

Takara Bio USA, Inc.

Shasta™ Total RNA-Seq Kit - 2 Chip User Manual for the ICELL8® cx Single-Cell System

Cat. No. 640288
for ICELL8 cx CELLSTUDIO™ v2.6 Software
(052925)

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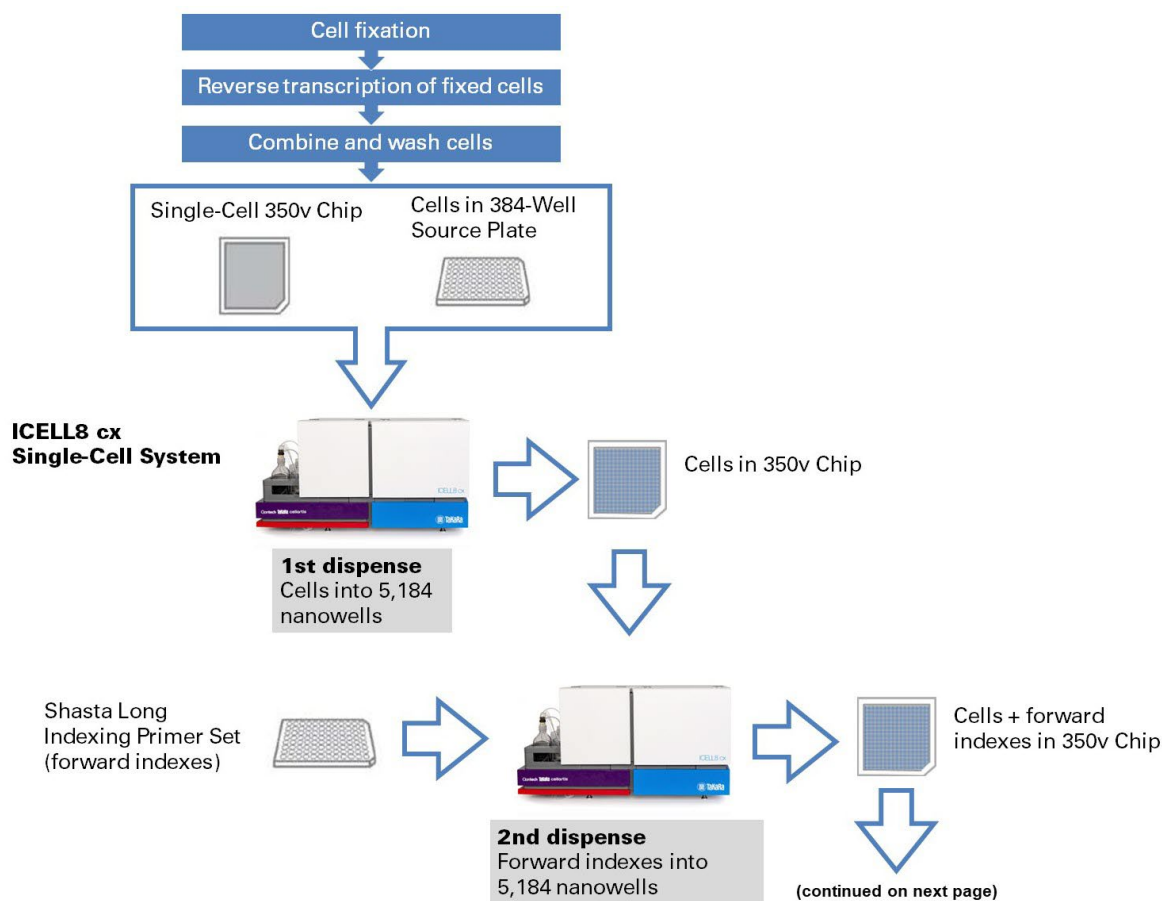
I. Introduction

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 ICELL8® cx Single-Cell System (Cat. No. 640188 or 640189). By leveraging the capabilities of the ICELL8 cx system along with SMART® (Switching Mechanism at 5' end of RNA Template) 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 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 ICELL8 cx system and ICELL8 cx CELLSTUDIO Software (v2.6.43 or later).

After cell dispense, an 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 ICELL8 cx instrument. 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.



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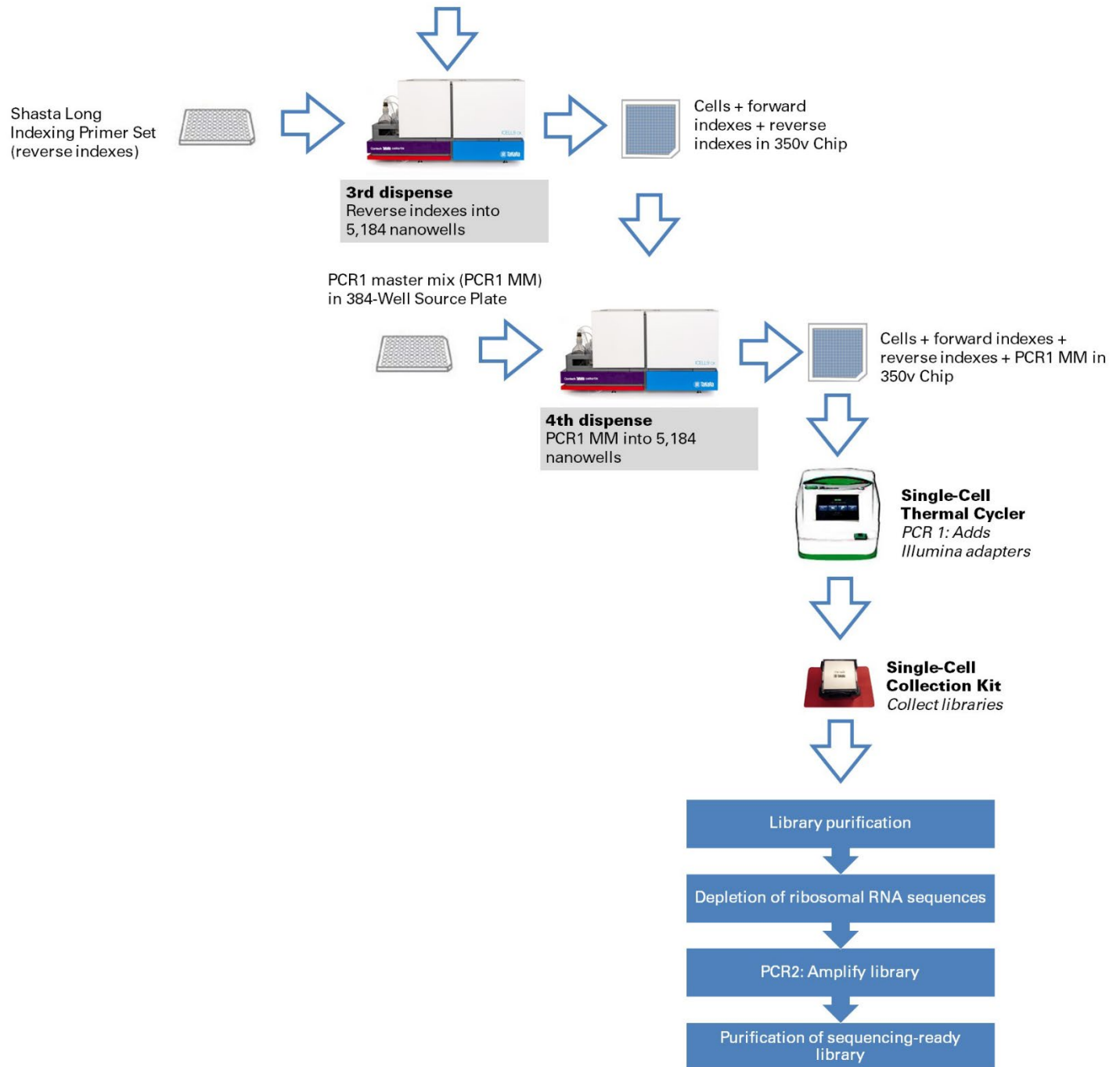


Figure 1. Complete Shasta Total RNA-Seq Kit workflow.

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

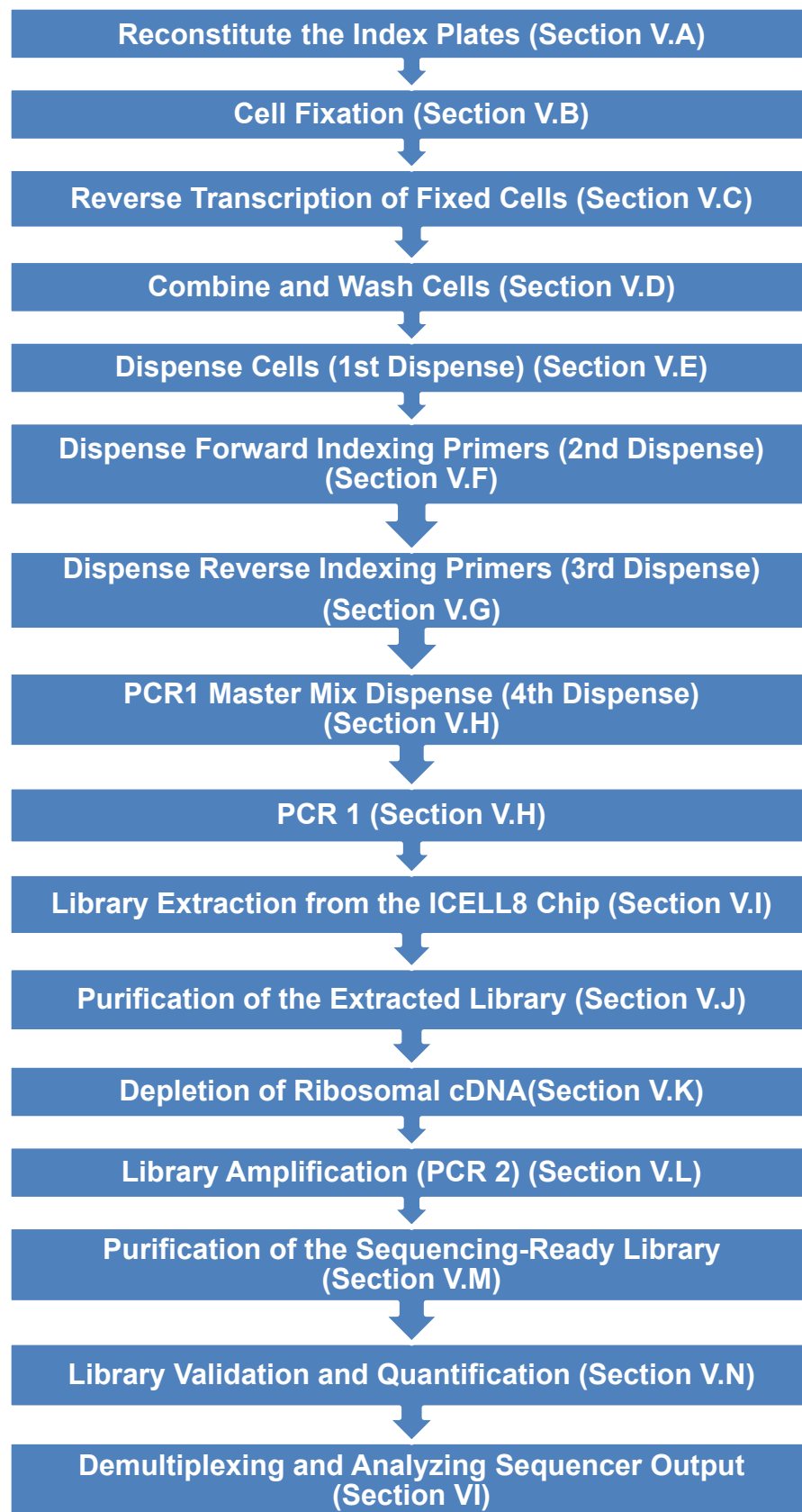


Figure 2. Protocols in the Shasta Total RNA-Seq workflow.

II. List of Components

The Shasta Total RNA-Seq Kit workflow requires use of an ICELL8 cx Single-Cell System (Takara Bio, Cat. No. 640188 or 640189) 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 ICELL8 cx instrument, additional chip holders can be ordered separately
- Plate Seal Applicator, included with the ICELL8 cx instrument
- 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), $\geq 2,600g$, 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 μ l, 10 μ l, 20 μ l, 200 μ l, and 1,000 μ l
- Multi-channel pipettes: 20 μ l and 200 μ l
- Filter pipette tips: 2 μ l, 20 μ l, 200 μ l, and 1,000 μ l
- Wide-bore pipette tips: 200 μ l and 1,000 μ l
- 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.J and Section V.M)

- NucleoMag NGS Clean-up and Size Select is preferred (Takara Bio; 5 ml size: Cat. No. 744970.5; 50ml 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.N)

- 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 $>2 \times 10^6$ 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 ICELL8 cx 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. ICELL8 cx System Application Notes

Refer to the [ICELL8 cx Single-Cell System User Manual](#) for full details. Included below are general reminders.

- All dispensing steps in the ICELL8 cx 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 ICELL8 cx 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).

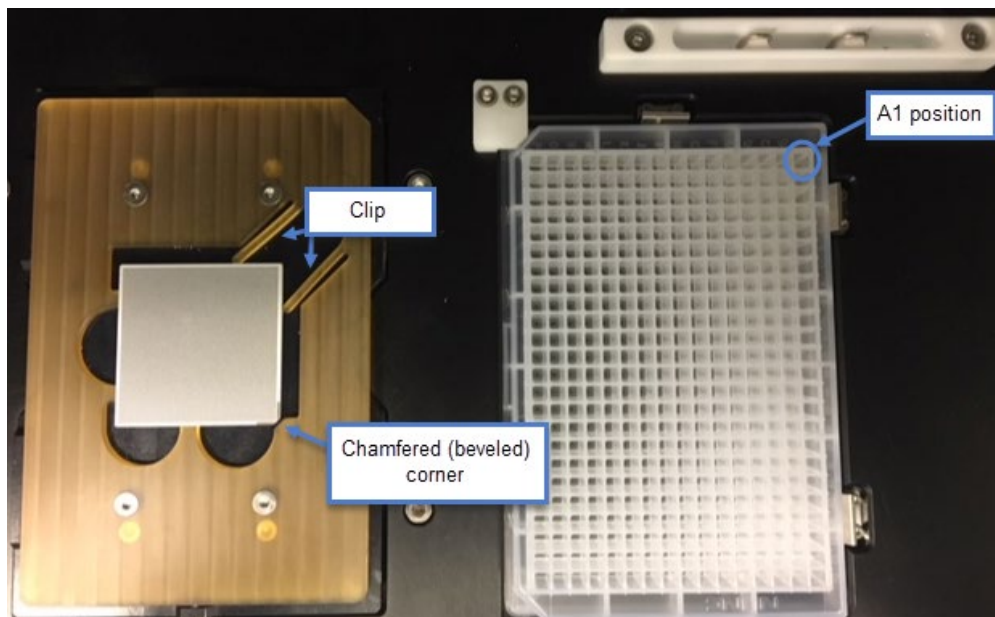


Figure 3. ICELL8 cx Single-Cell System Stage Module. (Left) chip nest. (Right) source plate nest.

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.

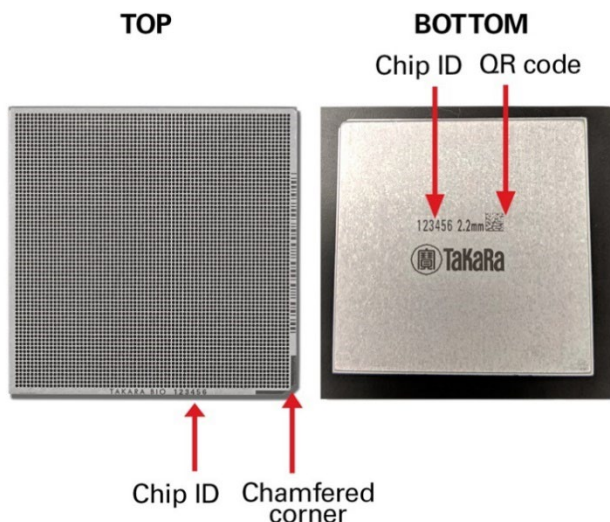


Figure 4. Single-Cell 350v Chip features. (Left) Top view of the Single-Cell 350v Chip. Note the chamfered (beveled) corner at the bottom right. The "TaKaRa" logo and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. (Right) Bottom view of the Single-Cell 350v 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 CELLSTUDIO v2.6.43 and CellSelect v2.6.52 Software. Please refer to the [ICELL8 cx Single-Cell System User Manual](#) and the [ICELL8 cx CellSelect v2.6 Software User Manual](#) for more detailed information.

Please contact [technical support](#) if you need to upgrade your ICELL8 software.

V. Procedure

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 3,000g at room temperature for 10 min.
2. Add 7 µl 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 foil sealing and centrifuge at 1,000g 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 3,000g 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	(5.00 ml)	Nuclease-Free Water
4,000 µl	(4.00 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 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).

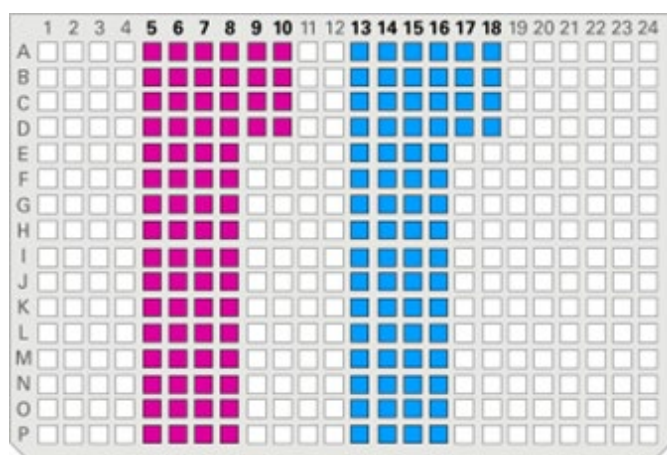


Figure 5. Rehydration buffer-dispense pattern for the Shasta Long Indexing Primer Set - A plate.

9. Seal the plate with sealing film and centrifuge the plate at 1,000g 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 an 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 and Chip Handling notes

- This protocol requires several milliliters of healthy cell suspensions with a cell density $\geq 0.7 \times 10^6$ cells/ml.
- Keep cells at 37°C with 5% CO₂ in a cell culture incubator when not performing manipulations.
- Perform all wash steps in an exhaust UV hood. Avoid exposing the cell culture 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.
- Put 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 ($\geq 2 \times 10^6$ cells) to a fresh 5 ml centrifuge tube.

NOTE: 2×10^6 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 $0.5\text{--}1 \times 10^6$ cells so that sufficient cells can be recovered after RT for the 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 $0.5\text{--}1 \times 10^6$ cells.

2. Centrifuge at 200g 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: 150–200g for 5 min.
- PBMCs or smaller cells: 300–500g 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 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 200g at 4°C for 5 min.
6. Carefully remove all supernatant without touching the pellet.
7. Tap the tube bottom to completely loosen the pellet, then add 200 μl 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 cells to a clean 5 ml tube. Add 50 μl 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 cold Quenching Solution to the cell solution. Mix by inverting the tube 2–3 times.
10. Centrifuge at 300g 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: 450–750g 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 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 $1.5\text{--}2.5 \times 10^6$ cells/ml in 500 µl of solution.

NOTE: This cell concentration will allow the whole RT plate to have $0.5\text{--}1 \times 10^6$ 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 1.5×10^6 cells/ml after fixation, more cells will be needed from other samples to ensure the total cell number of the RT plate is $0.5\text{--}1 \times 10^6$ cells.

If the cell concentration is higher than 2.5×10^6 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). 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 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 300g 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: 150–200g for 10 min.
- PBMCs or smaller cells: 300–500g for 10 min.

6. Add 4 ml of cold PBS and transfer the cells to a new 5 ml centrifuge tube. Centrifuge at 300g 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 to 95°C, and volume to 9 μl.
- Centrifuge the reconstituted TSO plate (from Section V.A) at 1,000g 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 decided 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

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

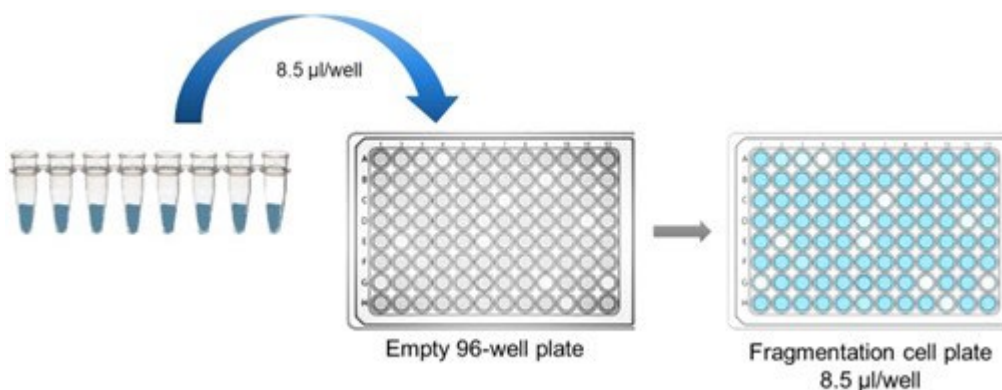


Figure 7. Creating the fragmentation cell plate. The workflow illustrates Steps 2–3.

- 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.
- Preheat the thermocycler according to the RT program in Step 13: cycler temperature at 10°C, lid temperature to 55°C, and volume to 20 µl.
- 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.

- 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 μ l 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 1,000g for 1 min at 4°C.

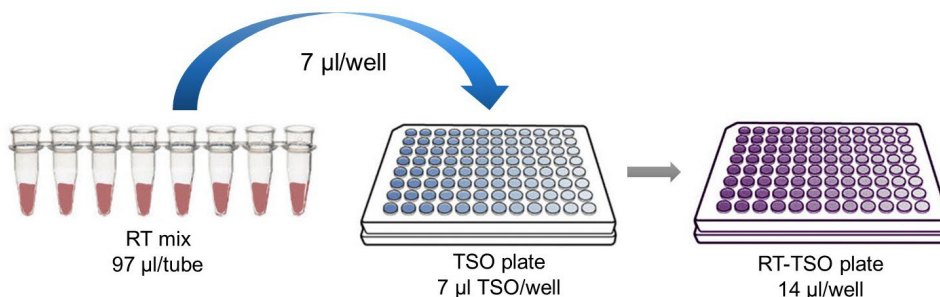


Figure 8. Creating the RT-TSO plate. The workflow illustrates Steps 8–9.

10. Transfer 11.5 μ l 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.**

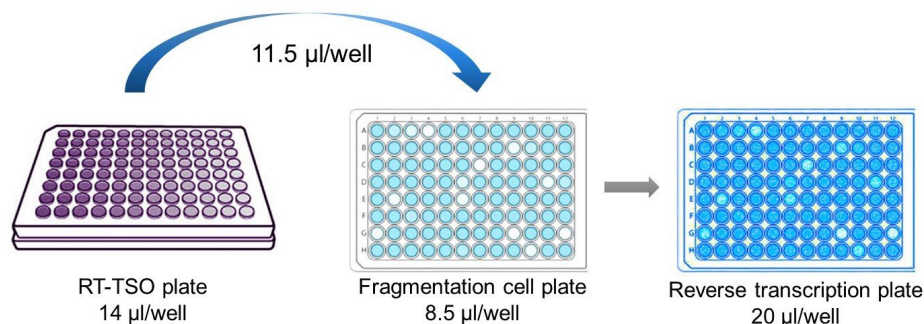


Figure 9. Creating the final plate used in the reverse transcription reaction.

11. Seal the fragmented cell (reverse transcription) plate with the sealing film. Do not vortex.
12. Centrifuge the plate at 100g 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:

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).

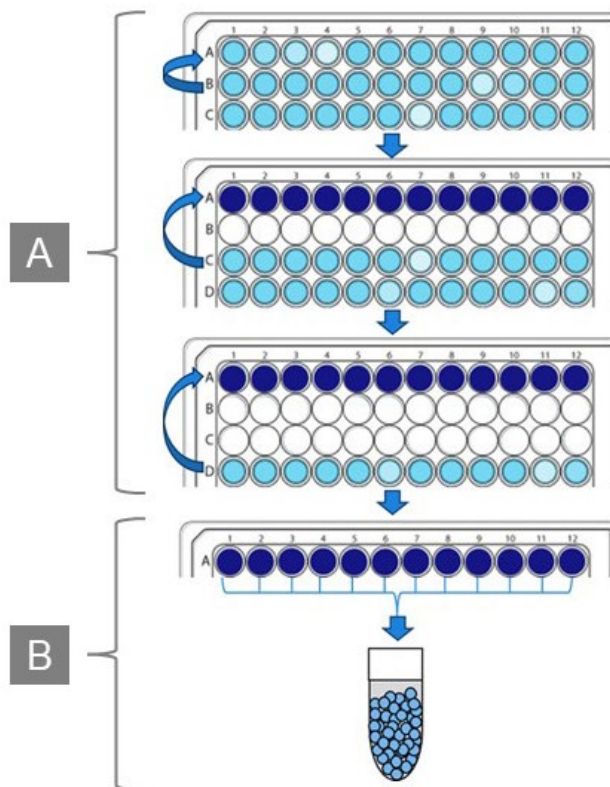


Figure 10. Combining RT products from Rows A–D of the plate into a 5 ml centrifuge tube. Panel A. Moving the contents of Rows B, C, and D on the plate to pool in the Row A wells. **Panel B.** All wells of Row A are further pooled into the 5 ml centrifuge tube.

- From the same RT products plate, transfer the contents of Row G, Row F, and Row E into Row H (Figure 11, Panel A).
- 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).

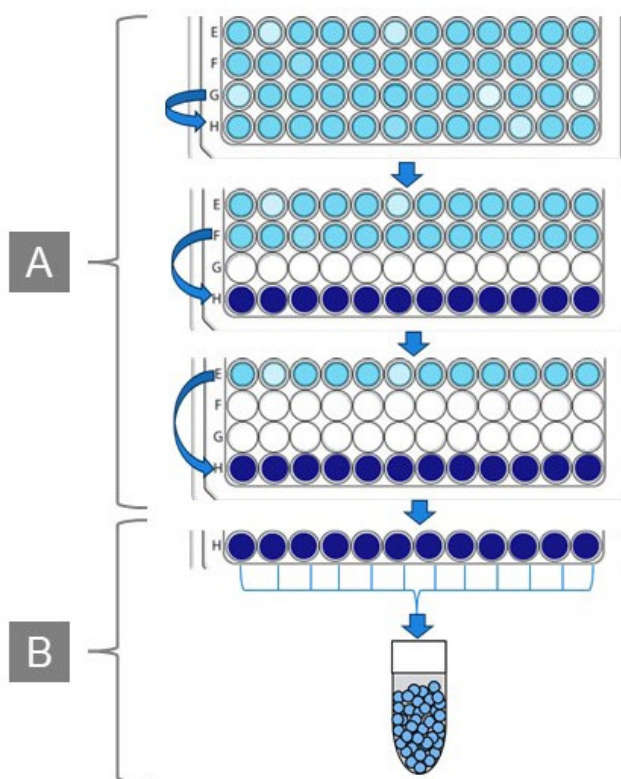


Figure 11. Combining RT products from Rows E–H of the plate into a 5 ml centrifuge tube. Panel A. Moving the contents of Rows G, F, and E on the plate to pool in the Row H wells. **Panel B.** All wells of Row H are further pooled into the 5 ml centrifuge tube.

- 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.

- Use a multichannel pipette to add 30 µl of wash buffer to each well of the RT products plate.
- 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.

- Invert the 5 ml tube five times. Centrifuge the tube at 1,000g at 4°C for 10 min.
- Gently remove the tube from the centrifuge and pipette out 4.4–4.9 ml supernatant, leaving about 100 µl 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 dispense the cells on the ICELL8 cx system.

E. Protocol: Dispense Cells on the ICELL8 cx 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
- Nanodispenser Chip Holder(s) (Figure 12)



Figure 12. Nanodispenser Chip Holder.

- Nanodispenser Chip Centrifuge Spinner and Nanodispenser Chip Centrifuge Balance (included with the ICELL8 cx instrument accessories)

2. Before You Start

- Perform a once-a-day warmup. Refer to the [ICeLL8 cx Single-Cell System User Manual](#) (Section VIII) for more details.

3. Procedure

IMPORTANT: Do not load cells into the source plate until the CELLSTUDIO software prompts with a window asking the user to mix the cells (Pause #1). If cells sit in the source plate wells before the cell dispensing starts, they will settle and cause nonuniform cell dispensing.

1. Select the protocol 'Shasta Total RNA-Seq'.

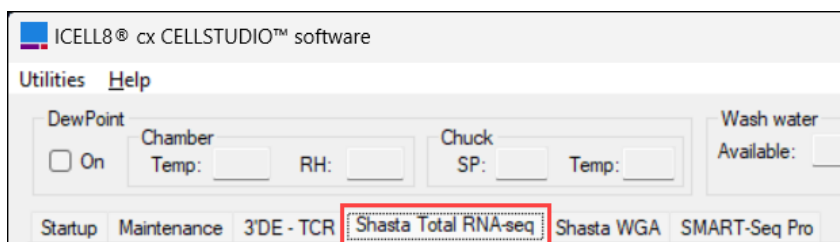


Figure 13. Selecting the Shasta Total RNA-Seq application in CELLSTUDIO software.

2. Click [Dispense Cells (50 nl) With Pause].

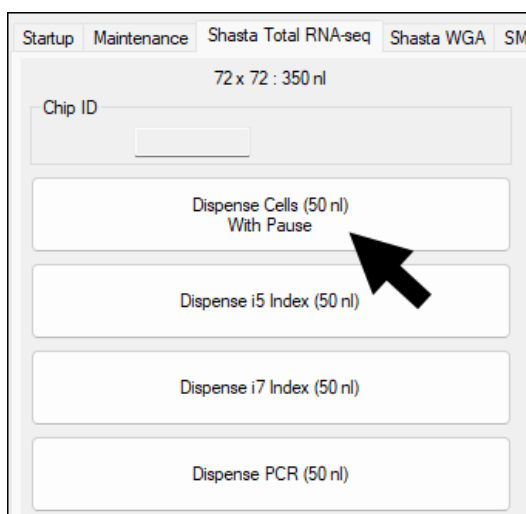


Figure 14. Use CELLSTUDIO software to dispense the cells.

3. A *Select index set* workflow window will display, asking you to indicate which indexing primer set you are using. Choose 'Shasta_Long_SetA' and click [Done].

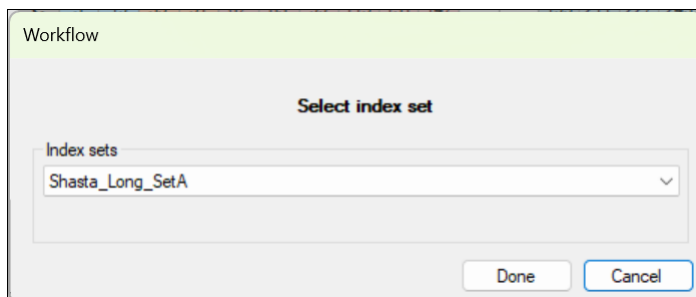


Figure 15. Select index set workflow prompt .

4. The software will pop up a window asking you to ensure the source plate and chip are correctly loaded on the instrument dispensing platform.

At this point, load the chip and an **empty** 384-Well Source Plate.

NOTE: Samples will not be loaded until Step 7.

5. In the software, click [Done].
6. Wait until the software prompts with a window asking the user to remix source plate (Pause #1).



Figure 16. Example dialog window when the dispense is paused prompting to remix the source plate.

- a. Open the door and take out the 384-Well Source Plate.
- b. Using a 1 ml pipette with a wide-bore tip, mix the recovered cells from Section V.D by slowly pipetting up and down five times to mix.
- c. Use a 200 μ l regular pipette tip to slowly and carefully load 35 μ l of cell solution into the bottom of wells A1, A2, B1, B2, C1, C2, D1, and D2 of the empty 384-Well Source Plate used in Step 4 (Figure 17). 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.

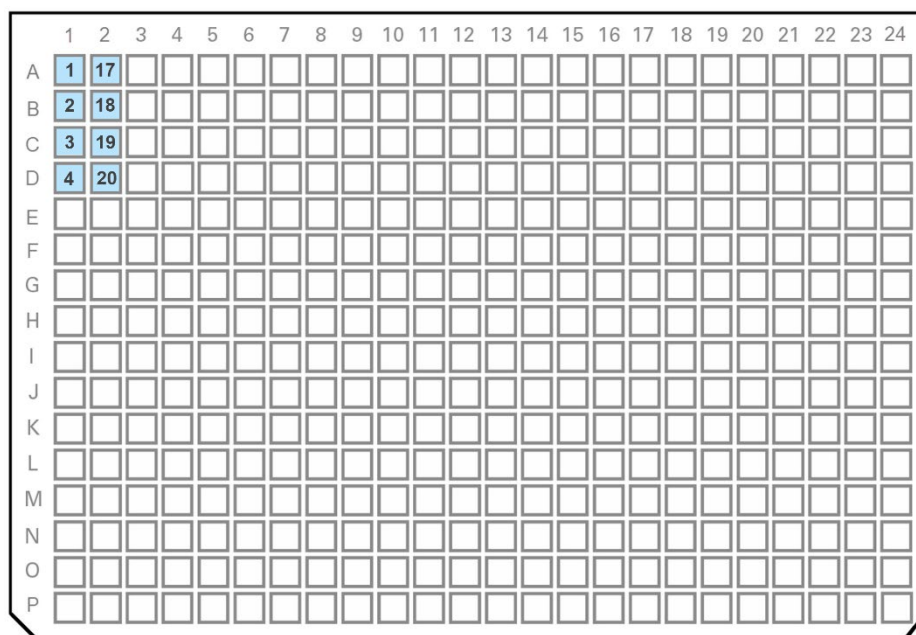


Figure 17. 384-Well Source Plate map for cell dispense. Wells A1–D2 (labeled 1–4, 17–20) are filled for Dispenses 2–4 (Steps 7–9)

- d. Place the 384-Well Source Plate back in the ICELL8 cx Single-Cell System plate nest.
7. In the software, click [OK]. Cell dispensing will start and continue until the next cell mix prompt (Pause #2). When prompted:
 - a. Open the door and discard the 384-Well Source Plate.
 - b. Using 1 ml pipette with the wide-bore tip, mix the recovered cells from Section V.D by slowly pipetting up and down five times to mix.
 - c. Using a 200 µl regular pipette tip and a new 384-Well Source Plate, slowly and carefully load 35 µl of cell solution into the bottom of wells A1, A2, B1, B2, C1, C2, D1, and D2 (Figure 17). Make sure no bubbles are trapped at the bottom of the wells.
 - d. Load the second 384-Well Source Plate in the ICELL8 cx Single-Cell System plate nest.
8. In the software, click [OK]. The cell dispensing will start again and continue until the next cell mix prompt (Pause #3). When prompted:
 - a. Open the door and discard the second 384-Well Source Plate.
 - b. Using 1 ml pipette with the wide-bore tip, mix the recovered cells from Section V.D by slowly pipetting up and down five times to mix.
 - c. Using a 200 µl regular pipette tip and a new 384-Well Source Plate, slowly and carefully load 35 µl of cell solution into the bottom of wells A1, A2, B1, B2, C1, C2, D1, and D2 (Figure 17). Again, make sure no bubbles are trapped at the bottom of the wells.
 - d. Load the third 384-Well Source Plate in the ICELL8 cx Single-Cell System plate nest.
9. Click [OK] on the screen. The cell dispensing will continue.
10. When dispensing is complete, remove the chip from the chip nest and blot with blotting paper. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.D "Blot and Centrifuge the Chip" for detailed instructions.

NOTE: Do not proceed if there are big droplets on the chip surface after dispensing. This indicates a problem with the instrument that needs to be checked.

11. Seal the loaded chip with the Chip Freezing Film following the directions in the system user manual.
12. Place the sealed chip on a Nanodispenser Chip Centrifuge Spinner and centrifuge the sealed chip at 3,000g at 4°C for 3 min with full acceleration and full brake. Balance the centrifuge with the Nanodispenser Chip Centrifuge Balance or a blank chip.
13. On the ICELL8 cx Single-Cell System, perform the [Tip Clean] procedure 3 times.

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

F. 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 protocol V.A).

1. Required Components

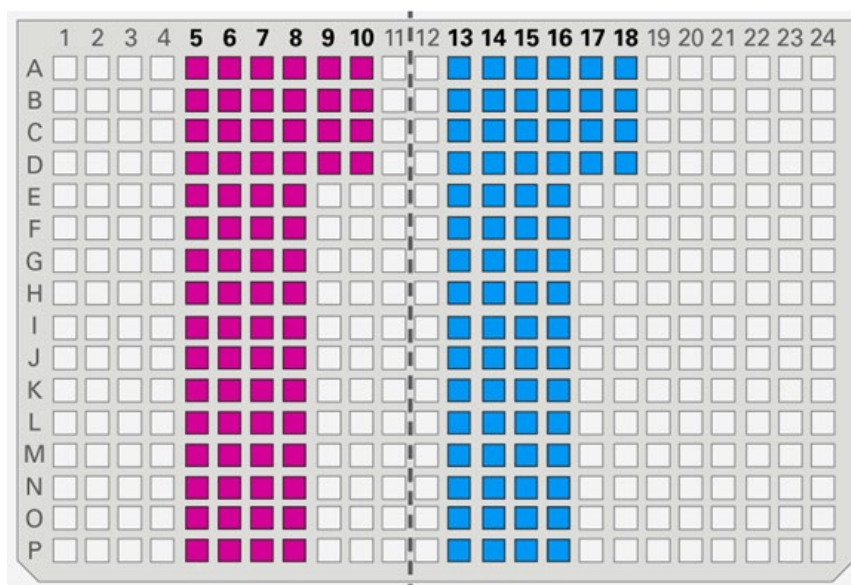
- From the Shasta Total RNA-Seq Kit - 2 Chip:
 - Single-Cell Loading Kit
 - Blotting Paper, RC Film
- Shasta Long Indexing Primer Set - A plate (prepared in Section V.A)
- A razor blade
- Film sealing roller

2. Before You Start

- Prepare the ICELL8 cx instrument.
 - If this step is done on the same day as the cell dispense, ensure the [Tip Clean] procedure was done 3 times after the previous protocol.
 - If this step is done on a different day from previous steps, perform a once-a-day warmup. Refer to the [ICELL8 cx Single-Cell System User Manual](#) (Section VIII) for more details.
- If the chip was stored frozen at -20°C , take the frozen chip out of the chip holder and thaw the chip facing right-side up on the benchtop at room temperature for at least 10 min.
- Centrifuge the thawed chip at 3,000g 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

1. Remove the Chip Freezing Film from the Single-Cell 350v Chip containing the dispensed cells. Place in the ICELL8 cx instrument chip nest per the directions for instrument use.
2. Centrifuge the reconstituted Shasta Long Indexing Primer Set - A plate at 1,000g at 4°C for 1 min before use.
3. Using the razor blade, cut the index plate seal into two halves along Column 11 and 12 (Figure 18).

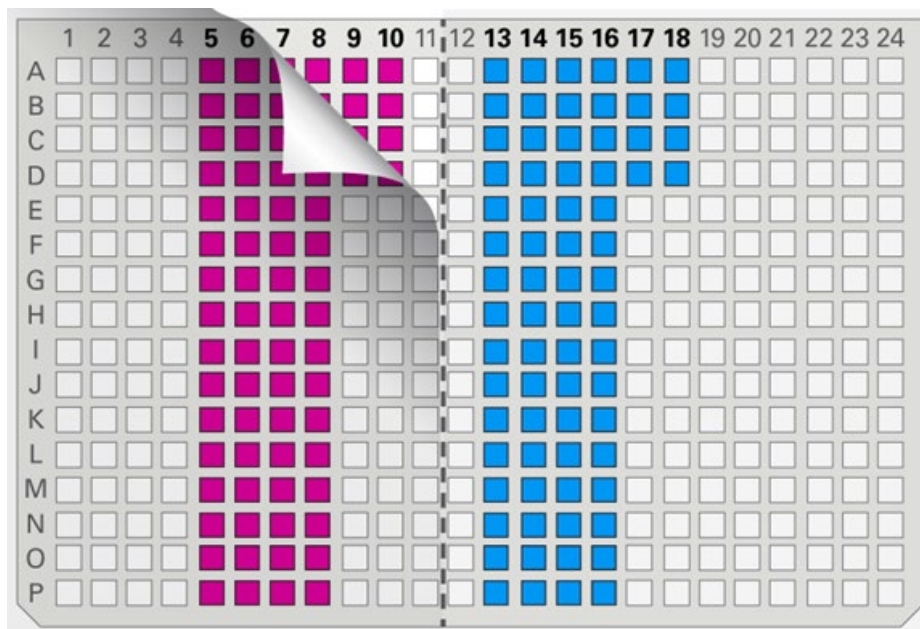


■ Forward indexing primers: A5 to P8, A9 to D9, and A10 to D10

■ Reverse indexing primers: A13 to P16, A17 to D17, and A18 to D18

Figure 18. 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.

- Remove the plate seal on the left-hand side to expose forward indexing primers (Columns 5–10, Figure 19).



■ Forward indexing primers: A5 to P8, A9 to D9, and A10 to D10

■ Reverse indexing primers: A13 to P16, A17 to D17, and A18 to D18

Figure 19. 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).

5. Refer to the [ICELL8 cx Single-Cell System User Manual](#), Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
 - a. Start in Section X.F, Step 5, with loading the index plate into the plate nest. Make sure that there are no air bubbles in the wells.
 - b. At Step 8, click the [Dispense i5 Index (50 nl)] button.
 - c. Follow Steps 9–14 as written in Section X.F.
 - d. For Step 15, seal with RC Film.
 - e. For Step 16, centrifuge the sealed Single-Cell 350v Chip at 3,000g at 4°C for 3 min with full acceleration and full brake.
6. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument.

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

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

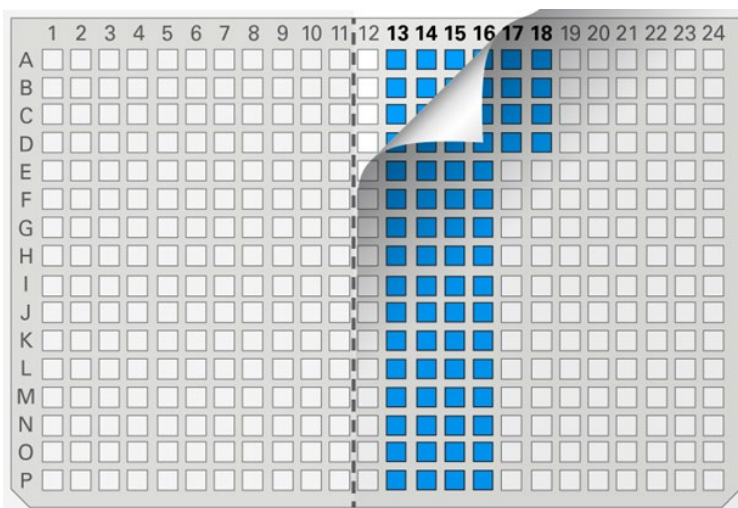
In this protocol, 72 reverse indexing primers are dispensed from the Shasta Long Indexing Primer Set - A plate used in the previous protocol.

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 20).



■ Reverse indexing primers: A13 to P16, A17 to D17, and A18 to D18

Figure 20. 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.F) and the plate was not centrifuged to remove them, remove the bubbles gently by pipette now.

2. Refer to the [ICELL8 cx Single-Cell System User Manual](#), Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
 - a. Start in Section X.F, Step 5, with loading the index plate into the plate nest.
 - b. At Step 8, click the [Dispense i7 Index (50 nl)] button.
 - c. Follow Steps 9–14 as written in Section X.F.
 - d. For Step 15, seal with RC Film.
 - e. For Step 16, centrifuge at 3,000g at 4°C for 5 min with full acceleration and full brake.
3. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument and prepare the Single-Cell Thermal Cycler (see next section).

NOT A SAFE STOPPING POINT: Immediately proceed to the next step (Section V.H, "PCR 1 Master Mix Dispense").

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

In this protocol, PCR reaction takes place inside nanowells of the chip and the second barcode will be 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 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

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

PCR 1 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 a fresh 384-Well Source Plate in wells A3, A4, B3, B4, C3, C4, D3 and D4 (Figure 21). Directly add to the bottom of the wells without trapping bubbles at the bottom or leaving the liquid on the well walls.

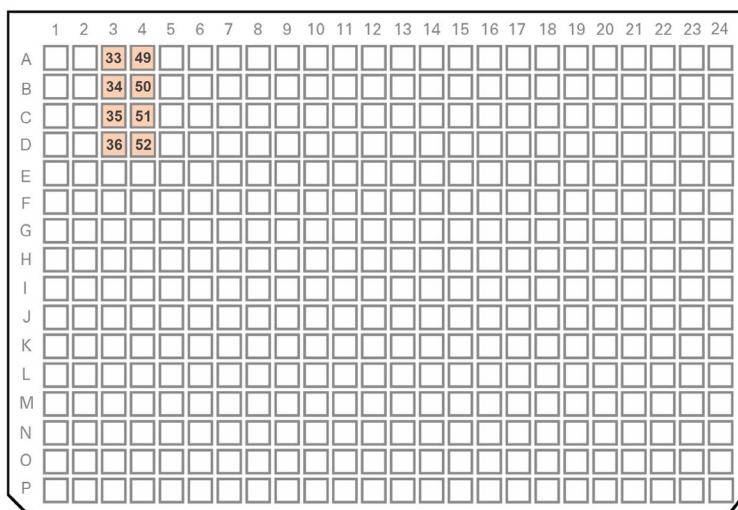


Figure 21. 384-Well Source Plate map for PCR1 master mix dispense. Wells A3–D4 (labeled 33–36, 49–52) are filled for the "Dispense PCR" step (Step 3).

3. Refer to the [ICELL8 cx Single-Cell System User Manual](#), Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
 - a. Start in Section X.F, Step 5, with loading the 384-Well Source Plate with the PCR1 master mix into the plate nest.
 - b. At Step 8, click the [Dispense PCR (50 nl)] button.
 - c. Follow Steps 9–14 as written in Section X.F.

NOTE: Do not proceed if there are big droplets on the chip surface after dispensing. This indicates a problem with the instrument that needs to be checked. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.D "Blot and Centrifuge the Chip" for detailed instructions.

- d. For Step 15, seal with RC Film.
 - e. For Step 16, centrifuge the sealed Single-Cell 350v Chip at 3,000g at 4°C for 5 min with full acceleration and full brake.
4. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument and prepare the thermal cycler. Run and immediately hold the PCR 1 program outlined in Step 6 below.
5. Make sure the thermal cycler lid temperature is at 72°C and the block temperature is at 96.5°C, volume is 0. After centrifugation, place the chip into the thermal cycler.

6. 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.

I. Protocol: Library Extraction from the Chip

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

Refer to the [ICELL8 cx Single-Cell System User Manual](#), Section XI "Protocol: Extract 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 protocol V.E, 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.J, 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.

J. 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.I, 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 (~2,000g) 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 ~1–2min 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.

K. 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 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.

- 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.

- Add 6.5 µl ZapR mix into four clean 0.2 ml PCR tubes.
- Add 15.5 µl of the purified library from the previous step (Section V.J) 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.
- 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.L, "Library Amplification") to perform the second PCR.

L. 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

- 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.K).
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.

M. 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 (~2,000g) 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.N).

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

N. 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. M). 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 22 for an example of a typical Bioanalyzer profile for a library generated by this protocol.

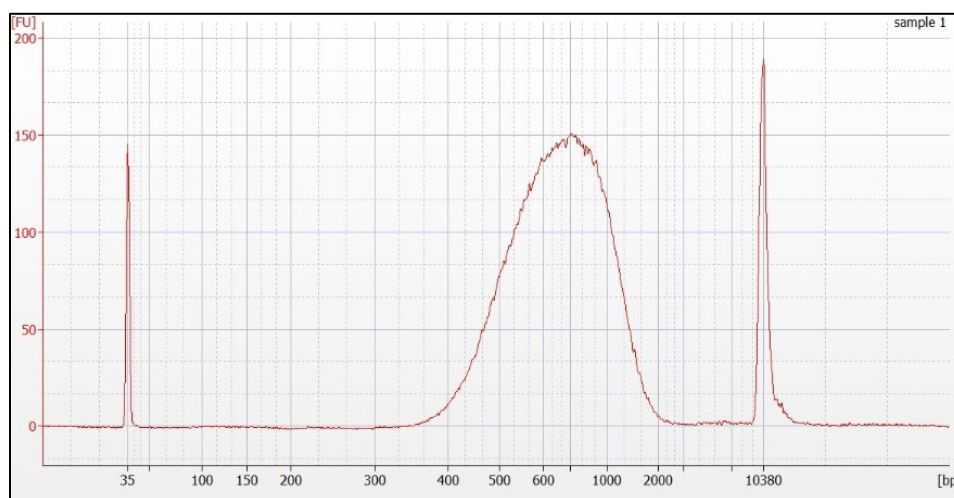


Figure 22. Example electropherogram results using the Agilent 2100 Bioanalyzer high sensitivity assay.

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.

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 22. Final library size may vary depending on cell types, cell number, and other factors.

Small peaks ≤ 200 bp (Figure 23, 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.K.

Large tails $> 2,000$ bp (Figure 23, 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>.

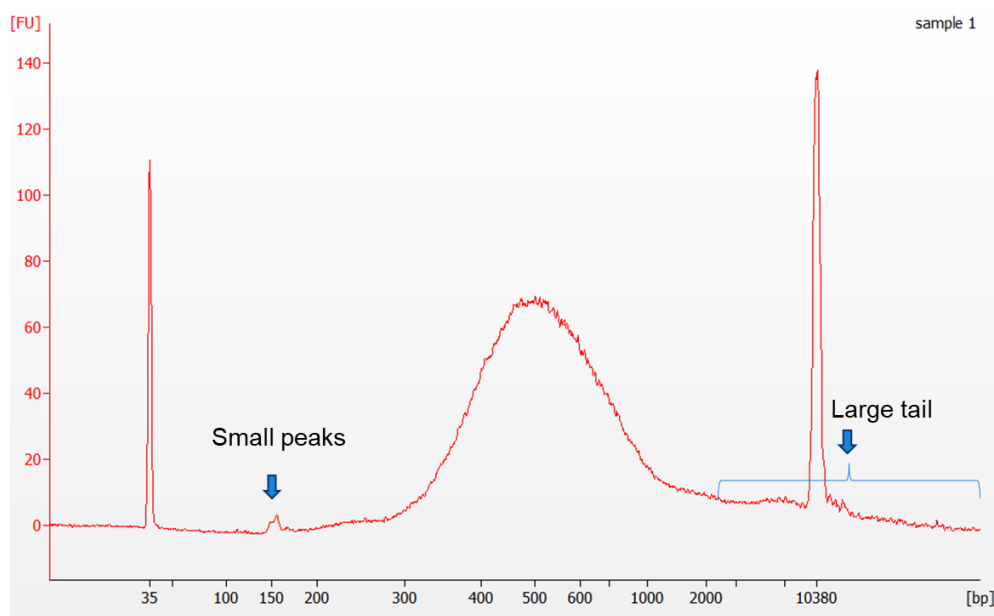


Figure 23. Example electropherogram results displaying abnormal peaks in the final library.

A sharp peak on the main curve (location may vary, example shown in Figure 24) indicates the rRNA is not sufficiently removed. Another round of ribosomal cDNA depletion (Section V.K) may help reduce the rRNA portion in the final library.

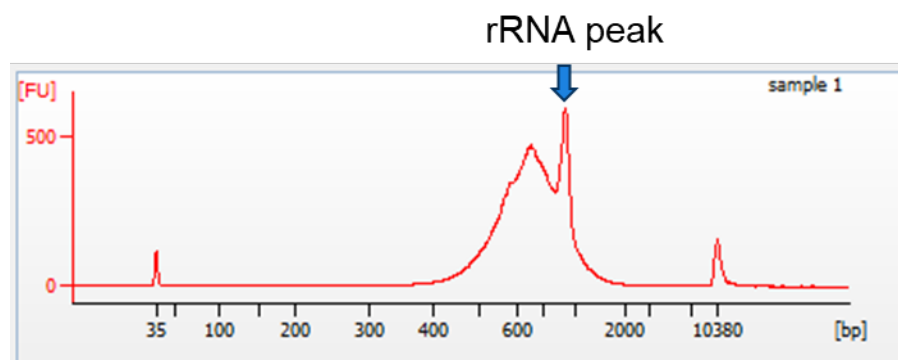


Figure 24. Example electropherogram results showing rRNA peaks in the final library.

Appendix B. PCR Cycling Guidelines

While 5 cycles of PCR during PCR 1 (Section V.H) will work to provide good transcript identification and sensitivity for many cell types, the number of PCR cycles for PCR 2 (Section V.L) 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 25.

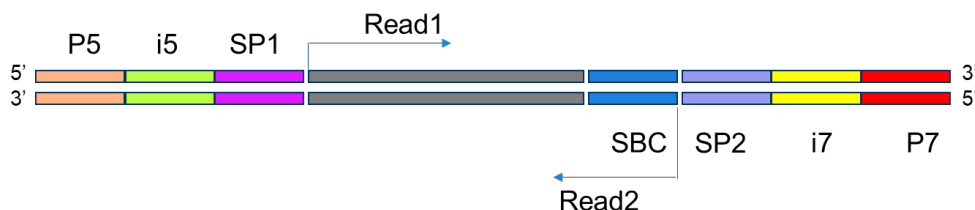


Figure 25. Structure of a final sequencing library. The final library is composed of the sample barcode (SBC), SP1 and SP2 (Illumina Truseq sequencing primer 1 and 2 sequences), i5 and i7 indexes, and P5 and P7 adapters.

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 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

Shasta Total RNA-Seq Kit uses TSO plate to add the sample barcode to differentiate samples. Sample barcode sequences are incorporated into Read2 after initial sequencing (see Figure 25, 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	ACGAACCT
14	B2	GGCTCTCT	46	D10	ATCCATTG	78	G6	ACGCAACC
15	B3	TCAATGCT	47	D11	ATGAATCT	79	G7	TTATCATC
16	B4	TG GTAATT	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	GTA CTATC
21	B9	AATAAGGA	53	E5	TATTGCTT	85	H1	AGCAAGGC
22	B10	ACCTTATT	54	E6	CGTAGGCA	86	H2	CATTCATC
23	B11	TGGTCCTG	55	E7	GA ACTAAG	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	G TAGAAGT	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	TG GTAATT	TG GTAATT	AATTACCA
Shasta Long Forward Index 17	A6	TTCTGAAC	TTCTGAAC	G TTCAGAA
Shasta Long Forward Index 18	B6	AACCAGAA	AACCAGAA	TTCTGGTT
Shasta Long Forward Index 19	C6	AACCGAAC	AACCGAAC	G TTCGGTT
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	GT TAAGTT	GT TAAGTT	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

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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 35	C7	AACGGTCT	AACGGTCT	AGACCGTT
Shasta Long Forward Index 36	D7	AACTCAAG	AACTCAAG	CTTGAGTT
Shasta Long Forward Index 37	E7	AACTCCGA	AACTCCGA	TCGGAGTT
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	AGATTTCAT
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	TCGTTCTGA	TCGAACGA
F14	Shasta Long Reverse Index 22	TCTCTACC	GGTAGAGA
G14	Shasta Long Reverse Index 23	TGGCGACG	CGTCGCCA
H14	Shasta Long Reverse Index 24	TTGATCCA	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
G15	Shasta Long Reverse Index 39	CCGCTAGG	CCTAGCGG

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
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	CGCGGTCTG
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	TCGTGCGC	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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This document has been reviewed and approved by the Quality Department.