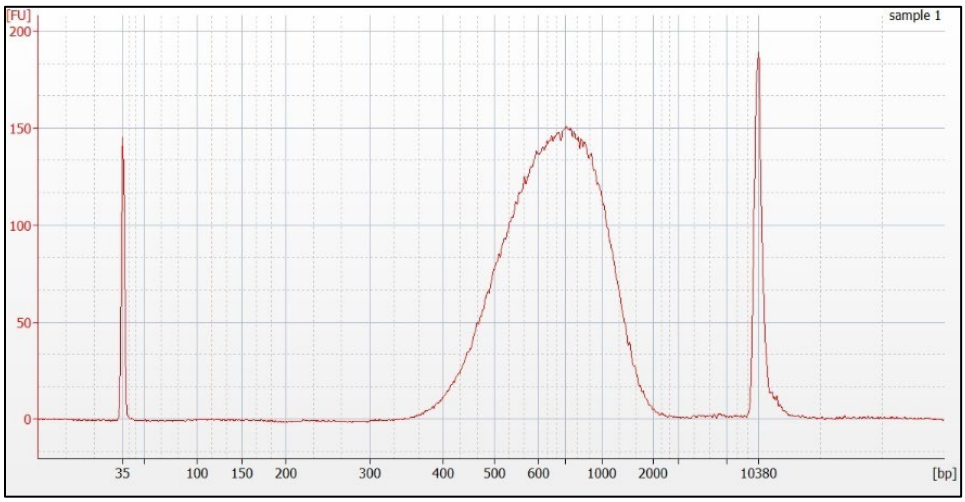


Figure 24. Example electropherogram results using the Agilent 2100 Bioanalyzer high sensitivity assay.

NOTE: If the Qubit reading is low, for example, 0.1–1 ng/µl, and the Bioanalyzer trace looks good, another round of 1X bead enrichment is recommended. Use 15 µl as the final elution volume to obtain a sufficient library yield for downstream Bioanalyzer, qPCR, and Illumina sequencing.

5. Based on the Bioanalyzer results, further dilute the "D1" library from Step 2 to 1 pmol/µl for the qPCR reaction.
6. Run qPCR using the Library Quantification Kit. Refer to the "Library Quantification Kit User Manual" on the takarabio.com website for instructions and use the average size as determined by the Bioanalyzer to calculate the molar library concentration.
7. Use the qPCR results to determine the final library quantity of the "D1" library for the sequencing.

SAFE STOPPING POINT: Store the sequencing library "D1" at –20°C until ready for sequencing. Refer to Appendix C for sequencing guidelines.

VI. Demultiplexing and Data Analysis

Use Cogent NGS Analysis Pipeline, Takara Bio's RNA-seq analysis pipeline

Cogent NGS Analysis Pipeline (CogentAP, version v3.1 and later) is required for demultiplexing the Shasta Total RNA-Seq data. Cogent NGS Discovery Software (CogentDS, version v2.1 and later) can be used for more in-depth data analyses and making plots. Sign up at takarabio.com to obtain CogentAP [link] or CogentDS [link] for no additional cost.

Make sure CogentAP and the reference genomes were installed correctly following the User Manuals (See Section V. in the Cogent NGS Analysis Pipeline User Manual).

A. bcl2fastq/BCL Convert.

Before using CogentAP for demultiplexing, raw paired FASTQ files are required as input. Sequencing output files from Illumina platforms can be converted to raw FASTQ files using bcl2fastq (v2.16) or BCL Convert (v4.1.5) software from Illumina (See Section VI. A in the Cogent NGS Analysis Pipeline User

Manuals). When preparing the sample sheet, dummy indices are provided, such as `TTTTTTTT` can be used for both i5 and i7 (See Figure 25 as an example). After feeding the bcl2fastq or BCL Convert software with this dummy sample sheet (for example, `--sample-sheet=DummySampleSheet.csv`.), the program will convert the sequencing files into two Undetermined FASTQs (See Figure 26 as an example).

[Header]								
IEMFileVersion	4							
Experiment Name	XXXXXXXX							
Date	mm/dd/yyyy							
Workflow	GenerateFASTQ							
Application	FASTQ Only							
Chemistry	Amplicon							
[Reads]								
75								
75								
[Data]								
Sample_ID	Sample_Name	Sample_P	Sample_W	I7_Index_I	index	I5_Index_I	index2	Sample_Project
	Dummy				TTTTTTTT		TTTTTTTT	

Figure 25. An example of the "dummy sample sheet" for bcl2fastq to convert sequencing files generated from an Illumina NextSeq® 500 sequencer.

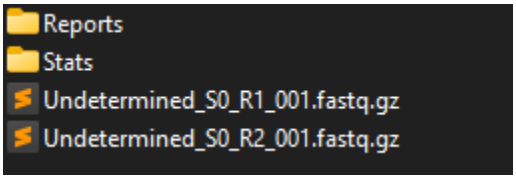


Figure 26. An example of the paired "Undetermined" FASTQ files generated from the bcl2fastq software using the dummy sample sheet.

1. Check if the raw FASTQ files have the correct format by running the following commands in the terminal.

   ```
   gunzip -c Undetermined_R1_001.fastq.gz | head -n4
   gunzip -c Undetermined_R2_001.fastq.gz | head -n4
   ```

2. Confirm that the first line of each FASTQ file ends with the format of *NNNNNNNN+NNNNNNNN*, where the *N*s represent the i7 and i5 index sequences (8-base). In Figure 27, the i7 and i5 index sequence `CATACTCC+TAGGCTCT` (corresponding to *NNNNNNNN+NNNNNNNN*) is highlighted as an example.

   ```
   @A00613:696:HLW5WDRX3:1:2101:1090:1000 1:N:0:CATACTCC+TAGGCTCT
   NGGGGTTGTTGAGACACACAGTGAGGGAGAAGGAGGGATATCTGAGTTGGCTCCCTGCTTTTATTGTAACCTTTT
   +
   #:F:F:FFF,FF:FFFF:FFFFFFFFFFFFFFFFFFFFFFFFF:FFFFF:FFFF:FFFFFFFFFFF,FFFF:FFF
   ```

Figure 27. An example of the format of the FASTQ files.

B. Demultiplex with CogentAP using `--dry_run`

1. Set up of the `$COGENT_AP_HOME` Environmental Variable is recommended (See Section V.D. in the Cogent NGS Analysis Pipeline User Manual).
2. Read counts for all the barcodes in the experiment must be estimated before running the demux process. To perform the estimation, demultiplex using the `--dry_run` mode. The WELLLIST file can be found here: `$COGENT_AP_HOME/config/well_list_shasta_total_rna.csv`.

```
$COGENT_AP_HOME/cogent rna demux --dry_run \
-f <FASTQ_R1> \
-p <FASTQ_R2> \
-b <WELLLIST> \
-t shasta_total_rna \
-o <OUTPUT>
```

3. A folder called `demultiplexed_fastqs/` will be created in the **<OUTPUT>** folder. A file called `demultiplexed_fastqs_counts_all.estimated.csv` can be found there. You will be using that CSV file as import into CogentDS in the next section.

C. Generate a Knee Plot Using CogentDS scRNA > Barcode Rank Plot

CogentDS is required for making the Barcode Rank Plot (or "knee plot") (see Section VI.C in the Cogent NGS Discovery Software User Manual, scRNA app > Barcode Rank Plot).

1. Upload the `demultiplexed_fastqs_counts_all.estimated.csv` file to the CogentDS software.
2. Wait for the confirmation message that your data has been successfully uploaded then click [Generate Barcode Rank Plot].
3. After the plot is generated, find the intersection point of the vertical and horizontal dotted lines with the knee plot (see Figure 28). At the intersection, there is text displayed that will be used in the next step:
   - <number> cells: This is the number of barcodes recommended to be demultiplexed
   - Total reads: This represents the minimum number of reads that should be analyzed.

   Make a note of these two values and proceed to the next section.

   Example:

   In Figure 28, the text at the intersection of the dotted lines shows:

   95862 cells; Total Reads 13643

   which means there are 95,862 barcodes with a read count of ≥13,643 reads/barcode.

   The values to note are therefore:

   - Number of barcodes (Barcodes): 95862
   - Minimum reads (Reads): 13643

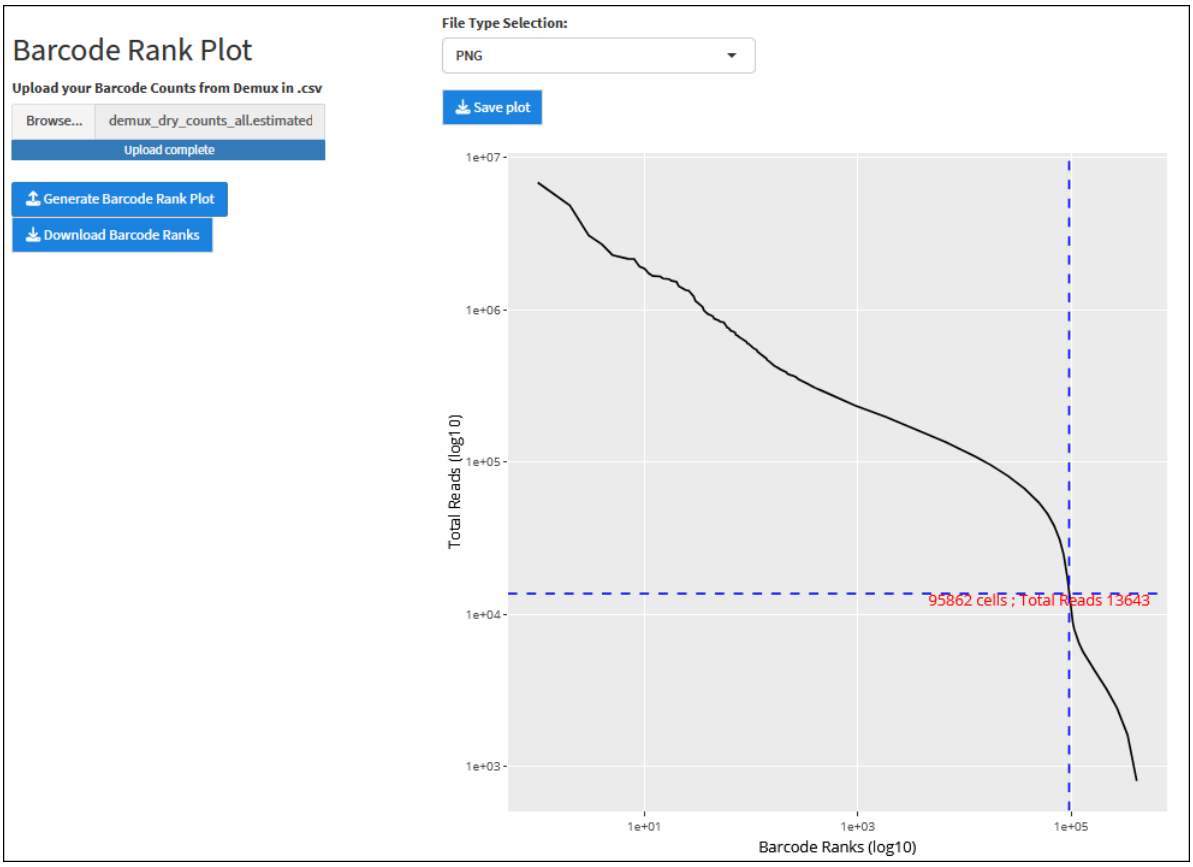


Figure 28. An example of the Barcode Rank plot generated by CogentDS.

D. Demultiplex on CogentAP Using the Calculated Threshold

Based on the values derived from the results of the Barcode Rank Plot, run the demux command using CogentAP.

```
$COGENT_AP_HOME/cogent rna demux \
--use_barcodes <BARCODES> \
--min_reads <READS> \
-f <FASTQ_R1> \
-p <FASTQ_R2> \
-b <WELLLIST> \
-t shasta_total_rna \
-o <OUTPUT>
```

Where **<BARCODES>** is the number of barcodes from the previous section and **<READS>** is the minimum number of reads.

Using the example from Figure 28, these arguments will be:

```
--use_barcodes 95862 --min_reads 13643
```

These two parameters can be adjusted based on your data. CogentAP will apply the more stringent of the two. The examples below will explain how the stringency of the two parameters is determined.

NOTE: These two values are automatically generated by the software. You may need to choose different values based on your experiments. By hovering the mouse over the black line, you can view and select a different region as the new cutoff from what is generated by the software.

1. Example: Stringent `min_reads`

In this example, the `min_reads` parameter is set to be more stringent.

Assuming you want to keep 100,000 barcodes based on the knee plot in Figure 28 and change the parameters to:

```
--use_barcodes 100000 --min_reads 13643
```

Since the `min_reads` parameter is set at 13,643, this will only keep barcodes with a read count of ≥ 13,643, and the result will only yield 95,862 barcodes. The barcodes with a ranking after 95,862 do not have a minimum read count of 13,643 and therefore will not be kept in the result.

2. Example: Stringent `use_barcodes`

In this example, the `use_barcodes` parameter is set to be more stringent.

Based on the knee plot in Figure 28, assuming you want to lower the `min_reads` cutoff and would like to keep barcodes with more than 10,000 reads, change the parameters to:

```
--use_barcodes 95862 --min_reads 10000
```

Since the barcodes with a read count fewer than 13,643 will be ranked after 95,862, but the `--use_barcodes` parameter is set to '95862', it will only keep 95,862 barcodes in the result.

Based on the knee plot in Figure 28, if you want to keep 100,000 barcodes for demultiplexing, the correct way of setting up the parameters is to make sure the `use_barcodes` parameter is more stringent than the `min_reads` parameter. An example of the command is below. This will result in 100,000 barcodes being demultiplexed.

```
$COGENT_AP_HOME/cogent rna demux \
--use_barcodes 100000 \
--min_reads 1000 \
-f <FASTQ_R1> \
-p <FASTQ_R2> \
-b <WELLLIST> \
-t shasta_total_rna \
-o <OUTPUT>
```

E. Analyze the Demultiplexed Data Files

1. Assuming the **`<OUTPUT>`** folder name is `demux` during the demultiplexing step, the resulting directory **`demultiplexed_fastqs`** generated inside the demux (previous section) is used as input to run the analyzer.

```
$COGENT_AP_HOME/cogent rna analyze \
-i <INPUT_FOLDER> \
-t shasta_total_rna \
-o <OUTPUT_FOLDER> \
-g <REFERENCE_GENOME>
```

NOTE: The analyze process may take longer than 24 hr depending on the data size.

Example:

```
$COGENT_AP_HOME/cogent rna analyze \
-i ./demux/demultiplexed_fastqs \
-t shasta_total_rna \
-o analyze \
-g hg38
```

2. After the analyze process completes, there will be a `report/` folder within the **<OUTPUT_FOLDER>** which contains two files of interest:
   - The `CogentDS_preliminary-analysis_report.html` will show a summary of the data analysis.
   - The `CogentDS_analysis.rds` file can be imported into CogentDS for more detailed analysis and plotting. See the CogentDS user manual for its detailed features.

Appendix A. Troubleshooting Guidelines

A. Final Cell Dispense Concentration

Things to consider when deciding on what concentration you want to use for your final cell dispense solution:

- If the cell concentration is lower than 20,000 cells/ml in a final volume of 1 ml, there will not be enough cells for the downstream steps. Increase the cell number per well during the RT step (up to 10,000 cells per well). If there are no intact cells with mostly cell debris, it indicates the cell fixation did not work well or the cells were handled too harshly.
- If the cell concentration is greater than 400,000 cells/ml, a higher number of doublets may occur. Therefore, cells should be diluted to 400,000 cells/ml or less.

B. Final Library Size

A typical library curve should contain one single peak as shown in Figure 29. Final library size may vary depending on cell types, cell number, and other factors.

Small peaks ≤200 bp (Figure 29, left) usually come from primer dimers, which indicate too many beads were used during the library purification steps. The small peaks can be further reduced or removed by another bead clean-up, using 0.6X or 0.65X bead ratio, rather than the 0.7X outlined in Section V.M.3.

Large tails >2,000 bp (Figure 29, right) usually indicate overamplification. The library can be further cleaned up by a double-sided size selection and bead clean-up (for example, first 0.5X or 0.6X beads, second 0.8X) before qPCR and sequencing. Refer to an explanation of double-sided size selection from the Illumina technical support page: https://support.illumina.com/bulletins/2020/07/library-size-selection-using-sample-purification-beads.html.

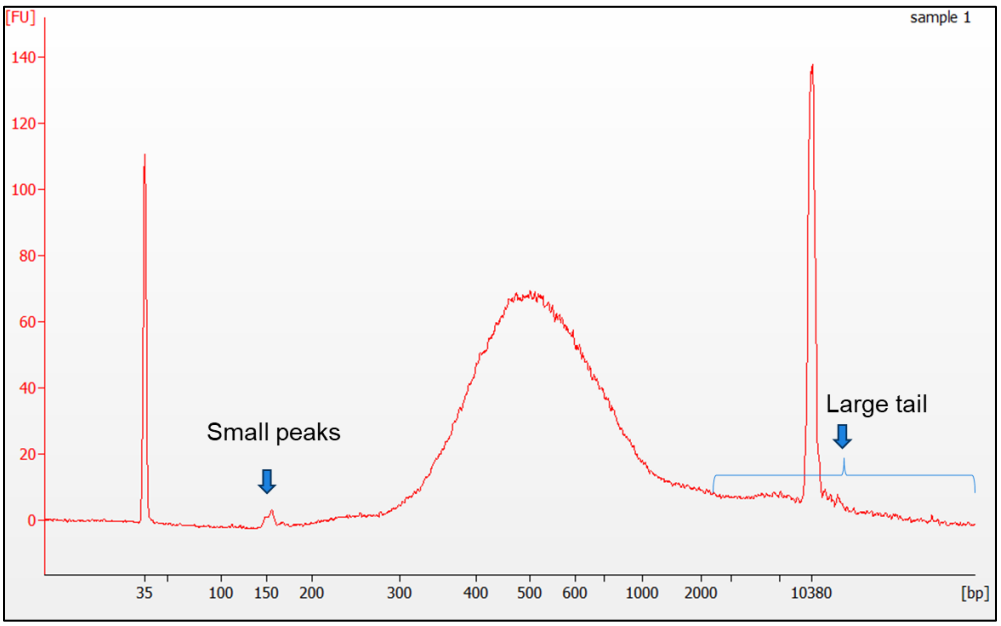


Figure 29. Example electropherogram results displaying abnormal peaks in the final library.

A sharp peak on the main curve (location may vary, example shown in Figure 30) indicates the rRNA is not sufficiently removed. Another round of ribosomal cDNA depletion (Section V.L) may help reduce the rRNA portion in the final library.

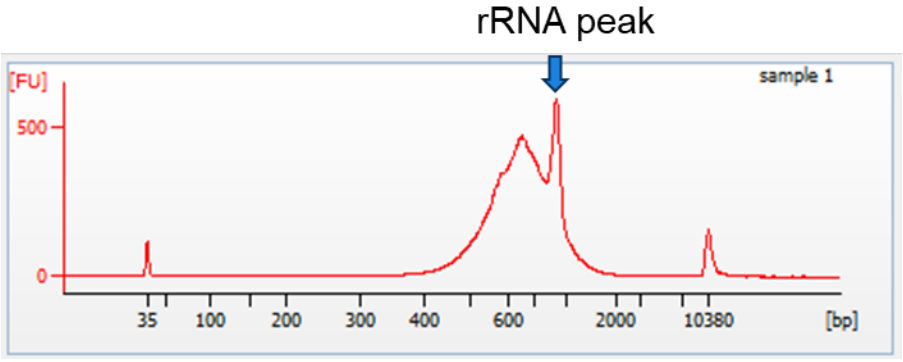


Figure 30. Example electropherogram results showing rRNA peaks in the final library.

Appendix B. PCR Cycling Guidelines

While 5 cycles of PCR during PCR 1 (Section V.I) will work to provide good transcript identification and sensitivity for many cell types, the number of PCR cycles for PCR 2 (Section V.M) may need to be adjusted depending on cell type and total cell numbers dispensed into the Single-Cell 350v Chip (Table 3).

- Cells with high mRNA content (such as the K-562 cell line) and in cases where more than 50,000 cells are dispensed on the chip may require 15 cycles to provide sufficient library yield.
- Cells with low mRNA content (such as PBMCs) or less than 50,000 cells on the chip may require 20–25 cycles or more to produce sufficient yield for sequencing.

Table 3. PCR 2 cycle number guidelines.

Cell type	Cell concentration for dispensing	Expected cell number output in final data per chip	PCR 2 cycles
High mRNA content cells	20,000–50,000	5,000–10,000	18 - 20
	50,000–80,000	10,000–20,000	17 - 18
	80,000–200,000	20,000–50,000	15 - 17
	200,000–400,000	50,000–100,000	15
Low mRNA content cells	20,000–50,000	5,000–10,000	22 - 25
	50,000–80,000	10,000–20,000	20 - 22
	80,000–200,000	20,000–50,000	18 - 20
	200,000–400,000	50,000–100,000	17-18

Table 3 provides general guidance for the number of PCR 2 cycles needed based on cell type and cell concentration. It is possible that the suggested number of cycles will not generate enough material for downstream sequencing. In these cases, we recommend using a 1:1 ratio of NucleoMag NGS Clean-up and Size Select beads to enrich the library with a final elution volume is 10–15 µl. If this still does not give sufficient yield, we recommend further increasing the number of PCR 2 cycles. We do not recommend adjusting the number of PCR 1 cycles.

Appendix C. Guidelines for Library Sequencing

A. Final Sequencing Library Structure

Libraries generated using the Shasta Total RNA-Seq Kit have standard Illumina TruSeq® adapters. The unique combinations of the sample barcode, i5, and i7 are required to discriminate between cells. The structure of a final sequencing library is shown in Figure 31.

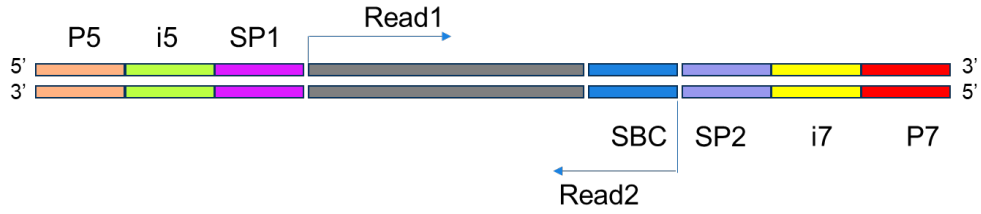


Figure 31. Structure of a final sequencing library. The final library is composed of the sample barcode (SBC), SP1 and SP2 (Illumina Truseq sequencing primer 1 and 2 sequences), i5 and i7 indexes, and P5 and P7 adapters.

B. Compatible Illumina Platforms

Libraries generated with the Shasta Total RNA-Seq Kit can be run on the following Illumina platforms with paired-end sequencing (Takara Bio has validated libraries using MiSeq®, NextSeq, and NovaSeq™):

- MiSeq
- MiniSeq®
- NextSeq series
- HiSeq® 2000/2500, 3000/4000
- HiSeq X series (Rapid Run and High Output)
- NovaSeq 6000

C. Recommendations for Library Sequencing

Refer to Illumina documentation for instructions on denaturing and diluting libraries.

1. Loading Concentration and PhiX Recommendations

For NextSeq instruments, we recommend a loading concentration of 1.5 pM as a good starting point. The concentration needs to be quantified by qPCR. Refer to information about individual Illumina instruments on Illumina's website for loading guidelines.

Libraries generated with this protocol do not require the inclusion of PhiX. However, particular sample types sometimes do not display the well-balanced nucleotide diversity required for base calling. If you are not sure about the performance of your sample then please include PhiX. Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh and reliable stock of the PhiX control library.

2. Sequencing Depth Recommendations

The sequencing depth depends on the purpose of the study. The library covers the 5′ to 3′ full gene body and therefore, deeper sequencing will recover more information. For example, identification of cell heterogeneity can be achieved with shallow sequencing of about 20,000–100,000 reads/cell. However, identification of rare genes or alternative splicing transcript isoforms may need higher sequencing depth, for example, more than 100,000 reads per cell.

3. Sequencing Run Parameters

Libraries generated with this protocol use standard Illumina sequencing primers and do not need custom primers. Dual indexes (2 x 8 cycles) ***must*** be sequenced, and we recommend the number of cycles in Table 4, below. However, cycles of Read 1 and Read 2 can be flexible (e.g., 2 x 100, 2 x 150, and 2 x 250) depending on your experiments. Longer read length (2 x 150 or longer) is recommended for certain analysis, such as gene fusion and isoform studies.

NOTE: Single-end sequencing is not compatible with the libraries produced by this workflow.

Table 4. Sequencing run parameters.

Sequencing read	Recommended # of cycles
Read 1	75 or longer
i7 Index	8
i5 Index	8
Read 2	75 or longer

Appendix D. Barcode Sequences

A. TSO Plate Sequences

The Shasta Total RNA-Seq Kit uses a TSO plate to add the sample barcode to differentiate samples. Sample barcode sequences are incorporated into Read2 after initial sequencing (see Figure 31, above). The primer sequences are shown in Table 5. The barcodes provide guidance for sample differentiation after demultiplexing.

Table 5. TSO plate index primer 1–96 barcodes.

Index name	Well position	Index sequence in primers	Index name	Well position	Index sequence in primers	Index name	Well position	Index sequence in primers
1	A1	AACCAACG	33	C9	AGTTGAAC	65	F5	TCCATAAC
2	A2	AACGATAG	34	C10	AACCTCAG	66	F6	GTCATCTA
3	A3	AAGAAGAC	35	C11	AACGGTCT	67	F7	GTCCGCAA
4	A4	AGAGCCTA	36	C12	AACTCAAG	68	F8	GTTCAATA
5	A5	ATAGTCAA	37	D1	AACTCCGA	69	F9	TAACGTCG
6	A6	CAACTGCA	38	D2	AAGGTTCA	70	F10	GATTCTAT
7	A7	CAGCATGA	39	D3	AATTCGGT	71	F11	CATTCTAC
8	A8	CCGCCTAA	40	D4	ACCAGACC	72	F12	TTACTTCT
9	A9	CCTAGCGA	41	D5	ACTTAGTA	73	G1	AACTCTCC
10	A10	ACCATTCT	42	D6	AGCGGCAA	74	G2	AACTGATA
11	A11	CTTGGCCT	43	D7	AGGTCGAA	75	G3	CTAGTCAA
12	A12	GCGGTTCT	44	D8	AGTCTGGA	76	G4	AAGTTGGA
13	B1	GCTTGATG	45	D9	ATAATGGT	77	G5	ACGAACTT
14	B2	GGCTCTCT	46	D10	ATCCATTG	78	G6	ACGCAACC
15	B3	TCAATGCT	47	D11	ATGAATCT	79	G7	TTATCATC
16	B4	TGGTAATT	48	D12	CAAGATTG	80	G8	ACTTACGT
17	B5	TTCTGAAC	49	E1	CCGAATTG	81	G9	ACTTCTAA
18	B6	AACCAGAA	50	E2	ATCAAGTT	82	G10	AAGTAATC
19	B7	AACCGAAC	51	E3	CCTTCAGG	83	G11	AGAGGTCC
20	B8	AACCGCCA	52	E4	CGAATATT	84	G12	GTACTATC
21	B9	AATAAGGA	53	E5	TATTGCTT	85	H1	AGCAAGGC
22	B10	ACCTTATT	54	E6	CGTAGGCA	86	H2	CATTCATC
23	B11	TGGTCCTG	55	E7	GAACTAAG	87	H3	AGGTTATG
24	B12	CAACGAGG	56	E8	TCAGTAGT	88	H4	AGTATAGT
25	C1	CCAATGGA	57	E9	GACTATTG	89	H5	ATGGTACT
26	C2	CGCCTATG	58	E10	GTTATATG	90	H6	ATTACGAA
27	C3	CTCTCCAA	59	E11	GCAGTCCA	91	H7	AGTAGGTA
28	C4	GGCTTGAA	60	E12	GCTCAAGG	92	H8	CATTAGAA
29	C5	GTTAAGTT	61	F1	AGTCTTAT	93	H9	CCAGGCAT
30	C6	TCAAGTAT	62	F2	GGTCAGAT	94	H10	CCGTACTA
31	C7	ACTACCAT	63	F3	GTAAGAAG	95	H11	GGTTAATA
32	C8	TGAGTCCT	64	F4	GTAGAAGT	96	H12	AACCTTCA

B. Shasta Long Indexing Primer Set - A Sequences

Tables 6 and 7 contain information on the forward and reverse index primers and sample sheet sequences, respectively. A shortened name is used for simplicity instead of using the full name of each index. For example, "Forward Index 1" below stands for "Shasta Long Forward Index 1".

Table 6. Shasta Long Indexing Primer Set - A forward indexing primer 1–72 indexes. Continued on the next page.

Forward indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index name	Well position	Index sequence in primers	MiSeq, HiSeq 2000/2500, NovaSeq	MiniSeq, NextSeq, HiSeq 3000/4000, HiSeq X
Shasta Long Forward Index 1	A5	AACCAACG	AACCAACG	CGTTGGTT
Shasta Long Forward Index 2	B5	AACGATAG	AACGATAG	CTATCGTT
Shasta Long Forward Index 3	C5	AAGAAGAC	AAGAAGAC	GTCTTCTT
Shasta Long Forward Index 4	D5	AGAGCCTA	AGAGCCTA	TAGGCTCT
Shasta Long Forward Index 5	E5	ATAGTCAA	ATAGTCAA	TTGACTAT
Shasta Long Forward Index 6	F5	CAACTGCA	CAACTGCA	TGCAGTTG
Shasta Long Forward Index 7	G5	CAGCATGA	CAGCATGA	TCATGCTG
Shasta Long Forward Index 8	H5	CCGCCTAA	CCGCCTAA	TTAGGCGG
Shasta Long Forward Index 9	I5	CCTAGCGA	CCTAGCGA	TCGCTAGG
Shasta Long Forward Index 10	J5	CGCAACGG	CGCAACGG	CCGTTGCG
Shasta Long Forward Index 11	K5	CTTGGCCT	CTTGGCCT	AGGCCAAG
Shasta Long Forward Index 12	L5	GCGGTTCT	GCGGTTCT	AGAACCGC
Shasta Long Forward Index 13	M5	GCTTGATG	GCTTGATG	CATCAAGC
Shasta Long Forward Index 14	N5	GGCTCTCT	GGCTCTCT	AGAGAGCC
Shasta Long Forward Index 15	O5	TCAATGCT	TCAATGCT	AGCATTGA
Shasta Long Forward Index 16	P5	TGGTAATT	TGGTAATT	AATTACCA
Shasta Long Forward Index 17	A6	TTCTGAAC	TTCTGAAC	GTTCAGAA
Shasta Long Forward Index 18	B6	AACCAGAA	AACCAGAA	TTCTGGTT
Shasta Long Forward Index 19	C6	AACCGAAC	AACCGAAC	GTTCGGTT
Shasta Long Forward Index 20	D6	AACCGCCA	AACCGCCA	TGGCGGTT
Shasta Long Forward Index 21	E6	AATAAGGA	AATAAGGA	TCCTTATT
Shasta Long Forward Index 22	F6	ACCTTATT	ACCTTATT	AATAAGGT
Shasta Long Forward Index 23	G6	TGGTCCTG	TGGTCCTG	CAGGACCA
Shasta Long Forward Index 24	H6	CAACGAGG	CAACGAGG	CCTCGTTG
Shasta Long Forward Index 25	I6	CCAATGGA	CCAATGGA	TCCATTGG
Shasta Long Forward Index 26	J6	CGCCTATG	CGCCTATG	CATAGGCG
Shasta Long Forward Index 27	K6	CTCTCCAA	CTCTCCAA	TTGGAGAG
Shasta Long Forward Index 28	L6	GGCTTGAA	GGCTTGAA	TTCAAGCC
Shasta Long Forward Index 29	M6	GTTAAGTT	GTTAAGTT	AACTTAAC
Shasta Long Forward Index 30	N6	TCAAGTAT	TCAAGTAT	ATACTTGA
Shasta Long Forward Index 31	O6	TCGCGGAT	TCGCGGAT	ATCCGCGA
Shasta Long Forward Index 32	P6	TGAGTCCT	TGAGTCCT	AGGACTCA
Shasta Long Forward Index 33	A7	AGTTGAAC	AGTTGAAC	GTTCAACT
Shasta Long Forward Index 34	B7	AACCTCAG	AACCTCAG	CTGAGGTT
Shasta Long Forward Index 35	C7	AACGGTCT	AACGGTCT	AGACCGTT
Shasta Long Forward Index 36	D7	AACTCAAG	AACTCAAG	CTTGAGTT
Shasta Long Forward Index 37	E7	AACTCCGA	AACTCCGA	TCGGAGTT

Forward indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index name	Well position	Index sequence in primers	MiSeq, HiSeq 2000/2500, NovaSeq	MiniSeq, NextSeq, HiSeq 3000/4000, HiSeq X
Shasta Long Forward Index 38	F7	AAGGTTCA	AAGGTTCA	TGAACCTT
Shasta Long Forward Index 39	G7	AATTCGGT	AATTCGGT	ACCGAATT
Shasta Long Forward Index 40	H7	ACCAGACC	ACCAGACC	GGTCTGGT
Shasta Long Forward Index 41	I7	ACTTAGTA	ACTTAGTA	TACTAAGT
Shasta Long Forward Index 42	J7	AGCGGCAA	AGCGGCAA	TTGCCGCT
Shasta Long Forward Index 43	K7	AGGTCGAA	AGGTCGAA	TTCGACCT
Shasta Long Forward Index 44	L7	AGTCTGGA	AGTCTGGA	TCCAGACT
Shasta Long Forward Index 45	M7	ATAATGGT	ATAATGGT	ACCATTAT
Shasta Long Forward Index 46	N7	ATCCATTG	ATCCATTG	CAATGGAT
Shasta Long Forward Index 47	O7	ATGAATCT	ATGAATCT	AGATTCAT
Shasta Long Forward Index 48	P7	CAAGATTG	CAAGATTG	CAATCTTG
Shasta Long Forward Index 49	A8	CCGAATTG	CCGAATTG	CAATTCGG
Shasta Long Forward Index 50	B8	CCGGAGTT	CCGGAGTT	AACTCCGG
Shasta Long Forward Index 51	C8	CCTTCAGG	CCTTCAGG	CCTGAAGG
Shasta Long Forward Index 52	D8	CGAATATT	CGAATATT	AATATTCG
Shasta Long Forward Index 53	E8	CGGAGACT	CGGAGACT	AGTCTCCG
Shasta Long Forward Index 54	F8	CGTAGGCA	CGTAGGCA	TGCCTACG
Shasta Long Forward Index 55	G8	GAACTAAG	GAACTAAG	CTTAGTTC
Shasta Long Forward Index 56	H8	GAAGCTCG	GAAGCTCG	CGAGCTTC
Shasta Long Forward Index 57	I8	GACTATTG	GACTATTG	CAATAGTC
Shasta Long Forward Index 58	J8	GAGTAACG	GAGTAACG	CGTTACTC
Shasta Long Forward Index 59	K8	GCAGTCCA	GCAGTCCA	TGGACTGC
Shasta Long Forward Index 60	L8	GCTCAAGG	GCTCAAGG	CCTTGAGC
Shasta Long Forward Index 61	M8	GGATATCG	GGATATCG	CGATATCC
Shasta Long Forward Index 62	N8	GGTCAGAT	GGTCAGAT	ATCTGACC
Shasta Long Forward Index 63	O8	GTAAGAAG	GTAAGAAG	CTTCTTAC
Shasta Long Forward Index 64	P8	GTAGAAGT	GTAGAAGT	ACTTCTAC
Shasta Long Forward Index 65	A9	GTATCTGA	GTATCTGA	TCAGATAC
Shasta Long Forward Index 66	B9	GTCATCTA	GTCATCTA	TAGATGAC
Shasta Long Forward Index 67	C9	GTCCGCAA	GTCCGCAA	TTGCGGAC
Shasta Long Forward Index 68	D9	GTTCAATA	GTTCAATA	TATTGAAC
Shasta Long Forward Index 69	A10	TAACGTCG	TAACGTCG	CGACGTTA
Shasta Long Forward Index 70	B10	TCGGAACG	TCGGAACG	CGTTCCGA
Shasta Long Forward Index 71	C10	CATTCTAC	CATTCTAC	GTAGAATG
Shasta Long Forward Index 72	D10	TTACTTCT	TTACTTCT	AGAAGTAA

Table 7. Shasta Long Indexing Primer Set - A reverse indexing primer 1–72 indexes. Continued on the next page.

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Well position	Index name	Index sequence in primers	MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
A13	Shasta Long Reverse Index 1	AACCGGTT	AACCGGTT
B13	Shasta Long Reverse Index 2	AACCTAGA	TCTAGGTT
C13	Shasta Long Reverse Index 3	AAGACCAG	CTGGTCTT
D13	Shasta Long Reverse Index 4	AGAACGAC	GTCGTTCT
E13	Shasta Long Reverse Index 5	AGAACTCT	AGAGTTCT
F13	Shasta Long Reverse Index 6	ATTCAGCT	AGCTGAAT
G13	Shasta Long Reverse Index 7	CATACGTC	GACGTATG
H13	Shasta Long Reverse Index 8	CCATTATG	CATAATGG
I13	Shasta Long Reverse Index 9	CCTTGAAT	ATTCAAGG
J13	Shasta Long Reverse Index 10	TCTTGCCT	AGGCAAGA
K13	Shasta Long Reverse Index 11	CTAGTTGC	GCAACTAG
L13	Shasta Long Reverse Index 12	CTCGCGTA	TACGCGAG
M13	Shasta Long Reverse Index 13	CTTGAGTC	GACTCAAG
N13	Shasta Long Reverse Index 14	GAACGTAT	ATACGTTC
O13	Shasta Long Reverse Index 15	GACTGCGG	CCGCAGTC
P13	Shasta Long Reverse Index 16	GCGTACGG	CCGTACGC
A14	Shasta Long Reverse Index 17	GCTTCTCC	GGAGAAGC
B14	Shasta Long Reverse Index 18	GGAGGCTC	GAGCCTCC
C14	Shasta Long Reverse Index 19	GGAGTATG	CATACTCC
D14	Shasta Long Reverse Index 20	GTCGCTAG	CTAGCGAC
E14	Shasta Long Reverse Index 21	TCGTTCGA	TCGAACGA
F14	Shasta Long Reverse Index 22	TCTCTACC	GGTAGAGA
G14	Shasta Long Reverse Index 23	TGGCGACG	CGTCGCCA
H14	Shasta Long Reverse Index 24	TTCGATGA	TCATCGAA
I14	Shasta Long Reverse Index 25	TTGATCCA	TGGATCAA
J14	Shasta Long Reverse Index 26	AACCTGCC	GGCAGGTT
K14	Shasta Long Reverse Index 27	AACGCATC	GATGCGTT
L14	Shasta Long Reverse Index 28	AACGCCAT	ATGGCGTT
M14	Shasta Long Reverse Index 29	AACGCGCA	TGCGCGTT
N14	Shasta Long Reverse Index 30	AAGAATGG	CCATTCTT
O14	Shasta Long Reverse Index 31	AAGACGCT	AGCGTCTT
P14	Shasta Long Reverse Index 32	ACCAACCG	CGGTTGGT
A15	Shasta Long Reverse Index 33	ACCGAATG	CATTCGGT
B15	Shasta Long Reverse Index 34	ACTCGCTA	TAGCGAGT
C15	Shasta Long Reverse Index 35	AGAAGAGC	GCTCTTCT
D15	Shasta Long Reverse Index 36	AGAATCTC	GAGATTCT
E15	Shasta Long Reverse Index 37	ATGCTTAG	CTAAGCAT
F15	Shasta Long Reverse Index 38	CAGACCTT	AAGGTCTG

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Well position	Index name	Index sequence in primers	MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
G15	Shasta Long Reverse Index 39	CCGCTAGG	CCTAGCGG
H15	Shasta Long Reverse Index 40	CCGGTTAG	CTAACCGG
I15	Shasta Long Reverse Index 41	CCTCGACG	CGTCGAGG
J15	Shasta Long Reverse Index 42	CGAAGCTG	CAGCTTCG
K15	Shasta Long Reverse Index 43	CGACCGCG	CGCGGTCG
L15	Shasta Long Reverse Index 44	CGTCATAA	TTATGACG
M15	Shasta Long Reverse Index 45	CTAGGAGA	TCTCCTAG
N15	Shasta Long Reverse Index 46	CTATTCAT	ATGAATAG
O15	Shasta Long Reverse Index 47	CTCTACTT	AAGTAGAG
P15	Shasta Long Reverse Index 48	CTGATTGA	TCAATCAG
A16	Shasta Long Reverse Index 49	CTTCGTTA	TAACGAAG
B16	Shasta Long Reverse Index 50	GAAGCAGC	GCTGCTTC
C16	Shasta Long Reverse Index 51	GAATAGGC	GCCTATTC
D16	Shasta Long Reverse Index 52	GCTCTGCT	AGCAGAGC
E16	Shasta Long Reverse Index 53	GGAGCGCA	TGCGCTCC
F16	Shasta Long Reverse Index 54	GGCGGTAT	ATACCGCC
G16	Shasta Long Reverse Index 55	GGTAACGC	GCGTTACC
H16	Shasta Long Reverse Index 56	GGTACGCC	GGCGTACC
I16	Shasta Long Reverse Index 57	GGTAGAAT	ATTCTACC
J16	Shasta Long Reverse Index 58	GGTTAGTC	GACTAACC
K16	Shasta Long Reverse Index 59	GTCTCGCG	CGCGAGAC
L16	Shasta Long Reverse Index 60	GTTCTACG	CGTAGAAC
M16	Shasta Long Reverse Index 61	TAGTATCT	AGATACTA
N16	Shasta Long Reverse Index 62	TAGTTAGG	CCTAACTA
O16	Shasta Long Reverse Index 63	TATTGCGC	GCGCAATA
P16	Shasta Long Reverse Index 64	TCAGTTAA	TTAACTGA
A17	Shasta Long Reverse Index 65	TCCGTATA	TATACGGA
B17	Shasta Long Reverse Index 66	TCCTGAGA	TCTCAGGA
C17	Shasta Long Reverse Index 67	TCGTCGCC	GGCGACGA
D17	Shasta Long Reverse Index 68	TGGCGTTA	TAACGCCA
A18	Shasta Long Reverse Index 69	TGGTATGA	TCATACCA
B18	Shasta Long Reverse Index 70	TTAAGCGT	ACGCTTAA
C18	Shasta Long Reverse Index 71	TTCGCGAC	GTCGCGAA
D18	Shasta Long Reverse Index 72	TTGCATAT	ATATGCAA

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