Takara Bio USA, Inc.

ICELL8® cx Human TCR a/b Profiling User Manual

Cat. Nos. 640200, 640179, 640180, 640181, 640182, 640197, 640212
for ICELL8 cx CELLSTUDIO™ v2.0 Software
(030920)

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I. Introduction

High-throughput TCR profiling of single cells

The ICELL8 cx Human TCR a/b Profiling workflow enables users to analyze T-cell receptor (TCR) diversity from single T cells isolated on the ICELL8 cx Single-Cell System (Cat. No. 640188 or 640189). The workflow consists of the ICELL8 cx TCR Chip (Cat. No. 640200), the ICELL8 Human TCR a/b Profiling - Indexing Primer Set (Cat. Nos. 640179, 640180, or 640181), the ICELL8 Human TCR a/b Profiling Reagent Kit (Cat. No. 640182), the ICELL8 Collection Kit – L (Cat. No. 640212), and the ICELL8 cx Loading Kit (Cat. No. 640197), each of which must be purchased separately. Our TCR profiling solution leverages SMART® technology (**S**witching **M**echanism **A**t 5′ End of **R**NA **T**emplate) and employs a 5′ RACE-like approach to capture complete V(D)J variable regions of TCR transcripts. As the name suggests, the workflow allows generation of libraries that provide information on both alpha- and beta-chain diversity. Included in the kit are primers that incorporate Illumina®-specific adaptor sequences during cDNA amplification for the generation of indexed libraries that are ready for sequencing on Illumina platforms.

The workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls, followed by the dispensing of the cells and controls using ICELL8 cx CELLSTUDIO™ v2.0 Software on the ICELL8 cx Single-Cell System into the wells of a 5,184-nanowell ICELL8 cx TCR Chip. A total of 1,728 unique barcoded oligos are preprinted in nanowells of each chip, and each barcode is printed three times on a chip. As many as eight different samples can be analyzed in a single run, and the dispensing process is completed in approximately 15 minutes. During this time, cells are maintained in the humidity- and temperature-controlled environment provided by the ICELL8 cx Single-Cell System.

Following the dispensing of the samples and controls, the ICELL8 cx Single-Cell System is used to acquire images of the 5,184 nanowells with both blue and red wavelength filters, enabling visualization of Hoechst 33342 and propidium iodide staining, respectively (Hoechst staining is used to visualize and identify candidate cells based on size/morphology criteria, while propidium iodide staining is used to identify dead cells). Following image acquisition, images are analyzed either automatically or manually using ICELL8 cx CellSelect® v2.0 Software, which identifies nanowells containing single, viable cells that meet user-defined criteria. Upon identification and selection of candidate nanowells, the ICELL8 cx CellSelect v2.0 Software generates a file that will be used to direct the dispensing of RT-PCR reagents to selected nanowells for downstream cDNA synthesis and amplification steps.

Following the freezing of the ICELL8 cx TCR Chip, the chip is returned to the ICELL8 cx Single-Cell System and RT-PCR reagents are distributed to nanowells selected by the software. The ICELL8 cx TCR Chip is then transferred to a thermal cycler (for Cat. No. 640188, the ICELL8 cx Thermal Cycler), which is programmed to perform first-strand synthesis and amplification of cDNA in a single run. During this process (Figure 1), first-strand cDNA synthesis is dT-primed (oligo dT amp primer) and performed by the MMLV-derived SMARTScribe™ Reverse Transcriptase (RT), which adds nontemplated nucleotides upon reaching the 5′ end of each mRNA template. The 5′ SMART barcoded primer, preprinted into each nanowell of the ICELL8 cx TCR Chip, anneal to these nontemplated nucleotides and with template-switching technology adds an adapter and nanowell-specific barcode to the 5′ ends of full-length cDNAs. The added adapter then serves as a priming site during the PCR amplification portion of the program, allowing for unbiased amplification of full-length cDNA. The barcoded cDNAs from each nanowell are pooled, concentrated, and purified off-chip using AMPure XP beads. The purified cDNA can also be used for generating Illumina sequencing libraries using the Nextera® XT DNA Library Preparation kit, to enrich for cDNA derived from mRNA 5′ ends as described in Appendix E. After

pooling and a cleanup step, two rounds of gene-specific PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of TCRa and/or TCRb transcripts:

- The first gene-specific PCR uses the amplified double-stranded cDNA as a template and includes a forward primer with complementarity to the SMART sequence—which also incorporates the Illumina Read 1 sequence (Primer P5)—and reverse primers that are complementary to the constant (i.e., nonvariable) region of TCRa and TCRb genes (TCRa and TCRb Human Primer 1). By priming from the SMART sequence and the constant region, the first PCR specifically amplifies the entire variable region and a considerable portion of the constant region of TCRa and TCRb cDNA.
- The second gene-specific PCR takes the product from the first PCR as a template and uses same forward primer and the reverse primers bind in the constant region, internal to the PCR1 primers (ICELL8 TCRa and TCRb Human Primer 2 forward HT Indexes) allowing amplification of the entire variable region and a portion of the constant region of TCRa and TCRb cDNA. The forward and reverse primers include adapter and index sequences that are compatible with the Illumina sequencing platform and allow for multiplexing of samples.

Following post-PCR purification, size selection, and quality analysis, the library is ready for sequencing.

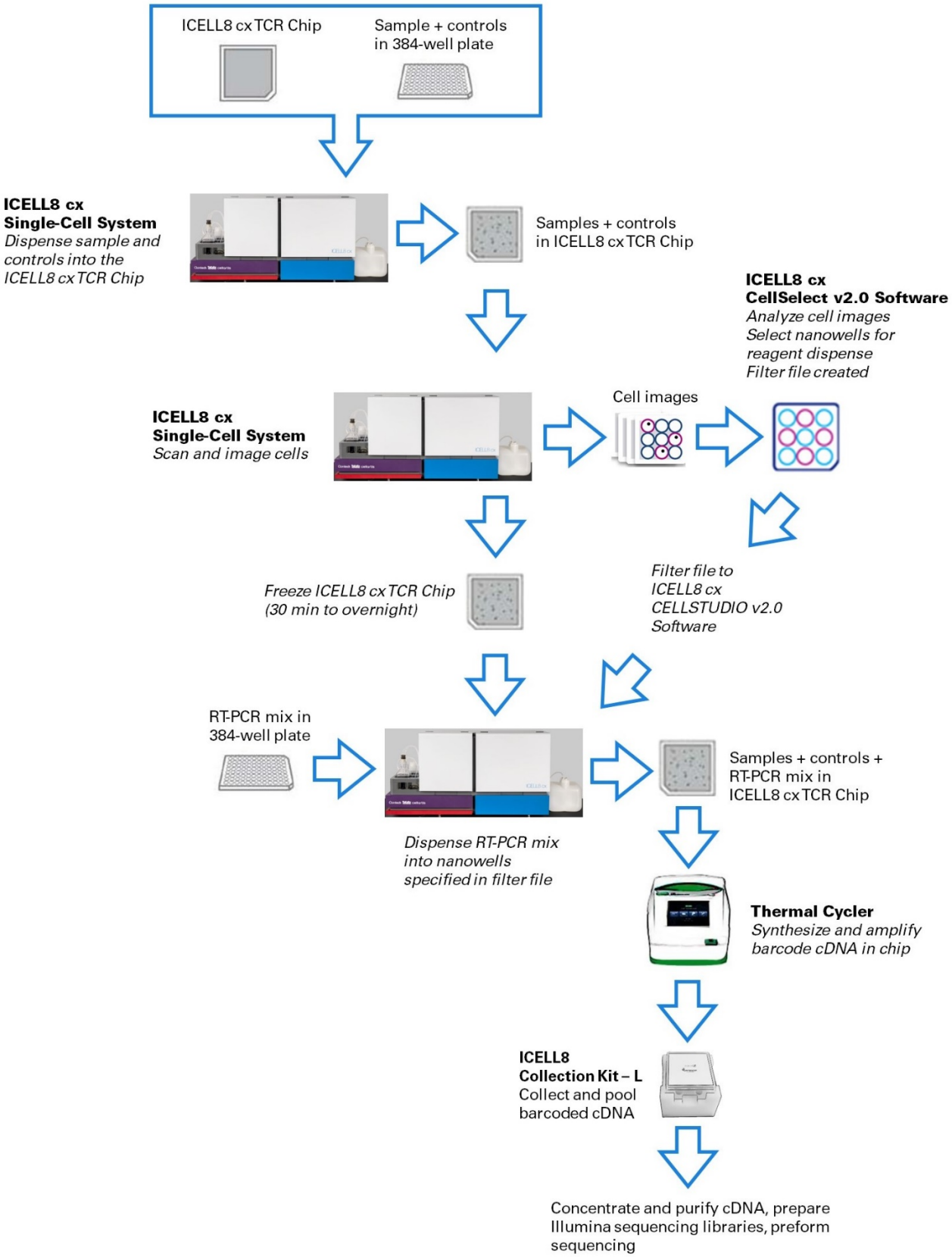

Figure 1. Workflow diagram for the ICELL8 cx Human TCR a/b Profiling workflow.

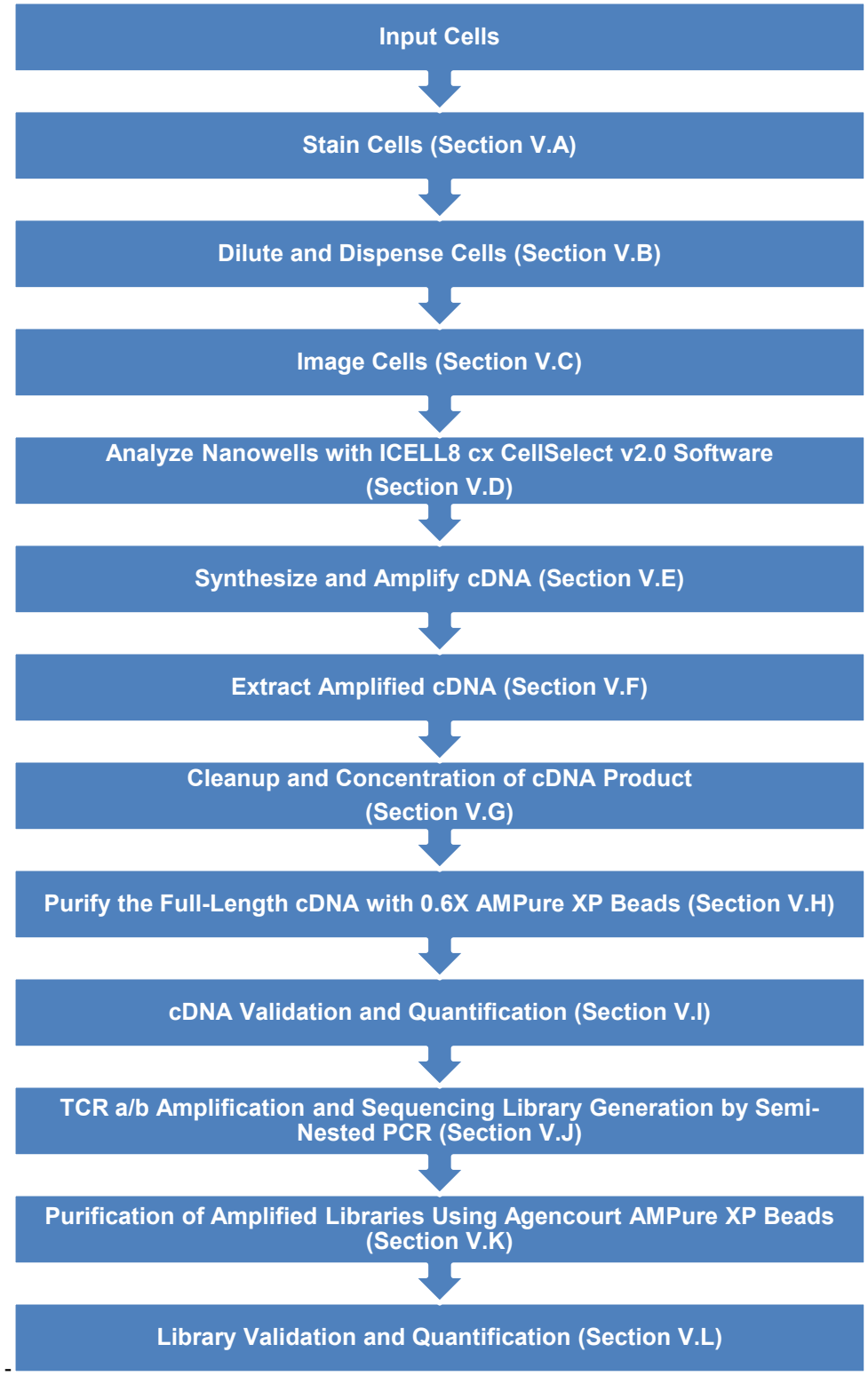

Figure 2. Protocols for ICELL8 cx Human TCR a/b Profiling.

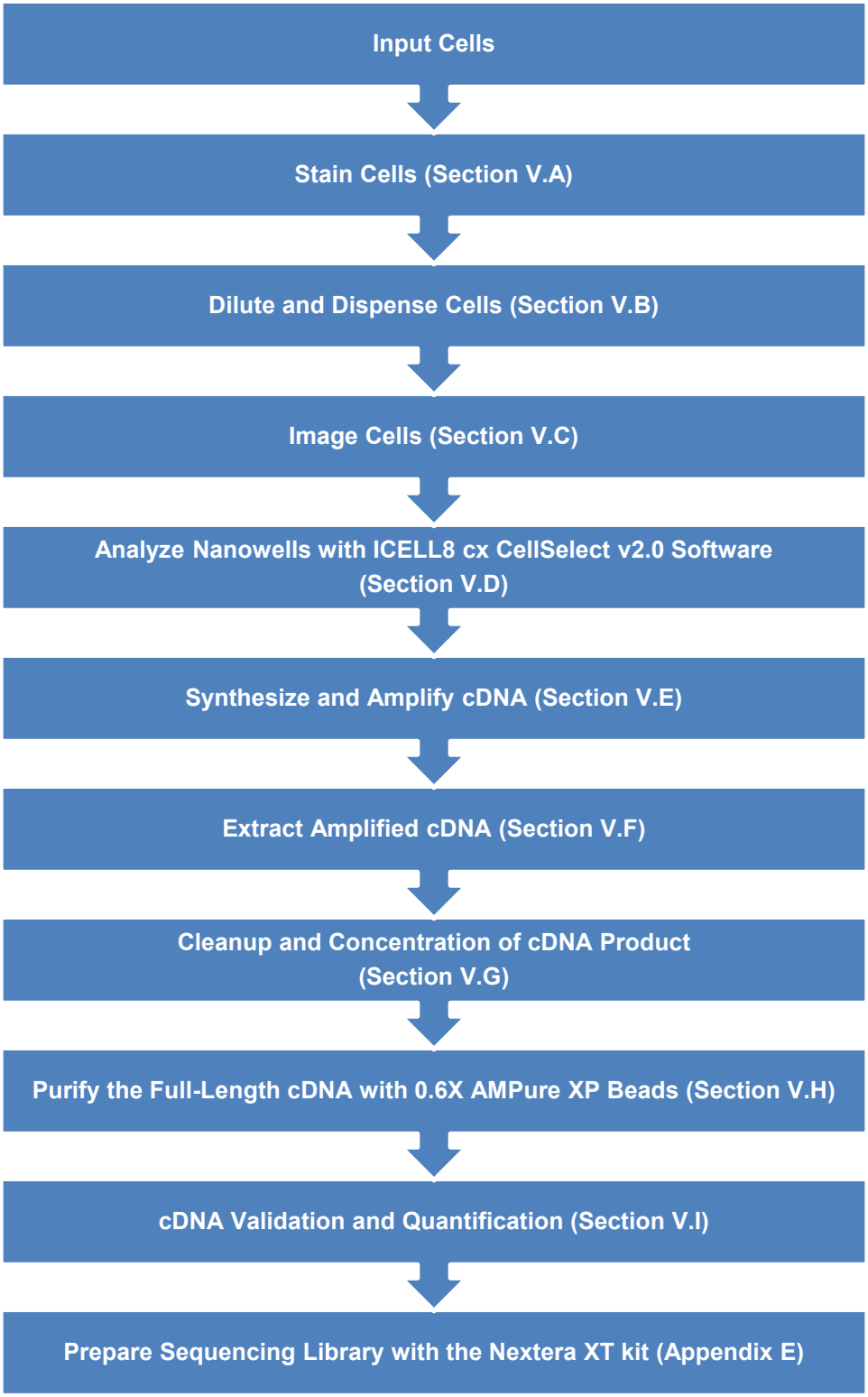

Figure 3. Protocols for 5′ DE.

II. List of Components

The ICELL8 cx Human TCR a/b Profiling workflow requires use of an ICELL8 cx Single-Cell System (Cat. No. 640188, 640189) and consists of the ICELL8 cx TCR Chip (Cat. No. 640200), the ICELL8 Human TCR a/b Profiling - Indexing Primer Set (Cat. Nos. 640179, 640180, or 640181), the ICELL8 Human TCR a/b Profiling Reagent Kit (Cat. No. 640182), the ICELL8 Collection Kit – L (Cat. No. 640212), and the ICELL8 cx Loading Kit (Cat. No. 640197), each of which must be purchased separately.

ICELL8 cx Human TCR a/b Profiling workflow components

Component Breakdown
ICELL8 cx TCR Chip (Cat. No. 640200, store at –20°C)

ICELL8 Human TCR a/b Profiling - Indexing Primer Set (Store at –20°C).			
	640179 (1 chip)	640180 (5 chips)	640181 (10 chips)
ICELL8 TCRa Human Primer 2 Forward HT Index 1 (aF1; 2 µM)	10 µl	10 µl	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 2 (aF2; 2 µM)	-	10 µl	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 3 (aF3; 2 µM)	-	10 µl	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 4 (aF4; 2 µM)	-	10 µl	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 5 (aF5; 2 µM)	-	10 µl	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 6 (aF6; 2 µM)	-	-	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 7 (aF7; 2 µM)	-	-	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 8 (aF8; 2 µM)	-	-	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 9 (aF9; 2 µM)	-	-	10 µl
ICELL8 TCRa Human Primer 2 Forward HT Index 10 (aF10; 2 µM)	-	-	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 1 (bF1; 2 µM)	10 µl	10 µl	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 2 (bF2; 2 µM)	-	10 µl	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 3 (bF3; 2 µM)	-	10 µl	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 4 (bF4; 2 µM)	-	10 µl	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 5 (bF5; 2 µM)	-	10 µl	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 6 (bF6; 2 µM)	-	-	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 7 (bF7; 2 µM)	-	-	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 8 (bF8; 2 µM)	-	-	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 9 (bF9; 2 µM)	-	-	10 µl
ICELL8 TCRb Human Primer 2 Forward HT Index 10 (bF10; 2 µM)	-	-	10 µl

Component breakdown continued on Page 10.

ICELL8 Human TCR a/b Profiling Reagent Kit (Cat. No. 640182)	**Cap color**	**Volume**
Package 1 (Store at –70°C)		
Control Jurkat Total RNA (1 µg/µl)	Yellow	5 µl
Package 2 (Store at –20°C)		
Second Diluent (100X)	Light blue	15 µl
RNase Inhibitor (40 U/µl)	Pink	15 µl
ICELL8 Fiducial Mix (1X)	-	25 µl
cDNA Amplification dNTP Mixture (25 mM each)	White	25 µl
Triton X-100 (3%)	Blue	10 µl
SMARTScribe Buffer (10X)	White	35 µl
GC Melt (5 M)*	White	60 µl
$MgCl_2$ (1 M)	Blue	10 µl
Oligo dT Amp Primer (100 µM)	Pink	10 µl
DTT (100 mM)	Red	10 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	50 µl
cDNA Amplification Buffer (2X)	White	35 µl
ICELL8 Amp Primer (10 µM)	Khaki	10 µl
cDNA Amplification Polymerase (50X)	Orange	12 µl
TCRa Human Primer 1 (2 µM)	Orange	10 µl
TCRb Human Primer 1 (2 µM)	Orange	10 µl
Primer P5 (5 µM)	Brown	15 µl
TCR Amplification Polymerase (1.25 U/µl)	Green	10 µl
TCR Amplification Buffer (5X)	White	25 µl
TCR Amplification dNTP Mixture (2.5 mM each)	Green	10 µl
Nuclease-Free Water	-	500 µl

*At times, precipitate may be observed in the GC Melt reagent. This precipitate does not affect the performance of the kit. The precipitate can be dissolved rapidly by mixing at room temperature or warming at 37°C for a few min.

ICELL8 Collection Kit – L (Cat. No. 640212, store at room temperature)	
Component	**Quantity per kit**
Collection Fixture – L	1
Collection Tube (2.0 ml)	2
Collection Film	1

ICELL8 cx Loading Kit (Cat. No. 640197, store at room temperature)	
Component	**Quantity per kit**
Blotting Paper	2
Optical Imaging Film	1
TE Sealing Film	1
SmartChip™ Intermediate Film	1

Details on the components included in these products are available for download at takarabio.com.

III. Additional Materials Required

The following reagents and materials are required but not supplied with the ICELL8 cx Human TCR a/b Profiling workflow products or the ICELL8 cx Single-Cell System:

General lab supplies:

- Personal protective equipment (PPE; e.g., powder-free gloves, safety glasses, lab coat, sleeve protector)
- Nuclease-decontamination solution
- Nuclease-free water
- Isopropanol
- Ethanol
- Thermal cycler with block for 0.2-ml tubes
- Centrifuges and rotors for conical tubes and plates (e.g., Eppendorf 5810R with swinging plate buckets, ≥2,600*g*, and room temperature & 4°C operation; Kubota 3740 with rotor SF-240 for cell preparation)
- 50-ml and 15-ml conical tubes
- Nuclease-free: 0.2-ml PCR tubes and nonstick 1.5-ml tubes
- ICELL8 384-Well Source Plate and Seal (Takara Bio, Cat. No. 640192)
- Single-channel pipettes: 10 µl , 20 µl , 200 µl , and 1,000 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 pipettes and controller
- Minicentrifuges for 1.5-ml tubes and 0.2-ml tubes or strips
- Vortex mixer
- Exhaust hood system with UV

For ICELL8 cx Single-Cell System general operation:

- Deionized water (for water reservoir and humidifier)
- Freshly mixed 0.2% sodium hypochorite solution

For staining and dispensing cells:

- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4; Thermo Fisher Scientific, Cat. No. 10010-023)
- ReadyProbes Cell Viability Imaging Kit, Blue/Red (contains Hoechst 33342 and propidium iodide; Thermo Fisher Scientific, Cat. No. R37610)
- TrypLE Express (Life Technologies, Cat. No. 12604-021)
- ICELL8 384-Well Source Plate and Seal (Takara Bio, Cat. No. 640192)
- Molecular biology-grade/PCR-grade/nuclease-free water

For cDNA concentration and quantification:

- NucleoSpin® Gel and PCR Clean-up Kit (Takara Bio, Cat. No. 740609.50)
- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Fluorometer (Thermo Fisher Scientific)
- Agilent 2100 Bioanalyzer (Sections V.H and V.K): High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)

For cDNA and sequencing library purification:

- Agencourt AMPure XP PCR purification system (5 ml ; Beckman Coulter, Cat. No. A63880)

NOTES:

- Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly recommend aliquoting the beads into 1.5-ml tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This aliquoting process is also essential for minimizing the chances of bead contamination.
- 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.

- 70% ethanol: prepared fresh from anhydrous ethanol for each experiment
- Agilent High Sensitivity DNA Kit (110 samples; Agilent, Cat. No. 5067-4626)
- Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)

For sequencing library validation:

- Thermal cycler with block for 0.2-ml tubes
- Agilent 2100 Bioanalyzer (Sections V.H and V.K): High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)

For cell counting:

- Recommended: MOXI Z Mini Automated Cell Counter Kit (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:

- Alternatively, you may use any preferred cell counter with demonstrated, accurate cell counting.
- Refer to a Moxi Z user manual for guidance in selecting an appropriate cassette size for the cells being analyzed.

IV. General Considerations

A. Sample Recommendations

This protocol is typically performed with several milliliters of healthy cell culture suspension as starting material. We recommend maintaining a cell density between 1 x 10^5 and 7.5 x 10^5 cells/ml prior to starting the protocol.

B. Protocol Best Practices

- 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 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 DNA-free, closed 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 ICELL8 cx Human TCR a/b Profiling workflow.
- Because of the large volume or viscosity of mixtures subject to purification using 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.

IMPORTANT: DO NOT UV treat preprinted chips.

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 Single-Cell 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 the chip oriented with the chamfered (beveled) corner positioned towards the lower-right corner of the chip nest (Figure 4, left).
- 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 source plate nest. (Figure 4, right).

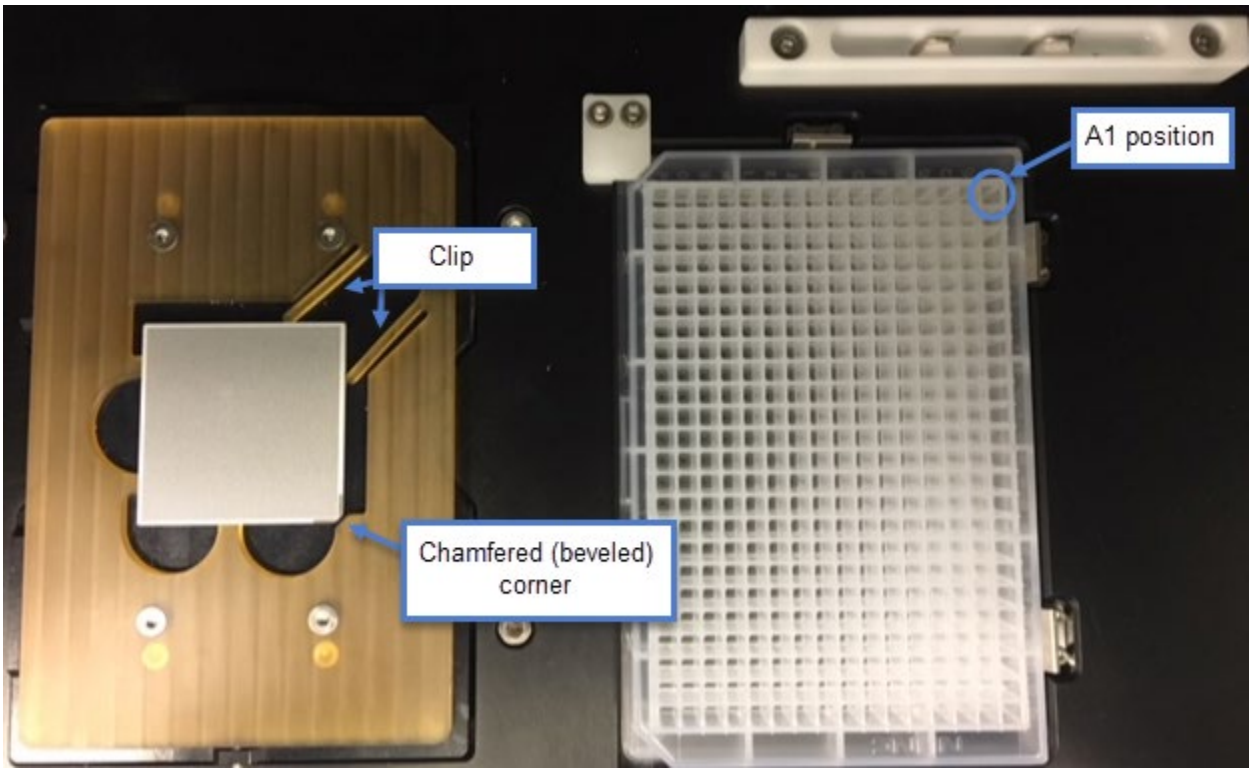

Figure 4. ICELL8 cx Single-Cell System Stage Module. **(Left)** chip nest. **(Right)** source plate nest.

E. ICELL8 cx TCR Chip

- Each ICELL8 cx TCR Chip is engraved with a unique number. You can use this number to link your chip images and other experimental record files.
- Oligos containing nanowell-specific barcodes were preprinted into each nanowell of the ICELL8 cx TCR Chip; the printing lot number is stamped onto the chip (See in Figure 5, below). A total of 1,728 unique nanowell barcodes are available; each nanowell barcode was printed once into a chip.

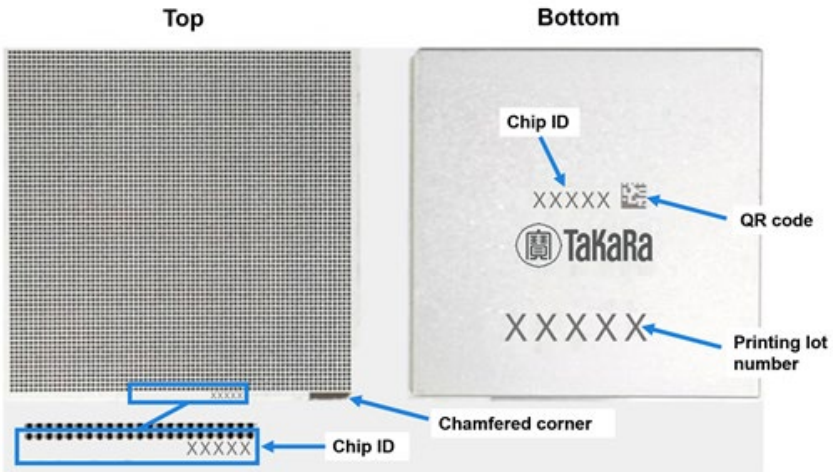

Figure 5. ICELL8 cx TCR Chip features. **(Left)** Top view of the chip. Note the chamfered (notched) corner at the bottom right. "TaKaRa" and chip ID (unique to each chip) are on the chip border, near the chamfered corner. **(Right)** Bottom view of the chip. The chip ID is also engraved on the other 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. The lot number corresponding to the printing of the barcode-containing oligos is also engraved on this side of the chip.

F. Software

The instructions in this manual are written for use with CELLSTUDIO and CellSelect v2.0 Software. Please refer to the ICELL8 cx Single-Cell System User Manual and the ICELL8 cx CellSelect v2.0 Software User Manual for more detailed information.

V. Procedure

A. Protocol: Stain Cells

In this protocol, sample cells are stained with Hoechst 33342 and propidium iodide dyes that enable imaging, analysis, and selection of candidates suitable for downstream analysis following dispensing into the ICELL8 cx TCR Chip. Start from Section V.A.1 if working with cells in suspension. Start from Section V.A.2 and then proceed to Section V.A.1 if working with adherent cells.

Prerequisites:

- Several milliliters of healthy cell culture suspension.
- A cell density maintained between 1 x 10^5 and 7.5 x 10^5 cells/ml.

Before you start:

- Perform a once-a-day warmup. Refer to the ICELL8 cx Single-Cell System User Manual (Section VIII) for more details.

- Prefreeze ICELL8 Chip Holder(s) (Figure 6) at –80°C.

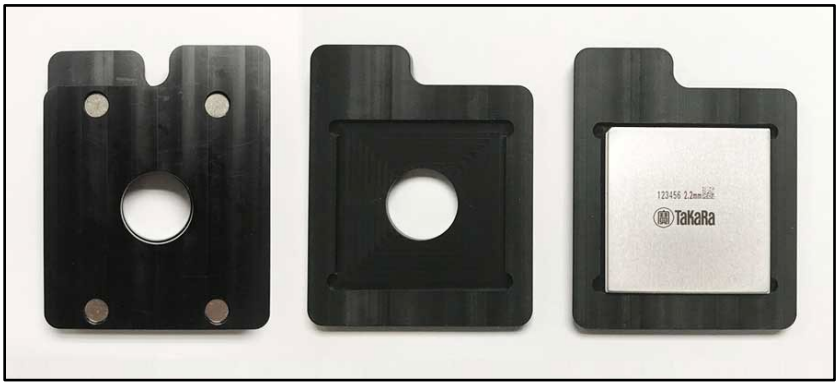

Figure 6. ICELL8 Chip Holder.

- Set the chip centrifuge to 22°C.
- Thaw Second Diluent (100X) and nuclease-free water on ice. Once thawed, keep on ice for the remainder of the protocol.
- Prewarm 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4), TrypLE Express*, and cell culture medium* to 37°C.

  *Required only if performing dissociation of adherent cells.

- Dilute Control Jurkat Total RNA (1 µg/µl) to 10 ng/µl for use in the next protocol (Section V.B, Table 1) as indicated in the following steps and keep the dilution on ice:
  a. Dilute Control Jurkat Total RNA to 50 ng/µl by mixing 38 µl of nuclease-free water with 2 µl of Control Jurkat Total RNA (1 µg/µl) in a sterile nuclease-free microcentrifuge tube.
  b. Dilute Control Jurkat Total RNA to 10 ng/µl by mixing 20 µl of nuclease-free water with 5 µl of the Control Jurkat Total RNA diluted to 50 ng/µl in the previous step.

NOTES:

- Return Control Jurkat Total RNA (1 µg/µl) stock solution to storage at –70°C.
- Diluted Control Jurkat Total RNA should be kept on ice at all times.

Cell and chip handling notes:

- Keep cells at 37°C with 5% CO_2 in a cell culture incubator when not performing manipulations.
- Some cell lines may require trypsinization to achieve a single-cell suspension.
- 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.

1. Preparation of Stained Suspension Cells (e.g., human Jurkat cells)

1. Prepare a 1:1 mixture of Hoechst 33342 and propidium iodide. Combine 80 µl of each dye per ml of cells to be stained. An example using 2 ml of cells is described below (e.g., prepare 320 µl of premixed dye solution by adding 160 µl each of Hoechst 33342 and propidium iodide).

   NOTE: Protect this mixture from light until ready for use.

2. Transfer ~2.1 ml of suspension cells to a fresh 5-ml tube.
3. Determine cell concentration using a Moxi automated cell counter and an appropriate Moxi Z cassette (e.g., use a Moxi Z cassette MF-M for Jurkat cells; refer to a Moxi Z user manual for guidance in selecting an appropriate cassette size for the cells being analyzed) or your preferred method. Use a pipette to transfer 75 µl of cell suspension to one end of the loading platform. Take a reading and record the cell concentration.
4. Add 320 µl of the premixed Hoechst 33342 and propidium iodide dye mix to the cells. Mix gently by inverting the tube 5 times. DO NOT vortex or overagitate the cells.
5. Incubate cells at 37°C for 20 min.
6. Add an equal volume of prewarmed 1X PBS (to 37°C; no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4) to stained cells. For the example described here, 2 ml of prewarmed 1X PBS is added to the 5-ml tube containing the stained cell suspension.
7. Mix the stained cell suspension and PBS by gently inverting the tube 5 times. DO NOT vortex or overagitate the cells.
8. Pellet the cells by centrifugation at 100*g* for 3 min at room temperature. Avoid over-centrifugation or pelleting into a firm pellet or clump.

   NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. For example, PBMCs or similarly sized cells require centrifugation at 500*g* for 3 min.

9. Gently remove tube from the centrifuge without disturbing the cell pellet.
10. Carefully decant the supernatant without disturbing the cell pellet.
11. Wipe remaining fluid from the tube top using a fresh Kimwipe such that the last remaining drop on the tilted tube top is gently removed.
12. Gently add 1 ml of 1X PBS (prewarmed to 37°C) to the tube side wall.
13. Use a wide-bore 1-ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.
14. Count the cells using a Moxi automated cell counter and an appropriate cassette (or your preferred method). Take two readings for each stained cell sample and average the results. The recommended concentration range of stained cell suspension is 1.2×10^5–5.0×10^5 cells/ml. If you end up with a concentration lower than 1.2×10^5 cells/ml, re-pellet the stained cell suspension and resuspend in a lower volume of 1X PBS to achieve a concentration in the recommended range.
15. Proceed to the next protocol (Section V.B).

2. Preparation of Adherent Cells in a 75-cm Culture Flask*

*Adjust volumes accordingly for different-sized flasks.

1. To a 75-cm flask containing adherent cells, exchange cell media with 10 ml of 1X PBS prewarmed to 37°C by dispensing the PBS on the side walls of the flask.

   NOTE: DO NOT pour PBS directly onto cells.

2. Wash the cells by tilting the flask gently. DO NOT mix by pipetting.
3. Remove the PBS from the cells using a standard large-bore tissue culture pipette.
4. Add 3 ml of TrypLE Express prewarmed to 37°C to the flask to dissociate the cells.
5. 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 media (containing 10% serum), prewarmed to 37°C. DO NOT vortex or overagitate cells.
7. Follow the procedure for Preparation of Stained Suspension Cells (Section V.A.1, above) starting from Step 1.

B. Protocol: Dilute and Dispense Cells

In this protocol, sample cells and controls are diluted and aliquoted into a 384-Well Source Plate and dispensed into the ICELL8 cx TCR Chip using the ICELL8 cx Single-Cell System. Refer to the ICELL8 cx Single-Cell System User Manual for detailed information about instrument setup and operation.

Before you start

- Confirm that initialization and setup procedures for the ICELL8 cx Single-Cell System have been completed (see "Before you start" in Section V.A, above).
- Remove the ICELL8 cx TCR Chip from storage at –20°C and equilibrate at room temperature ~10 minutes prior to opening the packet. After equilibration, put the chip onto the cold block until ready to dispense on the ICELL8 cx system (Section V.B, step 9, below).
- Prefreeze the empty ICELL8 Chip Holder(s) (Figure 6, above) at –80°C, if that was not done in the previous step (Section V.A).
- Aliquot 300–500 µl of 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4) on ice for positive and negative controls.
- This protocol requires the ICELL8 384-Well Source Plate and Seal, and the following components the ICELL8 Human TCR a/b Profiling Reagent Kit: Control Jurkat Total RNA (diluted to 10 ng/µl; Section V.B), stained cell suspension (Section V.A) Second Diluent (100X), RNase Inhibitor (40 U/µl); and from the ICELL8 cx Loading Kit: Blotting Paper and SmartChip Intermediate Film.
- Use the concentration of stained cell suspension measured at the end of the previous protocol (Section V.A) and the information in Table 1 (below) to calculate the volumes of stained cell suspension and 1X PBS that should be combined to obtain a final concentration of 1.4 cell/50 nl in a total volume of 1 ml .

Procedure

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Diluted Stained Cell Suspension	For 8 Samples (Per Sample Amounts)
Second Diluent (100X)	1.0 µl	1.0 µl	10.0 µl	1.0 µl
RNase Inhibitor (40 U/µl)	1.0 µl	1.0 µl	10.0 µl	1.0 µl
Control Jurkat Total RNA (10 ng/µl)	–	1.0 µl	–	–
Stained cell suspension	–	–	Dilute to 1.4 cell/50 nl *	Dilute to 1.4 cell/50 nl *
1X PBS (Ca^{2+} and Mg^{2+} free)	98.0 µl	97.0 µl	Up to 1,000.0 µl	Up to 100 µl
Total	100.0 µl	100.0 µl	1,000.0 µl **	100 µl

*Sufficient stained-cell suspension should be included such that the final concentration in the 1,000 µl volume of diluted stained cell suspension is 1.4 cell/50 nl (i.e., 28,000 cells/ml).

**The 1,000 µl total volume of diluted stained cell suspension is sufficient for distributing 80 µl of cell suspension in each of eight source wells (see Figure 7, below).

1. Vortex Second Diluent and RNase Inhibitor and spin the tubes briefly to collect contents at the bottom.

Prepare positive and negative controls

2. Prepare negative and positive controls in separate 1.5-ml microcentrifuge tubes using the volumes indicated in Table 1 (above).

NOTES:
- Keep the positive control sample on ice.
- Mix well, but do not vortex the positive and negative control samples.
- The final concentration of Control Jurkat Total RNA should be 5 pg/50 nl (equivalent to the total RNA content from one T cell).

Prepare diluted stained cell suspension

3. In a 1.5-ml microcentrifuge tube, combine the volumes of prewarmed 1X PBS, RNase Inhibitor, and Second Diluent indicated in the corresponding column of Table 1 (above). Mix the combined reagents by vortexing, then spin the tubes briefly to collect contents at the bottom.

NOTE: The amount of 1X PBS added will depend on the starting concentration of stained cell suspension.

4. To the 1.5-ml microcentrifuge tube from the previous step (containing 1X PBS, RNase Inhibitor, and Second Diluent), add the calculated volume of stained cell suspension that will yield a final concentration of 1.4 cell/50 nl in a total volume of 1 ml and invert gently to mix (refer to the ICELL8 cx Cell Dilution Tool spreadsheet provided by the Field Application Specialist during training). DO NOT use the pipette to mix at this step.

NOTES:
- Mix the stained cell suspension gently by inverting the tube several times before adding the cells to the reagents.
- Take the required volume of stained cell suspension from the center of the tube using a 20 µl or 200 µl pipette tip and add it slowly to the tube containing the other reagents.
- Work quickly to avoid settling of cells.

Prepare cell dispense source plate

Refer to the ICELL8 cx Single-Cell System User Manual for detailed information about instrument setup and operation.

5. Using a wide-bore 1-ml pipette tip, gently mix the diluted stained cell suspension prepared in Step 4 of this protocol (above) by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.

IMPORTANT: Proceed to the next step quickly to avoid settling of cells.

6. Using a 100-µl pipette tip, slowly and carefully load 80 µl of cell suspension into wells A1, A2, B1, B2, C1, C2, D1, and D2 of a 384-Well Source Plate as indicated in Figure 7 (below).

NOTES:
- Make sure to take each aliquot from the center of the tube containing the diluted stained cell suspension.
- Be careful not to splash liquid into neighboring wells.
- Make sure not to introduce bubbles when adding the cell suspension to the 384-well source plate.
- DO NOT vortex or spin down the 384-well source plate.
- DO NOT tap plate. If any bubbles are present, remove using a pipette tip.

7. Add Positive and Negative Controls to the 384-Well Source Plate as indicated in the following steps and in Figure 7 (below):
   - Add 25 µl of prepared Positive Control to Well P24
   - Add 25 µl of prepared Negative Control to Well A24

NOTES:
- Be careful not to splash liquid into neighboring wells.
- Make sure not to introduce bubbles when adding reagents to the 384-well source plate.
- DO NOT vortex or spin down the 384-well source plate.
- DO NOT tap plate. If any bubbles are present, remove using a pipette tip.

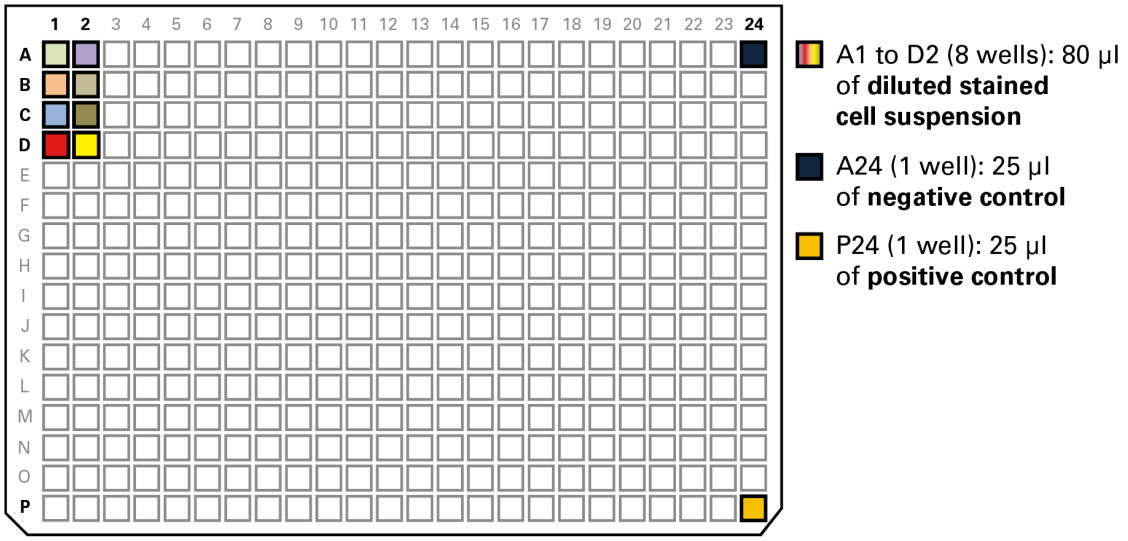

Figure 7. Setting up the 384-well source plate for dispensing cell samples and controls.

8. Seal the 384-Well Source Plate with a plate seal. DO NOT centrifuge the plate.

9. Open the packet containing the sealed ICELL8 cx TCR Chip and remove the seal from the preprinted chip. The chip is sealed along with a silica packet. Be careful not to cut the silica packet when opening the pouch.

   IMPORTANT: Ensure that the chip has equilibrated ~10 minutes at room temperature before opening the packet. After equilibration, put the chip onto the cold block until this step.

10. Place the chip on the chip nest in the ICELL8 cx Single-Cell System. The chamfered (beveled) corner of the chip should align with the chamfered corner of the chuck of the chip nest (refer to the ICELL8 cx Single-Cell System User Manual, Section X.A).

11. Place the 384-Well Source Plate in the ICELL8 cx Single-Cell System with the A1 corner positioned at the top-right corner of the plate nest. The beveled corners of the 384-Well Source Plate should be on the left side (refer to the ICELL8 cx Single-Cell System User Manual, Section X.B).

12. In CELLSTUDIO Software, click the [Dispense cells and Controls (50 nl)] button (Figure 8, below).

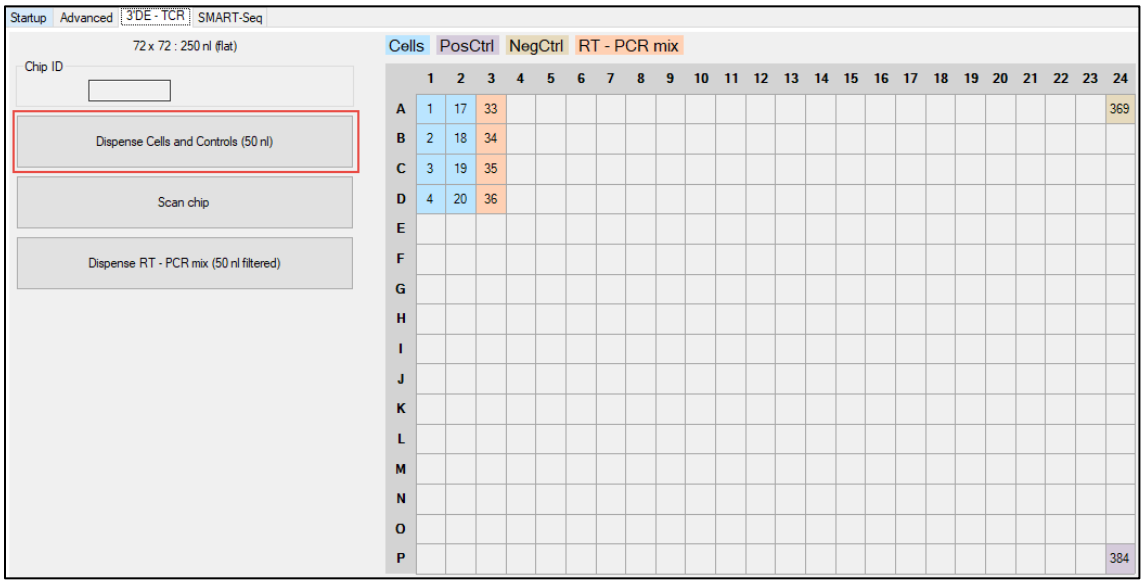

Figure 8. Using CELLSTUDIO Software to dispense cells into the ICELL8 cx TCR Chip.

13. Follow the software prompts and check the orientation of the source plate and ICELL8 cx TCR Chip to ensure they are correctly loaded on the dispensing platform. Click [Done] to proceed. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.C "Dispense the Sample Cells and Experimental Controls into the Chip" for details about the dispense step.

14. After the sample and control dispense is completed, 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 instructions to do that.

15. Seal the loaded chip with the SmartChip Intermediate Film (Figure 9, left) by removing the liner and applying the exposed side of the film to the blotted chip.

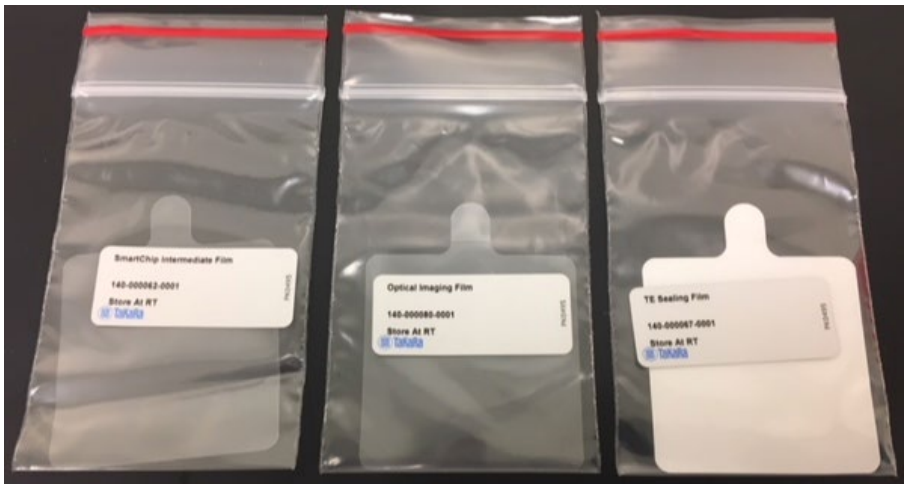

Figure 9. TE Sealing Film, Optical Imaging Film, and SmartChip Intermediate Film. **(Left)** SmartChip Intermediate Film is single-sided with a clear backing. **(Center)** Optical Imaging Film is double-sided and is provided between two layers of clear backing. **(Right)** TE Sealing Film is provided with a white backing.

16. Seal the blotted chip with the film using a film applicator (Figure 10, below).

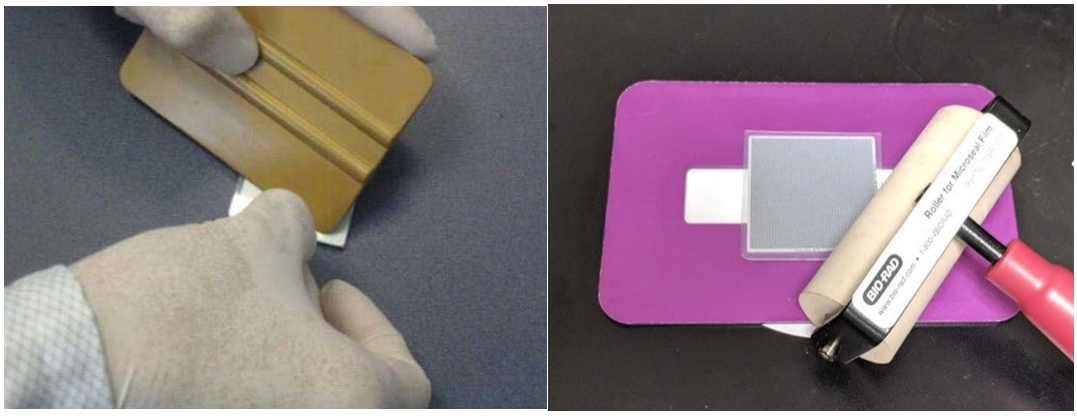

Figure 10. Sealing the blotted chip. Seal the blotted chip using **(Left)** a film applicator or **(Right)** a film sealing roller.

17. Place the sealed chip on a centrifuge spin plate and centrifuge the sealed chip at 300*g* for 5 min at 22°C with full acceleration and full brake (Figure 11, below). If you have one chip, balance the centrifuge with the supplied Balance Chip or a blank chip. Proceed to the next protocol (Section V.C) once the centrifugation is complete.

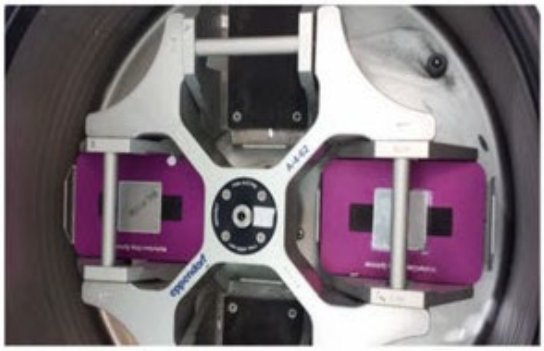

Figure 11. Centrifuge Chip Spinner.

C. Protocol: Image Cells

In this protocol, images of all 5,184 nanowells of the ICELL8 TCR cx Chip are acquired.

1. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.E "Scan Chip for Single Cells and Freeze the Chip" for detailed information about this protocol, with the following guidelines:
   a. Follow steps 1–5.
   b. For step 6, the *New stack info* dialog window will display (Figure 12, below). The "Analysis settings" should have the value '`AnalysisSetting_250nL_chip.XML`' selected and greyed out.

      For "Barcodes", choose '`TCR.xml`' from the drop-down list, located in `C:\ProgramData\Takara\CellSelect\AssayMaps\`.

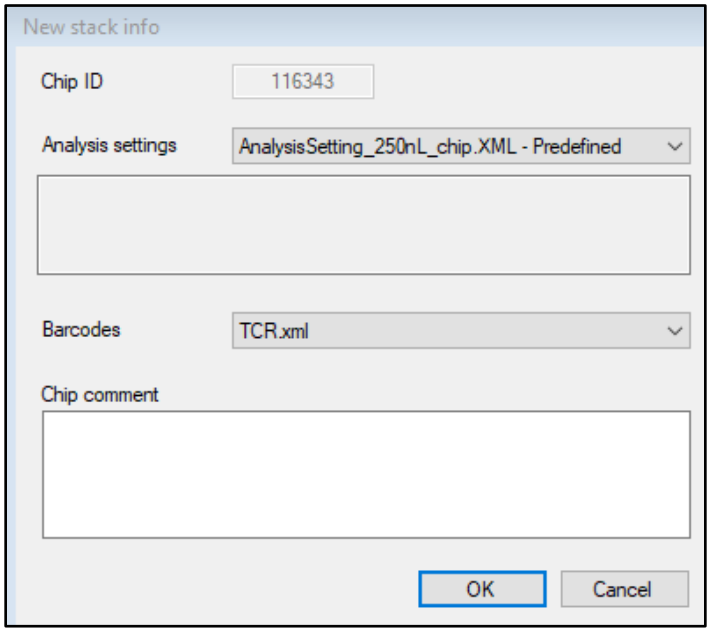

Figure 12. *New stack info* dialog window.

   c. Continue with the procedure from steps 7–12.
   d. At Step 13, remove the liner from only one side of the Optical Imaging film and apply the exposed sticky side of the imaging film to the chip, sealing carefully (similar to Figure 10, above).
2. Place the imaged chip into an empty Chip Holder that has been prechilled at –80°C. The chip holder should click closed and should close evenly, indicating a proper magnetic seal. Make sure that the Imaging Film is well sealed over the chip.
3. Freeze cells at –80°C for a minimum of 30 min before proceeding to RT-PCR (Section V.E, below).

SAFE STOPPING POINT: The chip can be frozen and stored in the chip holder at –80°C for subsequent processing.

D. Protocol: Analyze Nanowells with ICELL8 cx CellSelect v2.0 Software

Inspect the selected nanowells in CellSelect Software to exclude or include one or more candidate wells. Please refer to the ICELL8 cx CellSelect v2.0 Software User Manual for more information about this process.

E. Protocol: Synthesize and Amplify cDNA

In this protocol, first-strand cDNA synthesis is primed by an oligo dT primer (Oligo dT Amp Primer) and uses the SMART-Seq® barcoded oligos for template switching at the 5′ end of the transcript, which are deposited in the ICELL8 cx TCR Chip. Each oligo in the chip contains a unique nanowell barcode that allows cell identification after sequencing of the pooled cells.

For this protocol, you will need the following reagents from the ICELL8 Human TCR a/b Profiling Reagent Kit:

GC Melt (white cap), cDNA Amplification dNTP Mixture (white cap), $MgCl_2$ (blue cap), DTT (red cap), SMARTScribe Buffer (white cap), SMARTScribe Reverse Transcriptase (purple cap), cDNA Amplification Polymerase (orange cap), cDNA Amplification Buffer (white cap), Triton X-100 (blue cap), Oligo dT Amp Primer (pink cap), ICELL8 Amp Primer (khaki cap), and Nuclease-Free Water**.**

Before you start:

- If needed, perform a once-a-day warmup on the ICELL8 cx instrument.
- Set the centrifuge(s) used for spinning the chip and 384-Well Source Plate to 4°C.
- This protocol requires a 384-Well Source Plate (with seal); the following components from the ICELL8 cx Loading Kit: Blotting Paper, TE Sealing Film; and the following components from the ICELL8 Collection Kit – L: Collection Fixture, Collection Tube, and Collection Film.
- Thaw all reagents on ice except for SMARTScribe Reverse Transcriptase, cDNA Amplification Polymerase, and Triton X-100. Thaw and keep Triton X-100 at room temperature. Remove the enzymes from –20°C storage just prior to use and keep them on ice at all times. Gently vortex and spin down all thawed reagents besides the Triton X-100 and enzymes.

Procedure:

1. Remove the chip holder containing the ICELL8 cx TCR Chip from the –80°C freezer. Take the chip out of chip holder and thaw the chip at room temperature for 10 min to lyse cells.
2. Use a Kimwipe to dry any liquid on the chip surface, and then centrifuge the chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C. Keep the chip on ice until ready for dispensation of RT-PCR mix.
3. Combine RT-PCR reagents in a microcentrifuge tube on ice in the order listed in the recipe below, up to and including the Triton X-100. Vortex the mixture after addition of the Triton X-100 until it is completely dissolved. Keep the reagents on ice.

4. Add the SMARTScribe Reverse Transcriptase and cDNA Amplification Polymerase to the mixture immediately prior to use.

IMPORTANT: Remove the reverse transcriptase and PCR polymerase from the freezer, gently tap the tubes to mix, and add to the RT-PCR reaction mix. Mix by gently vortexing for 1–2 sec and spin the tube briefly in a minicentrifuge to collect contents.

56 µl	GC Melt (5 M)
24 µl	cDNA Amplification dNTP Mixture (25 mM each)
3.2 µl	$MgCl_2$ (1 M)
8.8 µl	DTT (100 mM)
30.9 µl	SMARTScribe Buffer (10X)
33.3 µl	cDNA Amplification Buffer (2X)
5.3 µl	Triton X-100 (3%)*
3.8 µl	Oligo dT Amp Primer (100 µM)
8.8 µl	ICELL8 Amp Primer (10 µM)
48 µl	SMARTScribe Reverse Transcriptase** (100 U/µl)
9.6 µl	cDNA Amplification Polymerase**
8.3 µl	Nuclease-Free Water
240 µl	Total volume per reaction

*Vortex the mixture after addition of the Triton X-100 until it is completely dissolved. Keep the reagents on ice.
**Add the enzymes and mix by pipetting up and down.

5. Pipette 50 µl of the RT-PCR reaction mix into wells A3, B3, C3, and D3 of a 384-Well Source Plate, as shown in Figure 13.

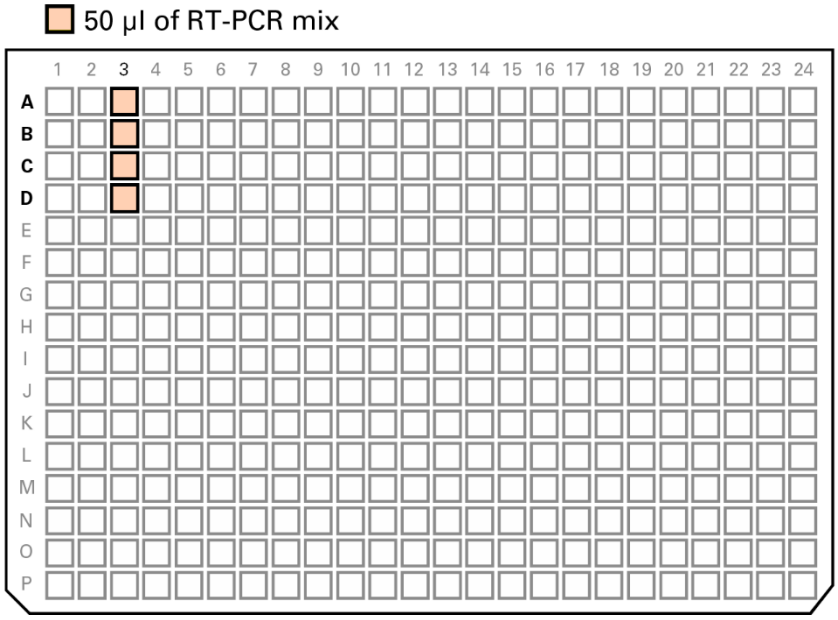

Figure 13. Setting up the source plate for dispensing RT-PCR mix.

6. Seal the 384-well source plate with 384-Well Plate Seal.

7. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C.
8. 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 3, with loading the source plate into the plate nest.
   b. At step 6, click the [Dispense RT - PCR mix] button (Figure 14).

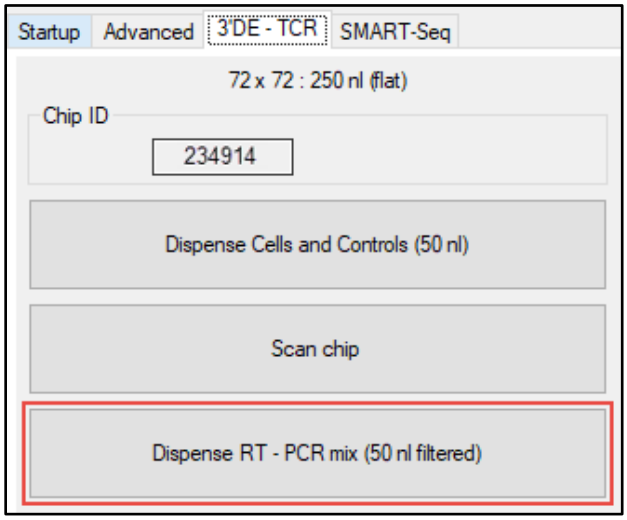

Figure 14. Using CELLSTUDIO Software to dispense the RT - PCR mix.

   c. Blot chip with blotting paper and blotter (steps 7–12 as written in Section X.F).
   d. Seal with the TE Sealing Film (step 13).
   e. Centrifuge the sealed chip at 3,220*g* for 3 min at 4ºC (step 14).
   f. Do step 15.

9. Place the ICELL8 cx TCR Chip into the thermal cycler to perform the RT-PCR reaction.

NOTES:

- For Cat. No. 640188, the RT-PCR program described below is preinstalled as the `2DIS-PCR` thermal profile program on the ICELL8 cx Thermal Cycler provided with the ICELL8 cx System. DO NOT alter the preinstalled program or substitute a different program without first consulting with a member of the Field Support Team; the `2DIS-PCR` program uses temperatures that are calibrated individually for each machine and that may vary slightly from the temperatures indicated below.
- If you are using the ICELL8 cx Single-Cell System JPN (Cat. No. 640189), use a thermal cycler of your choice. See the manufacturer's user manual for further instructions on thermal cycling the filled chip.

RT-PCR reaction

Temperature	Duration	# of cycles
50°C	3 min	
4°C	5 min	
42°C	90 min	
50°C	2 min	2 cycles
42°C	2 min	
70°C	15 min	
95°C	1 min	
98°C	10 sec	24 cycles
65°C	30 sec	
68°C	3 min	
72°C	10 min	
4°C	forever	

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

F. Protocol: cDNA Extraction from the Chip

This step extracts the amplified cDNA from the 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 85% of the Maximum Potential Volume.

SAFE STOPPING POINT: The cDNA eluent can be frozen at –20°C.

G. Protocol: Cleanup and Concentration of cDNA Product

In this protocol, extracted cDNA is cleaned-up using NucleoSpin Gel and PCR Clean-up Kit (Takara Bio, Cat. No. 740609.50).

Before you start:

- If this is the first use of the NucleoSpin Gel and PCR Clean-up Kit, make sure that the recommended amount of ethanol has been added to make the Buffer NT3 concentrate.
- Equilibrate an aliquot of AMPure XP beads to room temperature for ~30 min prior to use.
- Prepare fresh 70% (v/v) ethanol from a stock of anhydrous ethanol.
- This protocol requires the Nuclease-Free Water provided with the ICELL8 Human TCR a/b Profiling Reagent Kit.

Procedure:

1. Transfer the cDNA eluent obtained in the previous protocol to a 1.5-ml nuclease-free microcentrifuge tube and measure the volume of extracted eluent with a pipette tip.
2. Measure the extracted volume with a pipette tip. Use the NucleoSpin Gel and PCR Clean-up Kit to purify the cDNA product by following the manufacturer's protocol below:
    a. Mix 1 volume of sample with 2 volumes of Buffer NTI.
    b. Place a NucleoSpin Gel and PCR Clean-up Column into a 2-ml collection tube and load up to 700 µl of sample.
    c. Centrifuge for 30 sec at 11,000*g*. Discard the flowthrough and place the column back into the collection tube. Load the remaining sample if necessary and repeat the centrifugation step.
    d. Add 700 µl of Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column. Centrifuge for 30 sec at 11,000*g*. Discard the flowthrough and place the column back into the collection tube.
    e. Repeat the wash step once as per Step 2d for a total of two washes.
    f. Centrifuge for 1 min at 11,000*g* to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flowthrough while removing it from the centrifuge and the collection tube.
    g. Place the NucleoSpin Gel and PCR Clean-up Column into a new 1.5-ml microcentrifuge tube (not provided). Add 26 µl of water and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000*g*.
    h. Repeat the elution step twice using the same microcentrifuge tube used in the previous step, add 26 µl of DNase-RNase free water directly to the column matrix and incubate at room temperature for 1 min and centrifuge at maximum speed (≥ 10,000*g*) for 30 sec to elute the cDNA. You will end up with ~50 µl of total eluent.

H. Protocol: Purify the Full-Length cDNA with 0.6X AMPure XP Beads

Before you start:

- Ensure the aliquot of AMPure XP beads has been equilibrated to room temperature for ~30 min prior to use.
- Prepare fresh 70% (v/v) ethanol from a stock of anhydrous ethanol.

Procedure:

IMPORTANT: Be careful to avoid bead carryover into the purified cDNA sample.

1. 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.

   NOTE: The beads are viscous, so pipette them slowly.

2. Add 0.6X volume of well-vortexed DNA beads to the cDNA product.

   EXAMPLE: To a 50 µl of cDNA suspension obtained in Section V.G, Step 2h, add 30 µl of DNA beads.

3. Vortex to mix. Spin down briefly to collect all liquid.
4. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥2 min until the beads are completely separated from the solution.

   NOTE: During the ~5-minute incubation, if beads are not suspended or have settled in the tube, resuspend by pipetting to obtain a homogenous bead mixture.

5. Carefully remove the supernatant with a pipette. Discard the supernatant.
6. Wash the pellet with 200 µl of 70% (v/v) ethanol. Wait for 10 sec.
7. To sufficiently wash the cDNA-bound magnetic beads, turn the tube such that the opposite tube side faces the magnet, allowing the bead pellet to migrate from one side of the tube to the opposite side. Wait for 10 sec and turn the tube back to its original position. Repeat this process one more time for a total of two cycles. Once the bead pellet has reformed at the bottom of the tube, carefully remove and discard the supernatant, which contains contaminants. cDNA amplicons of the desired molecular weight will remain bound to the beads during the washing process.
8. Repeat Steps 5 and 6, for a total of two washes.
9. Spin the tube briefly in a minicentrifuge to collect the remaining ethanol at the bottom.
10. Place the tube on the magnetic stand and remove all remaining ethanol.

    NOTE: It is important to ensure all ethanol is removed for efficient recovery of nucleic acid.

11. Air-dry the washed beads at room temperature for ~2 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry the pellet.
12. Add 15 µl of Nuclease-Free Water and pipette up and down to mix. Make sure that all the beads are suspended.

13. Centrifuge briefly to collect all the liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 1 min or longer until the solution is completely clear.

NOTE: During the incubation on the magnet, a small population of beads may not pellet against the magnet. Use the supernatant to resuspend these products by pipetting up and down, and pipet them onto the magnet with the rest of the beads. Continue this incubation until there are no beads visibly remaining in the supernatant.

14. Carefully transfer 14 µl of clear supernatant containing purified the double-stranded cDNA to a nuclease-free tube. Label the tube with sample information and store at –20°C.

CAUTION: Do not transfer any of the beads.

I. Protocol: cDNA Validation and Quantification

In this protocol, the success of cDNA synthesis, purification, and size selection are evaluated using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). Please refer to the user manual for detailed instructions

Quantify cDNA with the Qubit fluorometric assay

1. Dilute the purified cDNA product 1:10 and use 2 µl of the dilution for quantitation with a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit (Life Technologies, Cat No. Q32851). Please refer to the user manual for the Qubit dsDNA HS Assay Kit for sample prep instructions.

Analyze cDNA quality with the Agilent Bioanalyzer

2. Based on the Qubit measurement obtained in the previous step, normalize the purified cDNA product to ~2 ng/µl.
3. Use 1 µl of the normalized cDNA product along with the Agilent High Sensitivity DNA Kit to load the Agilent 2100 Bioanalyzer. Please refer to the user manual for the Agilent High Sensitivity DNA Kit for instructions.
4. Compare the results for your samples with Figure 15 (below) to verify whether each sample is suitable for further processing. Successful cDNA synthesis and purification should yield a broad peak spanning ~400 bp to ~5,000 bp.

NOTE: If the Bioanalyzer profile indicates the presence of adapters, repeat the bead purification procedure and analysis of the purified cDNA (Sections V.G and V.H).

5. To quantify the cDNA yield, set the region table to measure between 400 and 5,000 bp and obtain the yield in pg/µl.

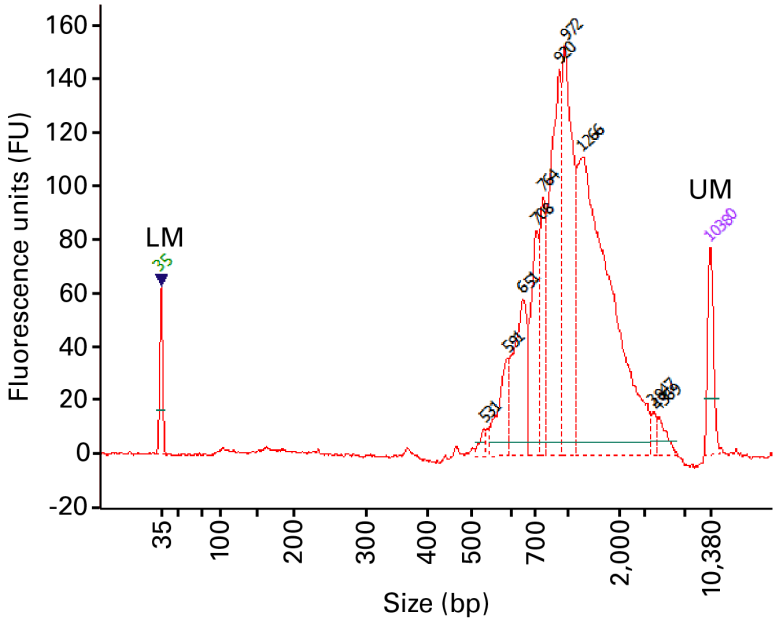

Figure 15. Typical Bioanalyzer trace for purified full-length cDNA that has been normalized to 2 ng/µl. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis.

J. Protocol: TCR a/b Amplification and Sequencing Library Generation by Semi-Nested PCR

IMPORTANT: This PCR protocol has been optimized based on using 500 pg of cDNA template into TCR-specific PCR 1. Do not adjust the input amount or recommended PCR cycle numbers.

1. TCR-specific PCR 1

This PCR selectively amplifies TCR sequences from the cDNA generated in Section V.H. Primer P5 anneals to the SMART sequence (incorporated during first-strand cDNA synthesis) and adds Illumina P5 and Read 1 sequences. TCRa Human Primer 1 and TCRb Human Primer 1 anneal to sequences in the constant regions of TCRa and TCRb cDNA, respectively.

For this protocol, you will need the following components:

TCR Amplification Buffer (white cap), TCR Amplification dNTP Mixture (green cap), Primer P5 (brown cap), TCRa Human Primer 1 (orange cap), TCRb Human Primer 1 (orange cap), TCR Amplification Polymerase (green cap), and Nuclease-Free Water.

1. Based on the quantification from the Bioanalyzer (Section V.I), dilute the cDNA (obtained in Section V.H, Step 13) in a labeled tube to a final concentration of 500 pg/µl. Transfer 1 µl of cDNA for each chip into a clean PCR tube. Store on ice.
2. Thaw all reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
3. To make the TCRa + TCRb Human Primer 1 Mix, add the primers at 2:1 ratio (TCRa Human Primer 1: TCRb Human Primer 1) in a clean PCR tube. Mix well by vortexing gently and then spin the tube briefly in a microcentrifuge.

EXAMPLE: For one chip (one PCR reaction), 3 µl of primer mix would be required, so mix 4 µl of TCRa Human Primer 1 with 2 µl of TCRb Human Primer 1 and use 3 µl of the mixture in each PCR.

4. Combine the following reagents (amounts are for one reaction, scale up as required) in the order shown below. Prepare enough PCR Master Mix for all the reactions, plus an additional 10% of the total reaction mix volume.

10 µl	TCR Amplification Buffer (5X)
4 µl	TCR Amplification dNTP Mixture (2.5 mM each)
1.25 µl	Primer P5 (5 µM)
3 µl	TCRa + TCRb Human Primer 1 Mix (from Step 3)
1 µl	TCR Amplification Polymerase
29.75 µl	Nuclease-Free Water
49 µl	Total volume added per reaction

NOTE: Remove the TCR Amplification Polymerase from the freezer, gently mix the tube **without** vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and then spin the tube briefly to collect the contents at the bottom of the tube.

5. Add 49 µl of PCR Master Mix to each tube containing 500 pg of cDNA in 1 µl from Step 1. Mix well and briefly spin to collect the contents at the bottom of the tubes.

6. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

TCR-specific PCR 1

95°C	1 min	
16 cycles:		
98°C	10 sec	]
60°C	15 sec	
68°C	45 sec	]
4°C	forever	

STOPPING POINT: The tubes may be stored at 4°C overnight.

2. TCR-specific PCR 2

This PCR further amplifies sequences corresponding to full-length TCR variable regions and adds Illumina HT sequencing adapters using a semi-nested approach. The Primer P5 is similar to the one used in TCR-specific PCR 1. The nested ICELL8 TCRa and/or TCRb Human Primer 2 Forward HT Index primers anneal to sequences in TCR constant regions that are internal to the sequences bound by TCRa Human Primer 1 and TCRb Human Primer 1 and add both the Illumina Read 2 sequence and P7-i7 index sequences.

IMPORTANT: In order to pool multiple libraries for Illumina sequencing run, be sure to use a different TCRa (aF1–aF10) and TCRb (bF1–bF10) Human Primer 2 Forward HT indexes for each library you wish to pool (See Appendix A).

For this protocol, you will need the following components:
TCR Amplification Buffer (white cap), TCR Amplification dNTP Mixture (green cap), Primer P5 (brown cap), ICELL8 TCRa Human Primer 2 Forward HT Indexes (white caps), ICELL8 TCRb Human Primer 2 Forward HT Indexes (white caps), TCR Amplification Polymerase (green cap), and Nuclease-Free Water.

1. Thaw all reagents needed for PCR (except the enzyme which should be kept in the –20°C freezer until just before use) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. To make the ICELL8 TCRa + TCRb Human Primer 2 Forward HT Index Mix, add the primers at 2:1 ratio (TCRa Human Primer 2 Forward HT Index 1: TCRb Human Primer 2 Forward HT Index 1) in a clean PCR tube. Mix well by vortexing gently and then spin the tube briefly in a microcentrifuge.

   EXAMPLE: For one chip (one PCR reaction), 3 µl of primer mix would be required, so mix 4 µl of the appropriate TCRa Human Primer 2 Forward HT Index with 2 µl of Human Primer 2 Forward HT Index and use 3 µl of the mixture in each PCR.
3. Prepare enough PCR Master Mix for all of the reactions, plus 10% of the total reaction mix volume per reaction. Combine the following reagents in the order shown:

10 µl	TCR Amplification Buffer (5X)
4 µl	TCR Amplification dNTP Mixture (2.5 mM each)
1.25 µl	P5 Primer (5 µM)
1 µl	TCR Amplification Polymerase (1.25 U/µl)
29.75 µl	Nuclease-Free Water
46 µl	Total volume added per reaction

   EXAMPLE: Remove the TCR Amplification Polymerase from the freezer, gently mix the tube without vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and then spin the tube briefly to collect the contents at the bottom of the tube.
4. For each reaction, add 46 µl of PCR Master Mix to a clean 0.2-ml tube.
5. Add 1 µl of PCR product from PCR 1 (Section V.J) to each tube.
6. Add 3 µl of the appropriate ICELL8 TCRa + TCRb Human Primer 2 Reverse HT Index Mix (from Step 2), noting that each chip sample requires a different index. Mix well and briefly spin to collect the contents at the bottom of the tube(s).
7. Place the tube(s) in a preheated thermal cycler with a heated lid and run the following program:

TCR-specific PCR 2

95°C	1 min	
14 cycles:		
98°C	10 sec	]
60°C	15 sec	
68°C	45 sec	]
4°C	forever	

STOPPING POINT: The tubes may be stored at 4°C overnight.

K. Protocol: Purification of Amplified Libraries Using Agencourt AMPure XP Beads

The TCR sequencing library is size-selected and purified using AMPure XP beads. This approach involves two rounds of size selection, which together remove primers, primer dimers, and PCR products containing undersized or oversized inserts. In the first round, fragments larger than ~900 bp are immobilized on beads and removed from the supernatant. In the second round, the supernatant is added to fresh beads, which immobilize fragments within the desired size range of ~400–900 bp. The beads are then washed with 80% ethanol, and the fragments are eluted with water. This approach preserves library yield and complexity while maximizing the relevance and consistency of downstream sequencing results.

NOTES:

- This is a second purification step and requires different volumes of reagents and incubation times than the protocol described in Section V.H. **DO NOT** interchange the protocols.
- Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse.
- You will need 400 µl of freshly prepared 70% ethanol per sample.

For this protocol, you will need the following components:
Agencourt AMPure XP (Beckman Coulter, Cat. No. A63880), 70% ethanol (provided by the user; made fresh), a magnetic separation device for 0.2-ml tubes/tube strips or a 96-well plate (provided by the user), and water.

1. Vortex AMPure XP beads until evenly mixed, then add 22.5 µl of AMPure XP beads to each sample.
2. Mix thoroughly by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly. **DO NOT vortex. Vortexing will generate bubbles, making subsequent handling of the beads difficult.**
3. Incubate at room temperature for 5 min to let the DNA bind to the beads.
4. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for ~2 min or longer, until the liquid appears completely clear and there are no beads left in the supernatant. The time required for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage in separating the samples for longer than 2 min.
5. While the reaction tubes are sitting on the magnetic separation device, use a pipette to transfer the supernatant (which contains your library) to clean PCR tubes.
6. Remove the tubes containing the beads from the magnetic separation device and discard them. Add 10 µl of AMPure XP beads to each tube containing supernatant.

   NOTE: Ensure that the beads are fully resuspended before use. If the beads appear to have settled at the bottom of the tube, **gently** vortex to ensure that they are completely mixed.
7. Mix thoroughly by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly. **Do not vortex. Vortexing will generate bubbles, making subsequent handling of the beads difficult.**

8. Incubate at room temperature for 5 min to let the DNA bind to the beads.
9. Place the tubes on the magnetic separation device for ~2 min or until the solution is completely clear.
10. While the tubes are sitting on the magnetic separation device, remove the supernatant with a pipette and discard it (the library is now bound to the beads.)
11. Keep the tubes on the magnetic separation device. Add 200 µl of freshly made 70% ethanol to each sample without disturbing the beads, to wash away contaminants. Wait for 30 sec and use a pipette to carefully remove the supernatant, which contains contaminants. The library will remain bound to the beads during the washing process.
12. Repeat the ethanol wash (Step 11) once.
13. Seal the tubes and briefly spin down to collect the liquid at the bottom of the well.
14. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol by using the eight-channel pipette. Take care to ensure that the bead pellet is not disturbed while removing the ethanol.

NOTE: It is important to make sure all ethanol is removed so the beads elute well and recovery is efficient

15. Let the sample tubes rest open on the magnetic separation device at room temperature for ~2–2.5 min until the pellet appears dry and is no longer shiny. You may see a tiny crack in the pellet.

NOTES: Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.

- If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the plate to sit at room temperature until the pellet is dry.
- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 5 min to rehydrate (Step 16) and may reduce library recovery and yield.
- Visit takarabio.com/rna-seq-tips to view examples of moist, dry, and overly dry pellets.

16. Once the bead pellet has dried, remove the tubes from the magnetic separation device and add 15 µl of water to cover the pellet. Remove the samples from the magnetic separation device and mix thoroughly by pipetting up and down to ensure complete bead dispersion.

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

17. Incubate at room temperature for at least 5 min.
18. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic separation device 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. Pipet these unpelleted beads up and down to resuspend them with the supernatant, and then pipet them towards the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

19. Transfer the clear supernatant containing the purified TCR library from each tube to a nuclease-free, low-adhesion tube. Label each tube with sample information and store at –20°C.

STOPPING POINT: The samples may be stored at –20°C.

L. Protocol: Library Validation and Quantification

To determine whether library production, purification, and size selection were successful, we recommend analyzing and quantifying the final libraries using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). Alternatively, the libraries can be quantified by qPCR using the NGS Library Quantification Kit (Takara Bio, Cat No. 638324). Please refer to the corresponding user manuals for detailed instructions.

1. Prepare a 1:5 dilution of the ICELL8-generated sequencing library for quantitation using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit. Refer to the Qubit dsDNA HS assay kit user manual for sample prep instructions.
2. Based on Qubit measurements, normalize the amplicon to 0.2–0.4 ng/µl samples by further diluting the 1:5 dilution prepared in Step 1. Use 1 µl of it to load the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit for validation. See the Agilent High Sensitivity DNA Kit user manual for instructions.

CAUTION: Be careful not to transfer beads along with your sample.

3. Compare the results for your samples with Figure 16 (below) to verify whether each sample is suitable for further processing. Successful library production and purification should yield a broad peak spanning 550 bp to 1,200 bp, with a maximum between ~700 bp and ~900 bp for samples generated from cells containing TCR libraries.

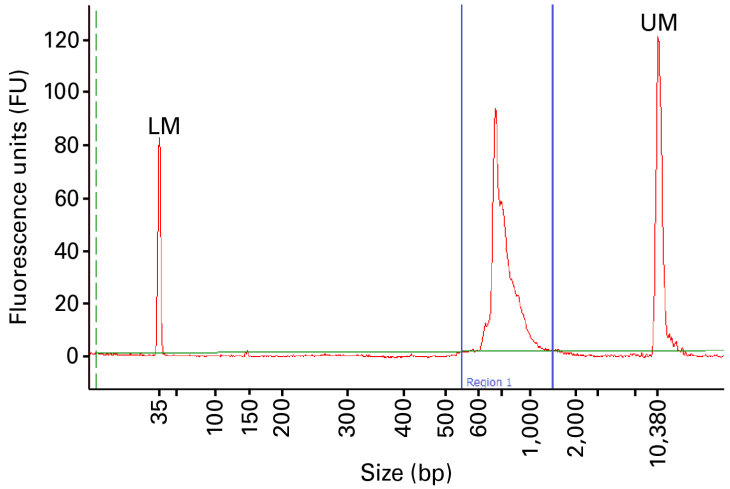

Figure 16. Example electropherogram results for TCRa and TCRb library. A library containing TCRa and TCRb sequences were generated using the ICELL8 cx Human scTCR a/b Profiling workflow. The library was produced using 24 cycles of amplification for the cDNA PCR, 16 amplification cycles for TCR PCR 1, and 14 amplification cycles for TCR PCR 2 (the cDNA profile for this library is shown in Figure 15). Following purification and size selection, the library was analyzed on an Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit. The libraries produced from the Jurkat Total RNA pool shows broad peaks between ~650 to 1,150 bp and maximal peaks in the range of ~700 to 900 bp. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. In this example, the library produced from 2 ng Jurkat RNA generated a broad peak spanning 550–1,500 bp. The Qubit measurement of the 1:5 diluted Control Jurkat RNA sample was 1.47 ng/ul. The positive control in this example (data not shown) is loaded in 1:25 dilution (normalized to 0.3 ng/µl based on a Qubit value of 1.47 ng/µl obtained from the 1:5 dilution). Set the region table to measure between 550 and 1,500 bp. After manual integration of the region between 550 and 1,500 bp, the sample in this example shows a concentration of 11.1 nmol/l. The yield should be ≥4.0 nmol/L.

4. To quantify the libraries, set the region table to measure between 550 and 1,200 bp. After manual integration of the region between 550 and 1,200 bp, the pool of Jurkat RNA in the example above (Figure 16) shows a library concentration of 11.1 nM.
5. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix B for sequencing guidelines.

Appendix A: Illumina HT Indexes

Unique combinations of Illumina indexes are required to discriminate between samples when sequencing a pool of two or more libraries on a single flow cell. Consult the Illumina literature (such as the TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

The ICELL8 TCRa and TCRb Human Primer 2 Forward HT Index primers contain the Read 2 sequence and i7 indexes. These primers are labeled sequentially ("aF1"–"aF10" and "bF1"–"bF10" for TCRa and TCRb primers, respectively), in correspondence with Illumina indexes D701–D710.

The P5 Primer contains the Read 1 sequence and i5 indexes.

Table 2. TCR a/b Human Indexing Primer Set HT for Illumina index sequences

TCR Primer 2 Forward HT Index			
Primer ID (cap label)	**Illumina ID**	**Index sequence**	**Included in indexing primer set**
aF1 & bF1	D701	ATTACTCG	640179, 640180, 640181
aF2 & bF2	D702	TCCGGAGA	640180, 640181
aF3 & bF3	D703	CGCTCATT	640180, 640181
aF4 & bF4	D704	GAGATTCC	640180, 640181
aF5 & bF5	D705	ATTCAGAA	640180, 640181
aF6 & bF6	D706	GAATTCGT	640181
aF7 & bF7	D707	CTGAAGCT	640181
aF8 & bF8	D708	TAATGCGC	640181
aF9 & bF9	D709	CGGCTATG	640181
aF10 & bF10	D710	TCCGCGAA	640181

Appendix B: Guidelines for Library Sequencing

Samples should be pooled to a final pool concentration of 4 nM. We recommend diluting the pooled libraries to a final concentration of 13.5 pM, including a 5–10% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in. While not essential, the addition of the PhiX control increases the nucleotide diversity and thus aids in high-quality data generation.

Sequencing should be performed on an Illumina MiSeq® sequencer using the 600-cycle MiSeq Reagent Kit v3 (Illumina, Cat. No. MS-102-3003) with paired-end, 2 x 300 base pair reads.

Appendix C: In-line Indexes

The `TCR.xml` file provided with ICELL8 cx CellSelect v2.0 Software contains the barcode sequence preprinted in each nanowell location on a chip. This allows every cell sequenced to be uniquely identified and its location on the chip known.

Appendix D: Data Analysis

The ICELL8 system is optimized for data analysis using the mappa™ Analysis Pipeline and hanta™ R kit.

- **mappa Analysis pipeline** interprets both ICELL8 and sequencing data, and outputs an HTML report. This report summarizes the complex read data into clear, simple charts.
- **hanta R kit** imports sequencing data and provides secondary analysis such as tSNE plots for user-friendly visualization.

More information on these tools can be found on our website at https://www.takarabio.com/products/automation-systems/icell8-system-and-software/bioinformatics-tools.

Data analysis can also be performed on a variety of other platforms. A detailed but non-exhaustive list of packages can be found at https://omictools.com/rep-seq-category.

Appendix E: 5′ DE Protocol: Prepare Sequencing Library with the Nextera XT kit

A. Additional Materials Required

The following reagents and materials are required to perform 5′ DE for the ICELL8 cx Human TCR a/b Profiling workflow, but are not supplied:

For sequencing library preparation and validation:

- Nextera XT DNA Library Preparation Kit (24 samples; Illumina, Cat. No. FC-131-1024)
- Nextera XT Index Kit (24 indexes, 96 samples; Illumina, Cat. No. FC-131-1001)
- NGS library quantification kit for Illumina platforms:
  - DNA Standards for Library Quantification (Takara Bio, Cat. No. 638325)
  - Library Quantification Kit (Takara Bio, Cat. No. 638324)

B. Procedure

1. cDNA synthesis, amplification, quantification, and validation

Please follow instructions as outlined in Sections V.E–V.H, above.

2. Protocol: Prepare Sequencing Library with the Nextera XT kit

In this protocol, the Nextera XT DNA Library Preparation Kit is used to add Illumina adapters and indexes to the purified cDNA via a tagmentation reaction followed by PCR.

Before you start:

- Thaw ATM (Amplicon Tagment Mix) and TD (Tagment DNA Buffer) reagents from the Nextera XT DNA Library Preparation Kit on ice.

- Thaw purified cDNA from Section V.H, Step 13 (if frozen). Dilute the cDNA to 0.2 ng/µl based on quantification in Section V.I, Step 5 and use 1 ng for library preparation. Set up multiple identical Nextera XT reactions to increase the final library yield if desired.
- Make sure that the NT (Neutralize Tagment Buffer) reagent is equilibrated to room temperature and does not contain precipitate; if precipitate is observed, vortex to resuspend the particulates.
- This protocol requires the Primer P5 (5 µM) provided with the ICELL8 Human TCR a/b Profiling Reagent Kit.

Procedure:

1. Prepare the Nextera XT tagmentation mix in a 0.2-ml nuclease-free tube as indicated below:

10.0 µl	TD (Tagment DNA Buffer)
5.0 µl	Purified full-length cDNA (0.2 ng/µl)
5.0 µl	ATM (Amplicon Tagment Mix)
20.0 µl	Total volume per reaction

2. Mix and centrifuge briefly to collect all liquid. Incubate the reaction in a conventional thermal cycler using the following program:

55°C	5 min
10ºC	forever

3. Immediately upon completion of the tagmentation reaction, remove the tube from the thermal cycler and spin it briefly. Add 5 µl of NT (Neutralize Tagment Buffer) and pipette up and down five times to mix. Briefly spin to collect contents at the bottom and incubate the tube at room temperature for 5 minutes.
4. Thaw one tube of i7 index primer (orange cap) from the Nextera XT Index Kit. Replace the orange cap with a new cap after each use to avoid index contamination. Mark the i7 index tube that you have used. **DO NOT** use the i5 index primer (white cap) supplied with the Nextera XT Index Kit.
5. Thaw Primer P5 (5 µM) provided with the ICELL8 Human TCR a/b Profiling Reagent Kit.
6. Prepare the Nextera XT PCR reaction mix in a 0.2-ml nuclease-free tube as indicated below:

15.0 µl	NPM (Nextera PCR Mastermix)
5.0 µl	i7 index primer (orange cap)
5.0 µl	Primer P5 (5 µM)
25.0 µl	Tagmented cDNA & NT mix (from Step 3)
50.0 µl	Total volume per reaction

7. Vortex to mix. Centrifuge briefly to collect all liquid. Incubate the reaction in a conventional thermal cycler using the following program:

72°C		3 min
95ºC		30 sec
12 cycles:		
	95°C	10 sec
	55°C	30 sec
	72ºC	30 sec
72°C		5 min
10ºC		forever

2. Protocol: Purify, Size Select, and Evaluate Sequencing Library

In this protocol, tagmented cDNA is purified and size selected via a series of treatments with AMPure beads, followed by quantitation and analysis with an Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). The libraries can be quantified by qPCR using the NGS Library Quantification Kit (Takara Bio, Cat No. 638324). The final output of this protocol is a sequencing-ready Illumina library.

Before you start:

- Equilibrate an aliquot of AMPure XP beads to room temperature for ~30 minutes prior to use.

  NOTE: 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 70% (v/v) ethanol from a stock of anhydrous ethanol.
- This protocol requires the Nuclease-Free Water provided with the ICELL8 Human TCR a/b Reagent Kit.

Procedure:

Purify and size select sequencing library

1. To each 50-µl volume of PCR product from the previous protocol (Appendix E, Section B.1, above), add 50 µl (1X) of AMPure XP beads.
2. Vortex to mix. Spin down briefly to collect all liquid.
3. Incubate the mixture at room temperature for ≥ 5 min and then on a magnetic stand for ≥ 2 min until the beads are completely separated from the solution.
4. Carefully remove the supernatant with a pipette. Discard the supernatant.
5. Wash the pellet with 200 µl of 70% (v/v) ethanol. Wait for 10 seconds.

6. To sufficiently wash the cDNA-bound magnetic beads:
   a. Turn the tube such that the opposite tube side faces the magnet allowing the bead pellet to migrate from one side of the tube to the opposite side.
   b. Wait for 10 sec and turn the tube back to its original position.
   c. Repeat this process one more time for a total of 2 cycles.

   Once the bead pellet has reformed at the bottom of the tube, carefully remove and discard the supernatant containing contaminants. Library amplicons of the desired molecular weight will remain bound to the beads during the washing process.
7. Repeat Steps 5 and 6, for a total of two washes.
8. Spin the tube briefly in a minicentrifuge to collect the remaining ethanol at the bottom.
9. Place the tube on the magnetic stand and remove all remaining ethanol.
10. Air-dry the washed beads at room temperature for ~5 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry the pellet.
11. Add 50 µl of Nuclease-Free Water then pipette up and down to mix. Make sure that all beads are suspended.
12. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 1 min or longer until the solution is completely clear.
13. Carefully transfer the supernatant containing the purified, size selected sequencing library to a new tube. You should end up with ~50 µl of sequencing-ready library.
14. To the eluent from the previous step (Step 13), add 25 µl (0.5X) of AMPure beads.
15. Vortex to mix. Spin down briefly to collect all liquid.
16. Incubate the mixture at room temperature for ≥ 5 min and then on a magnetic stand for ≥ 2 min until the beads are completely separated from the solution.
17. Carefully transfer the supernatant (~75 µl) to a clean PCR tube. **Save the supernatant and discard the beads**.
18. To the supernatant from the previous step (Step 17), add 10 µl (0.2X) of AMPure beads.
19. Vortex to mix. Spin down briefly to collect all liquid.
20. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥ 2 min until the beads are completely separated from the solution.
21. Carefully remove the supernatant with a pipette. Discard the supernatant.
22. Repeat Steps 5–9 from this protocol (above) to wash the pellet two times and remove the ethanol.
23. Air-dry the washed beads at room temperature for ~5 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry the pellet.
24. Add 13 µl of Nuclease-Free Water then pipette up and down to mix. Make sure that all beads are suspended.

25. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 1 min or longer until the solution is completely clear.
26. Carefully transfer the supernatant containing the purified, size selected sequencing library to a new tube. You should end up with ~12 µl of sequencing-ready library.

Evaluate sequencing library

27. Aliquot 1 µl of the final library obtained in the previous step (Step 26) for quantitation with a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit. Please refer to the user manual for the Qubit dsDNA HS Assay Kit for sample prep instructions.
28. Based on the Qubit measurement obtained in the previous step, normalize the sequencing library to 1 ng/µl, and use 1 µl of it along with the Agilent High Sensitivity DNA Kit to load the Agilent 2100 Bioanalyzer. Please refer to the user manual for the Agilent High Sensitivity DNA Kit for instructions.
29. Use the Bioanalyzer results to determine library quality (see Figure 17, below, for an example of a typical Bioanalyzer profile for an NGS library that has been successfully purified and size selected).
30. Quantify the diluted library with the NGS Library Quantification Kit (for Illumina).
31. Store the sequencing library at –20°C until ready for sequencing.

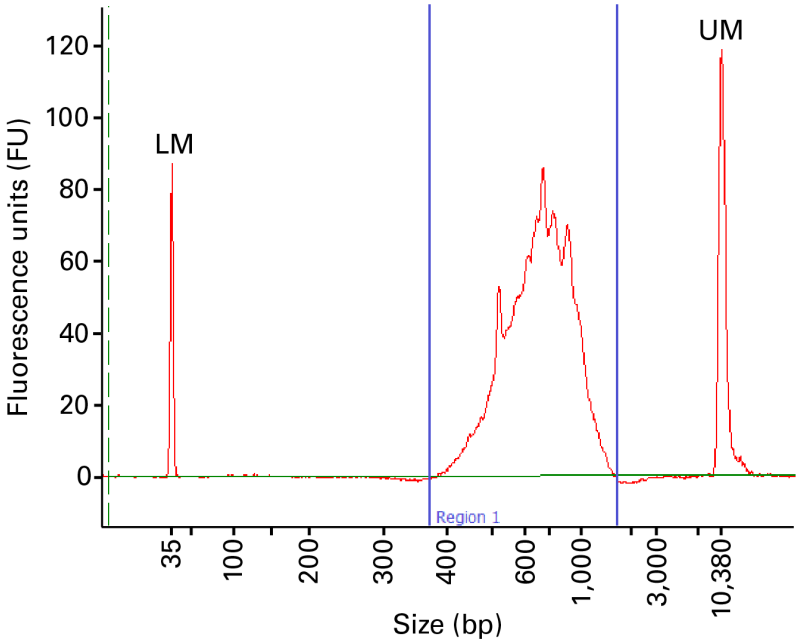

Figure 17. Typical Bioanalyzer trace for purified Nextera XT NGS library that has been normalized to 1 ng/µl.

3. Illumina Nextera XT Indexes

Unique combinations of Illumina indexes are required to discriminate between samples when sequencing a pool of two or more libraries on a single flow cell. Consult the Illumina literature (Nextera XT DNA Library Preparation Kit) for appropriate pooling guidelines.

4. Guidelines for Library Sequencing

Samples should be pooled to a final pool concentration of 4 nM.

- **NextSeq®**: We recommend diluting the pooled libraries to a final concentration of 1.8 pM, including a 20% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in.

  Sequencing can be performed on an Illumina NextSeq sequencer using the 150-cycle (mid or high output) with paired-end, 2 x 75 base pair reads.

- **MiSeq:** We recommend diluting the pooled libraries to a final concentration of 8 pM, including a 2-5% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in.

  Sequencing can be performed on an Illumina MiSeq sequencer using the 150-cycle with paired-end, 2 x 75 base pair reads.

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