

Takara Bio USA, Inc.

ICELL8® Human TCR a/b Profiling User Manual

Cat. Nos. 640178, 640179, 640180, 640181, 640182, 640109 & 640048
(071422)

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I. Introduction

High-throughput TCR profiling of single cells

The ICCELL8 Human scTCR a/b Profiling workflow enables users to analyze T-cell receptor (TCR) diversity from single T cells isolated on the ICCELL8 Single-Cell System (Cat. No. 640000). The workflow consists of the **ICCELL8 TCR Chip** (Cat. No. 640178), the **ICCELL8 Human TCR a/b Profiling - Indexing Primer Set** (Cat. Nos. 640179, 640180 & 640181), the **ICCELL8 Human TCR a/b Profiling Reagent Kit** (Cat. No. 640182), the ICCELL8 Collection Kit (Cat. No. 640048), and the ICCELL8 Loading Kit (Cat. No. 640109), each of which must be purchased separately. Our TCR profiling solution leverages SMART® technology (Switching Mechanism At 5' End of RNA Template) 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 the ICCELL8 MultiSample NanoDispenser (MSND) into the wells of a 5,184-nanowell ICCELL8 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 ICCELL8 MSND.

Following the dispensing of the samples and controls, the ICCELL8 Imaging System and Micro-Manager software are used to acquire images of the 5,184 nanowells with both Texas Red and Hoechst 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 CellSelect® Software, which identifies nanowells containing single, viable cells that meet user-defined criteria. Upon identification and selection of candidate nanowells, the CellSelect 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 ICCELL8 TCR Chip, the chip is returned to the ICCELL8 MSND and RT-PCR reagents are distributed to nanowells selected by the software. The ICCELL8 TCR chip is then transferred to the ICCELL8 Chip Cycler, which is programmed to perform first-strand synthesis and amplification of cDNA in a single run. During this process (Figure 1, below), 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 ICCELL8 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.

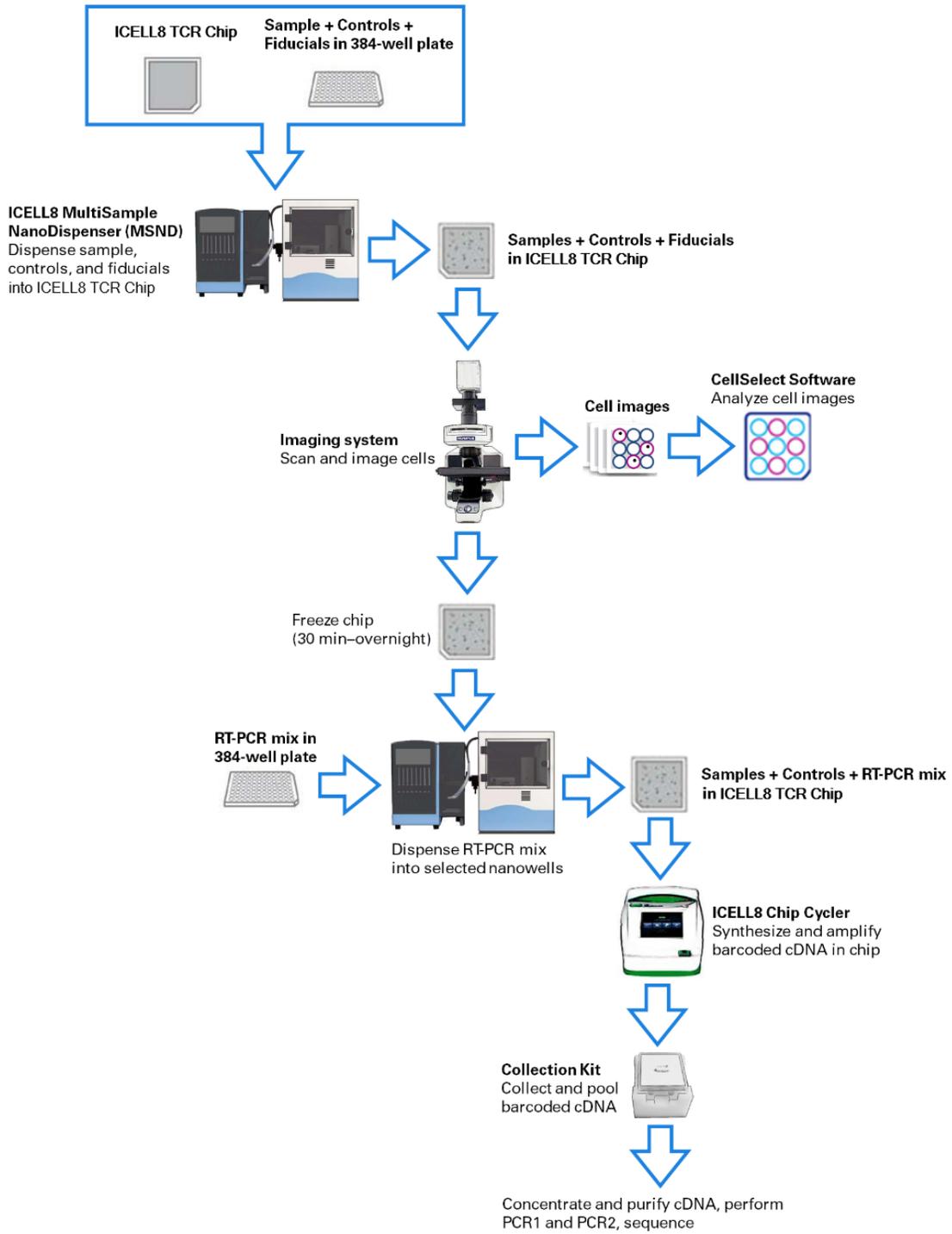


Figure 1. Diagram for the ICCELL8 Human TCR a/b Profiling workflow.

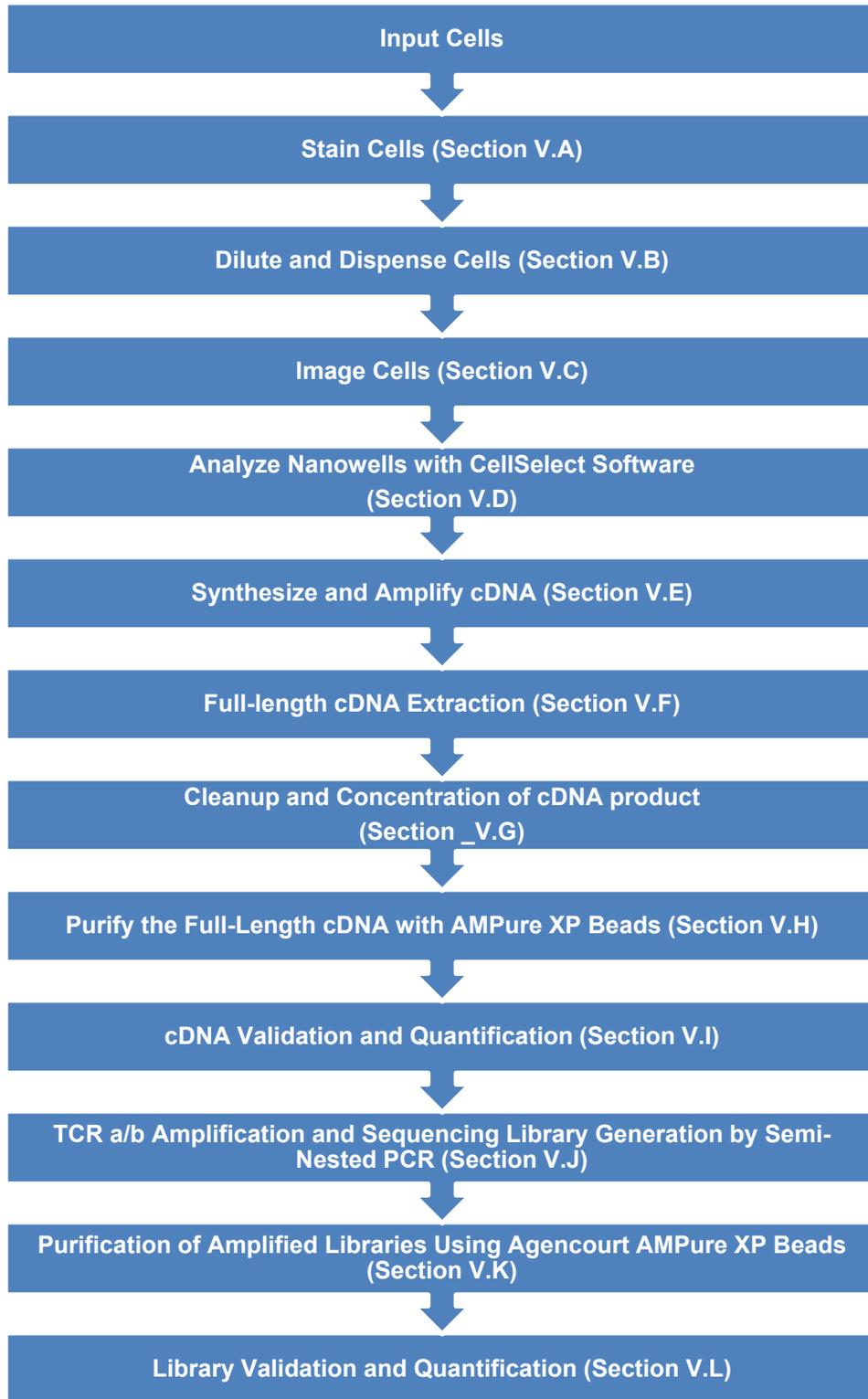


Figure 2. Protocols for ICELL8 Human TCR a/b Profiling.

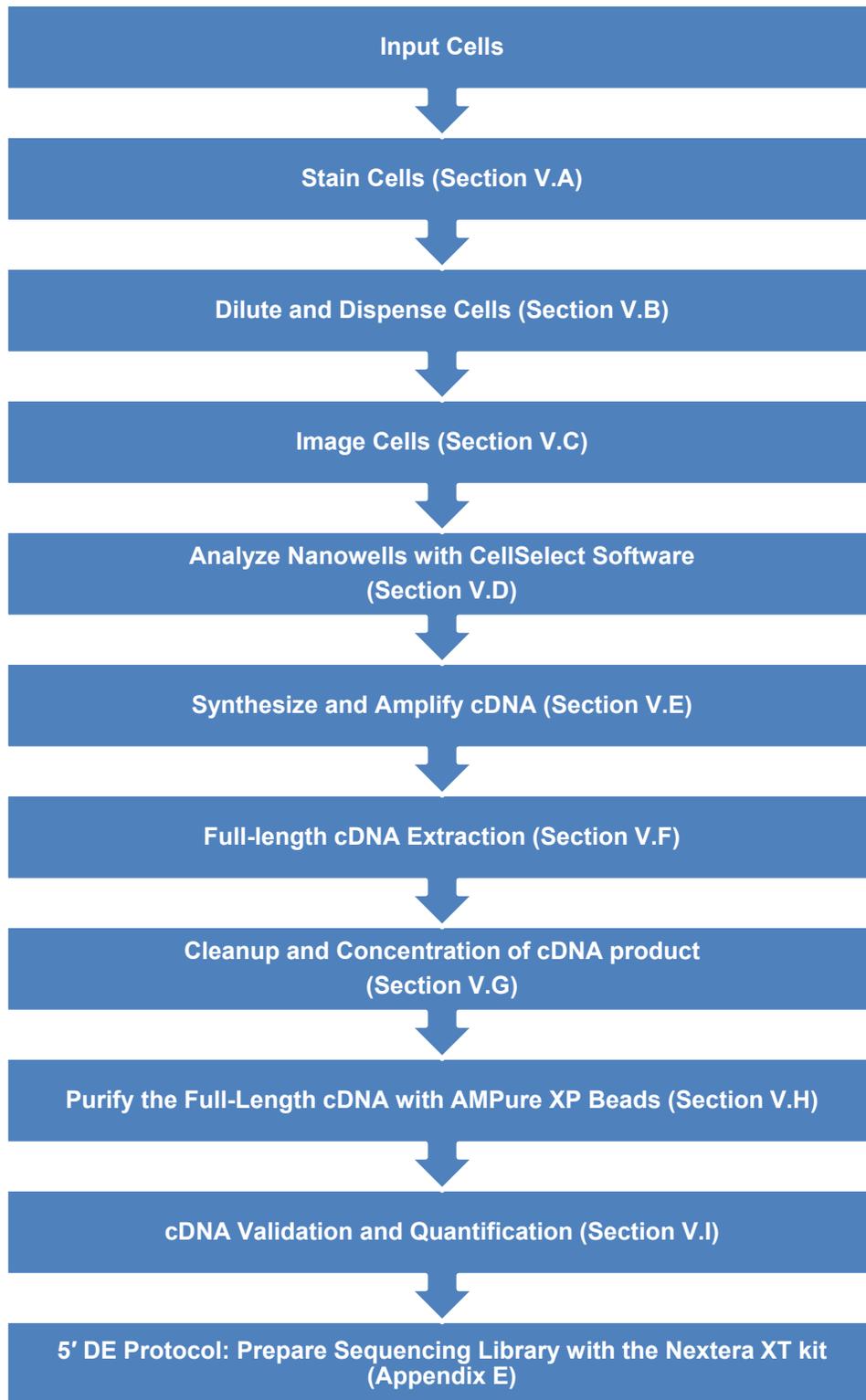


Figure 3. Protocols for 5' DE.

II. List of Components

The ICELL8 Human TCR a/b Profiling workflow consists of the ICELL8 TCR Chip (Cat. No. 640178), the ICELL8 Human TCR a/b Profiling - Indexing Primer Set (Cat. Nos. 640179, 640180 & 640181), the ICELL8 Human TCR a/b Profiling Reagent Kit (Cat. No. 640182), the ICELL8 Collection Kit (Cat. No. 640048), and the ICELL8 Loading Kit (Cat. No. 640109), each of which must be purchased separately.

ICELL8 Human TCR a/b Profiling Chip and Reagent Kit

Component Breakdown

ICELL8 TCR Chip (Cat. No. 640178, store at -20°C)

ICELL8 Human scTCR 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 8.

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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 (Cat. No. 640048, store at room temperature)	
Component	Quantity per kit
Collection Fixture	1
Collection Tube	2
Collection Film	1

ICELL8 Loading Kit (Cat. No. 640109, store at room temperature)	
Component	Quantity per kit
Blotting Paper	3
Optical Imaging Film	1
TE Sealing Film	1

III. Additional Materials Required

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

ICELL8 Single-Cell System

This protocol requires use of a ICELL8 Single-Cell System (Takara Bio, Cat. No. 640000). The ICELL8 system consists of the following components:

ICELL8 Single-Cell System (Cat. No. 640000)	
Component	Quantity per system
ICELL8 Single-Cell Instrument	1
ICELL8 150v Chip*	3
ICELL8 3' DE Chip*	3
ICELL8 Reagent Kit	6
ICELL8 Collection Kit	6
ICELL8 Loading Kit	6
MSND 384-Well Source Plate and Seals	1 (pack of 20)
ICELL8 Imaging Station Installation Kit	1
ICELL8 Imaging Film	1
Magnetic Tube Stand	1

*These chips are not compatible with the ICELL8 Human TCR a/b Profiling workflow. A ICELL8 TCR Chip must be purchased to perform the protocols detailed in this user manual.

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, $\geq 2,600g$, 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
- MSND 384-well Source Plates and Seals (Takara Bio, Cat. No. 640018)
- Single-channel pipettes: 10 μ l, 20 μ l, 200 μ l, and 1,000 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 pipettes and controller
- Minicentrifuges for 1.5-ml tubes and 0.2-ml tubes or strips
- Vortex mixer
- Exhaust hood system with UV
- Two pairs of tweezers for handling chips during imaging

For MSND general operation:

- Deionized water (for pressure reservoir and humidifier)
- Freshly mixed 0.2% bleach solution
- Helium (or argon) (>99.9% purity)

NOTE: The ICELL8 MSND has been qualified to work with either helium (preferred) or argon gas if helium is unavailable. References to 'noble gas' in this document should be interpreted to mean whichever of these two are being used in your system.

IMPORTANT: Other noble gases (neon, krypton, etc.) should NOT BE USED.

For staining and dispensing cells:

- 1X PBS (no Ca²⁺, Mg²⁺, 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)
- Molecular biology-grade/PCR-grade/nuclease-free water

For cleanup of PCR reactions:

- NucleoSpin Gel and PCR Clean-up Kit (Takara Bio, Cat. No. 740609.50)
- 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.

- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Fluorometer (Thermo Fisher Scientific)

For full-length cDNA amplification:

- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Fluorometer (Thermo Fisher Scientific)

For cDNA and sequencing library quality and quantity assessment:

- Agilent 2100 Bioanalyzer: High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Bioanalyzer instrument (Agilent) or similar

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)

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 1×10^5 and 7.5×10^6 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 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.
- Use deionized (e.g., Milli-Q) water for the MSND pressure bottle and humidifier; clean these reservoirs routinely. Use 0.2% bleach solution for the MSND wash bottle.
- 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 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 TCR Chip

- Each ICELL8 TCR Chip is engraved with a unique number. You can use this number to link your chip images and other experimental record files.
- Nanowell barcodes were preprinted onto each chip; the printing lot number is stamped onto the chip (see below, Figure 4). A total of 1,728 unique nanowell barcodes are available; each nanowell barcode was printed in triplicate onto a given chip.

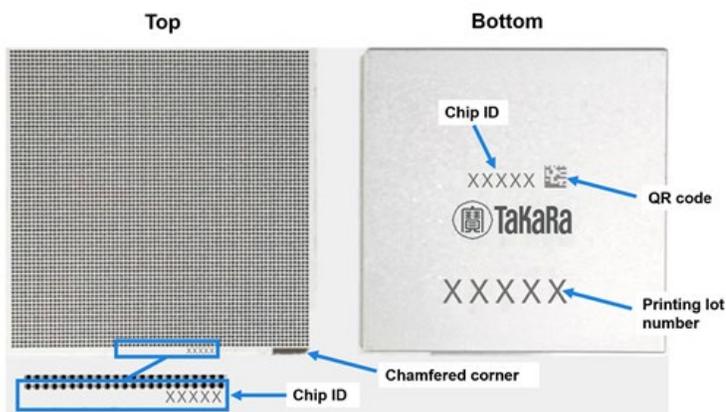


Figure 4. ICELL8 TCR Chip features and identifying information. **Left.** Note the chamfered (notched) corner at the bottom right. “TaKaRa” and Chip ID are on the chip border, near the chamfered corner. **Right.** Engraved on the back of each chip is the five-digit Chip ID (also called “Chip Number”) unique to each chip and the nanowell barcodes printing lot number. Next to the Chip ID is the corresponding QR code of the Chip ID that can be scanned by a barcode reader and entered into the software.

E. ICELL8 MSND Application Notes

Refer to the ICELL8 MSND User Manual for full details. Included below are general reminders.

- Before operating the instrument, check the water level in the pressure reservoir. Refill the pressure reservoir if the weighing scale below the pressure reservoir displays a value ≤ 4 kg (≤ 8 lb).
- Add deionized water (e.g., Milli-Q water) to the reservoir as indicated in the following steps and Figure 5 (below):

- Close the helium (or argon) in-line stopcock and open the vent stopcock.
- Fill the pressure reservoir with deionized water to the fill line (top of the protective cover rim). The weighing scale below the pressure reservoir should display a value of ~4 kg.
- Reattach the cap and let sit for ~20 min to degas. Make sure that the cap is properly threaded and securely fastened to prevent the noble gas from leaking out.
- Close the system by opening the noble gas in-line stopcock and closing the vent stopcock.

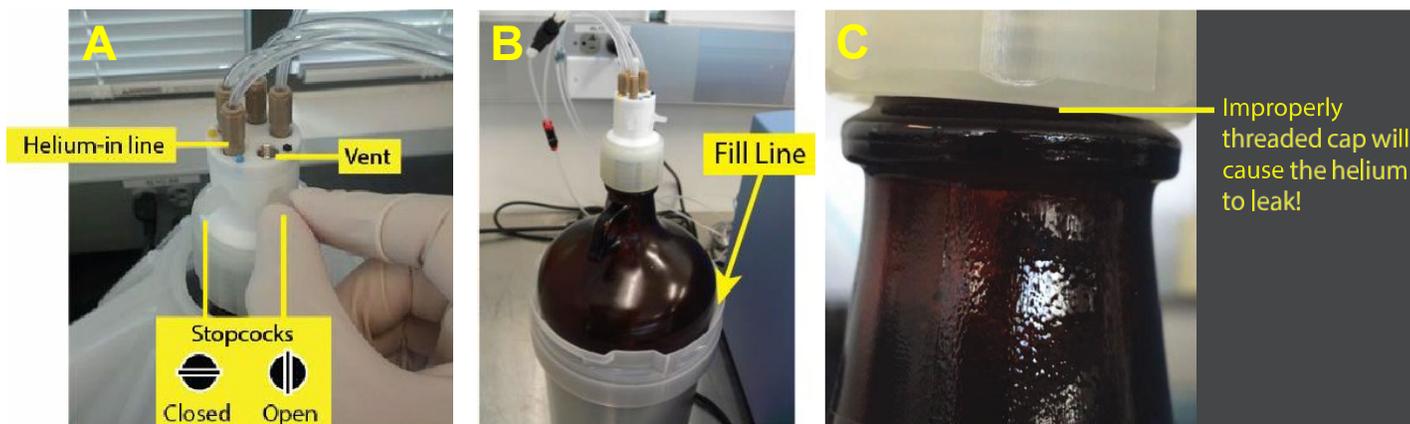


Figure 5. Filling the pressure reservoir. Panel A. Close the noble gas in-line stopcock and open the vent stopcock.

Panel B. Add Milli-Q water to the fill line of the pressure reservoir. Panel C. Make sure that the cap on the pressure reservoir is properly attached.

- Check the water level in the humidifier and fill if needed. Clean the humidifier regularly.
- Check the pressure on the noble gas tank. The regulator should be set to a supply input of >500 psi (3.5 MPa) and an output of 30–40 psi. If the tank pressure drops to <500 psi, replace it with a full tank.
- Check the bleach level in the wash bottle. If it is <25% full or ≥ 3 –4 days old, replace it with 500 ml of freshly made 0.2% sodium hypochlorite solution prepared from reagent-grade sodium hypochlorite in deionized, filtered water (e.g., Milli-Q, ELGA system, or equivalent with 0.2- μ m filtration).
- Check the waste container. If full, dispose of the liquid waste appropriately and replace with an empty container.
- All dispensing steps in the MSND Stage Module should be performed with the 384-well source plate oriented with the A1 well positioned at the top-right corner of the 384-well Plate Nest, and the chip oriented with the chamfered (notched) corner positioned towards the lower-right corner of the Dispensing Platform (Figures 6–8, below).

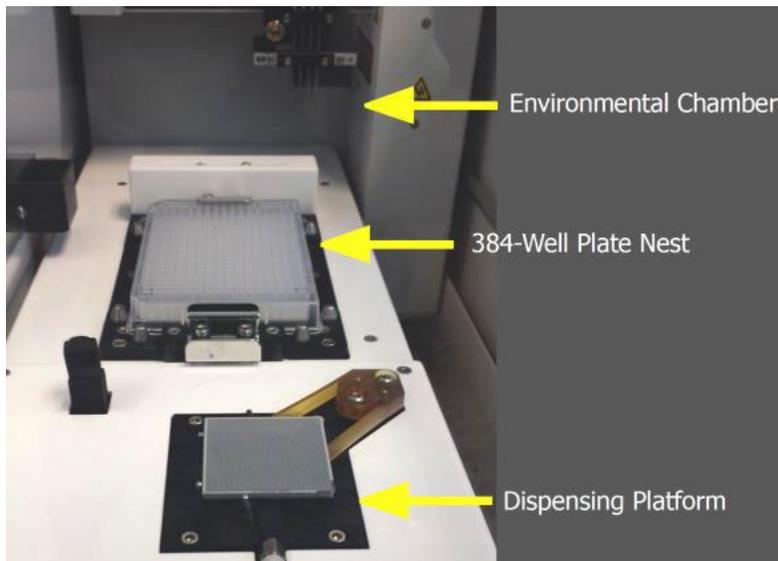


Figure 6. ICELL8 MSND Stage Module.

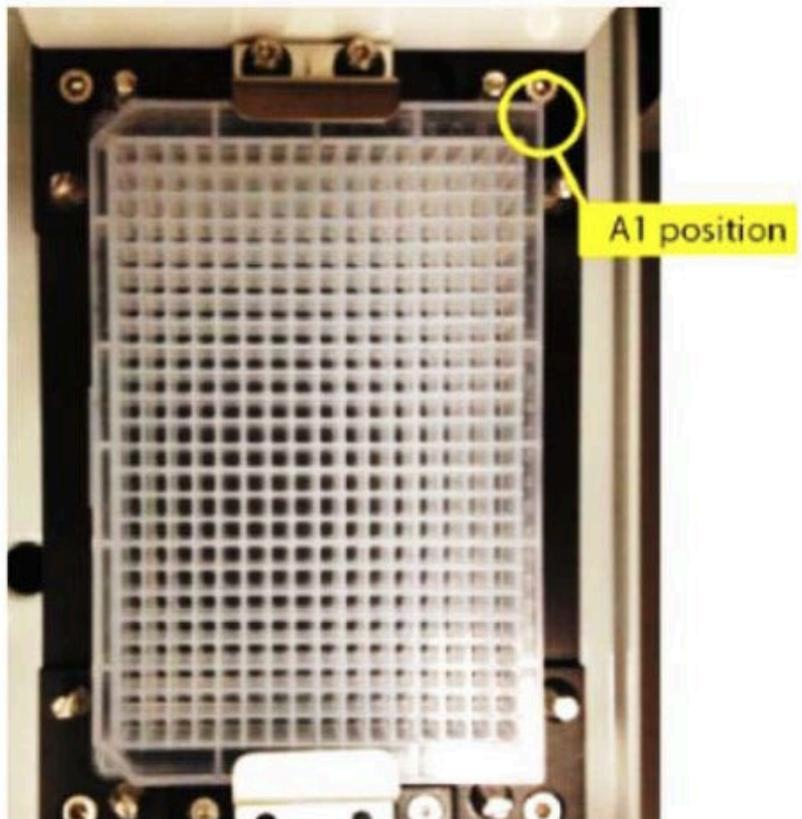


Figure 7. 384-well source plate in the Plate Nest.

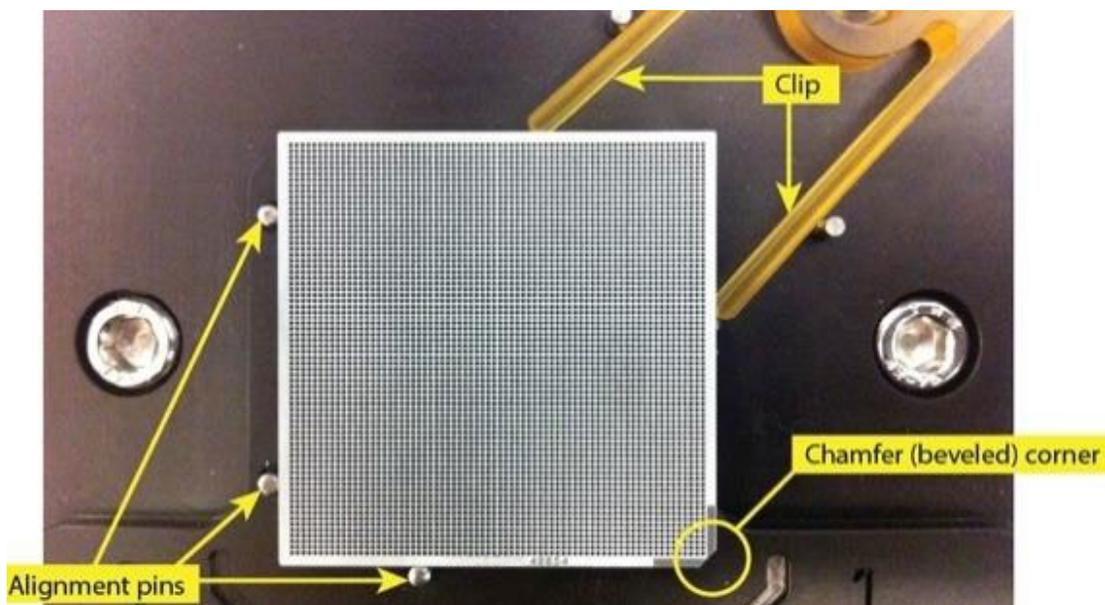


Figure 8. Chip seated in the Dispensing Platform. Stretch the arms of the clip apart and angle the chip onto the Dispensing Platform with the chamfered (notched) corner of the chip oriented towards the lower-right corner of the Dispensing Platform. The edges of the chip should be pressed against the three alignment pins.

IMPORTANT: Make sure that the entire chip lies perfectly flat and sits squarely on the Dispensing Platform. Make sure that the alignment pins do not improperly catch on the sidewalls of the chip.

F. ICELL8 Imaging System and CellSelect Software

Included below are instructions for using the ICELL8 Imaging System and CellSelect Software to perform the protocol described in this user manual. Please refer to the ICELL8 Imaging System User Manual and the CellSelect 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 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 1×10^5 and 7.5×10^6 cells/ml.

Before you start:

- Initialize the ICELL8 MSND; perform a daily warm-up via the *Startup* window, then pull up the *Advanced* window and execute the **Wash Prime** function followed by **Tip Clean**. Refer to the ICELL8 MSND User Manual for more details.
- Prefreeze Chip Holder(s) at -80°C .
- Set the chip centrifuge to 22°C .
- Start the ICELL8 Imaging System by turning on system components in the following order: the

fluorescence light source, the camera, the controllers for the microscope imaging filters and stage, and then the computer. Please note that the fluorescence light source requires a warmup period of ~5 min. Refer to the ICELL8 Imaging System User Manual for detailed setup instructions before first use.

- Thaw Second Diluent (100X), ICELL8 Fiducial Mix (1X), and Nuclease-Free Water (all provided with the ICELL8 Human TCR a/b Profiling Reagent Kit, Cat. No. 640182) 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 $\mu\text{g}/\mu\text{l}$) to 10 $\text{ng}/\mu\text{l}$ for use in the next protocol (Section V.B, Table 1) as indicated in the following steps and keep the dilution on ice:
 - Dilute Control Jurkat Total RNA to 50 $\text{ng}/\mu\text{l}$ by mixing 38 μl of nuclease-free water with 2 μl of Control Jurkat Total RNA (1 $\mu\text{g}/\mu\text{l}$) in a sterile nuclease-free microcentrifuge tube.
 - Dilute Control Jurkat Total RNA to 10 $\text{ng}/\mu\text{l}$ by mixing 20 μl of nuclease-free water with 5 μl of the Control Jurkat Total RNA diluted to 50 $\text{ng}/\mu\text{l}$ in the previous step.

NOTES:

- Return Control Jurkat Total RNA (1 $\mu\text{g}/\mu\text{l}$) 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., 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 Ca²⁺, Mg²⁺, 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 100g 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 500g 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 x 10⁵–5.0 x 10⁵ cells/ml. If you end up with a concentration lower than 1.2 x 10⁵ cells/ml, repellet 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 TCR Chip using the ICELL8 MSND. Refer to the ICELL8 MSND User Manual for detailed information about instrument setup and operation.

Before you start:

- Confirm that initialization and setup procedures for the ICELL8 MSND and ICELL8 Imaging System have been completed (see “Before you start” in [Section V.A](#), above).
- Prefreeze the empty Chip Holder (Figure 9, below) at -80°C .
- 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 an MSND 384-Well Source Plate (with seal) and the following components: Control Jurkat Total RNA (diluted to 10 ng/ μl ; [Section V.A](#)), stained cell suspension ([Section V.A](#)) Second Diluent (100X), ICELL8 Fiducial Mix, RNase Inhibitor (40 U/ μl), Blotting Paper, and Optical Imaging 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 cell/50 nl in a total volume of 1 ml.



Figure 9. Chip Holder.

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 cell/50 nl*	Dilute to 1 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 cell/50 nl (i.e., 20,000 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 10, 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 cell/50 nl in a total volume of 1 ml and invert gently to mix (refer to the ICELL8 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 MSND User Manual for detailed information about instrument setup and operation.

- 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.

- Using a 200 µl pipette tip, slowly and carefully load 80 µl of cell suspension into wells A1, A2, B1, B2, C1, C2, D1, and D2 of an MSND 384-Well Source Plate as indicated in Figure 10 (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.

- Add Positive Control, Negative Control, and ICELL8 Fiducial Mix (1X) to the 384-well source plate as indicated in the following steps and in Figure 10 (below):
 - Add 25 µl of prepared Positive Control to Well P24.
 - Add 25 µl of prepared Negative Control to Well A24.
 - Add 25 µl of ICELL8 Fiducial Mix (1X) to Well P1.

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.

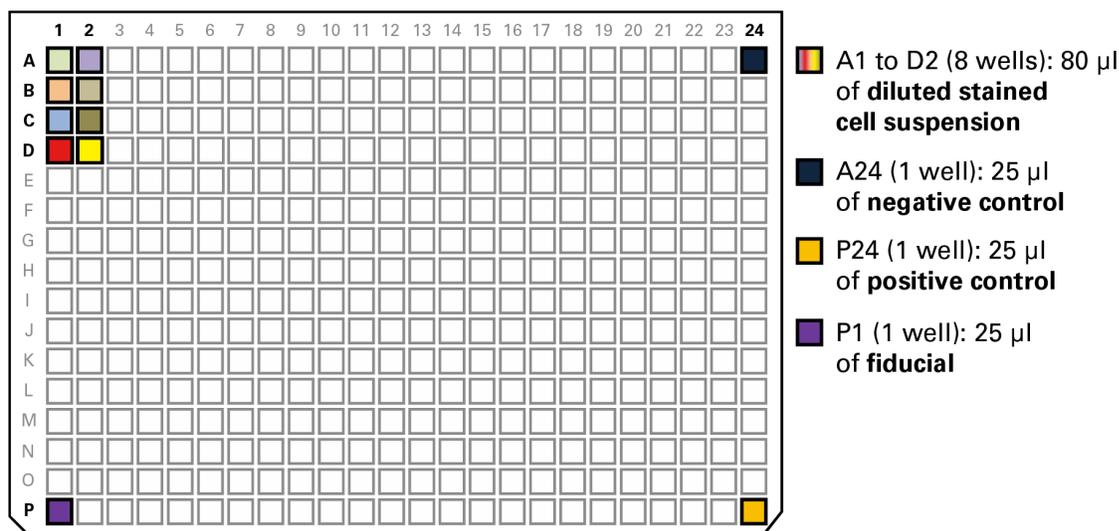


Figure 10. Setting up the 384-well source plate for dispensing cell samples and controls.

- Seal the 384-well source plate with a 384-well plate seal (Takara Bio, Cat. No. 640018). DO NOT centrifuge the plate.

9. Open the packet containing the sealed ICELL8 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.
10. Place the chip on the the Dispensing Platform in the MSND with the chamfered (notched) corner of the chip at the lower right corner of the Dispensing Platform, aligned with the chamfered corner of the chuck (see Figure 8, [Section IV.E](#)).

IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat on the chuck from all sides and is in the correct orientation** (see Figure 8).

11. Place the 384-well source plate in the MSND with the A1 corner positioned at the top-right corner of the Plate Nest and the beveled corners of the 384-well source plate on the left side (see Figure 7, [Section IV.E](#)).
12. Gently remove the seal from the 384-well source plate and lock the plate into position, then close the MSND environmental chamber doors.
13. In the ICELL8 MSND software GUI, click the *Single Cell* tab and then scan the barcode on the back of the chip or enter the chip ID into the text field.



CAUTION: Be sure to avert your eyes from the scanner light emitted by the Barcode Reader.

14. In the ICELL8 MSND Software GUI, click the [Dispense cells] button (Figure 11, below) then click **OK** after carefully going through all questions prompted. The ICELL8 MSND will start dispensing 50 nl of cell suspension, positive/negative control, and fiducial mix to the appropriate nanowells.

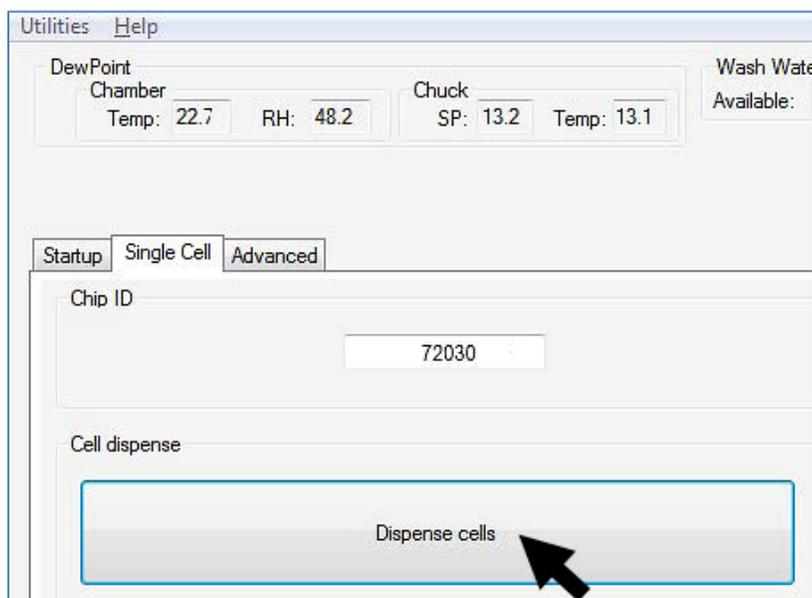


Figure 11. Using the MSND software to dispense cells into the ICELL8 TCR Chip.

15. After dispense of reagents is completed, remove the chip from the Dispensing Platform and gently blot for 2 sec with a fresh piece of blotting paper (provided in the ICELL8 Loading Kit) and a blotter (Figure 12, below).



Figure 12. Blot the chip with blotting paper and a blotter. A blotter is provided with the ICELL8 Single-Cell System (Cat. No. 640000).

16. Remove the liner from **only one side** of the imaging film and apply the exposed sticky side of the imaging film to the blotted chip (Figure 13, below).



Figure 13. Sealing film and imaging film. The sealing film (left) is provided with a white backing. The imaging film (right) has three layers; the intermediate layer is double sided and is provided between two layers of clear backing.

17. Use a Film Applicator to make sure chip is securely sealed to avoid well-to-well contamination (left panel of Figure 14). **Note the orientations of the chamfered corner and the imaging film tab** (right panel of Figure 14).

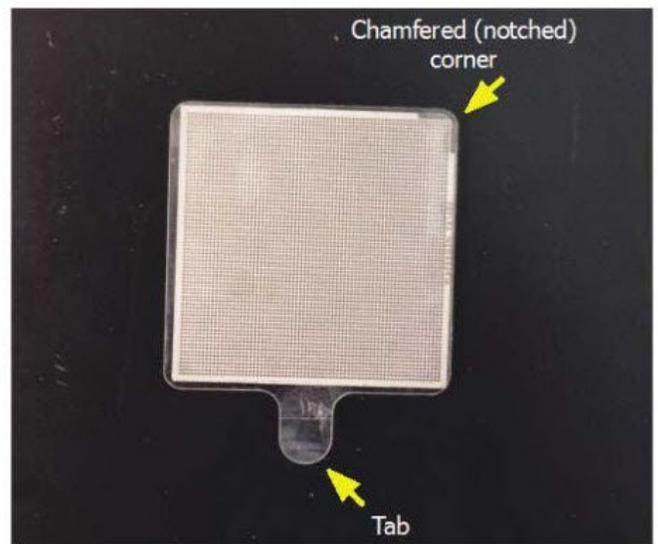
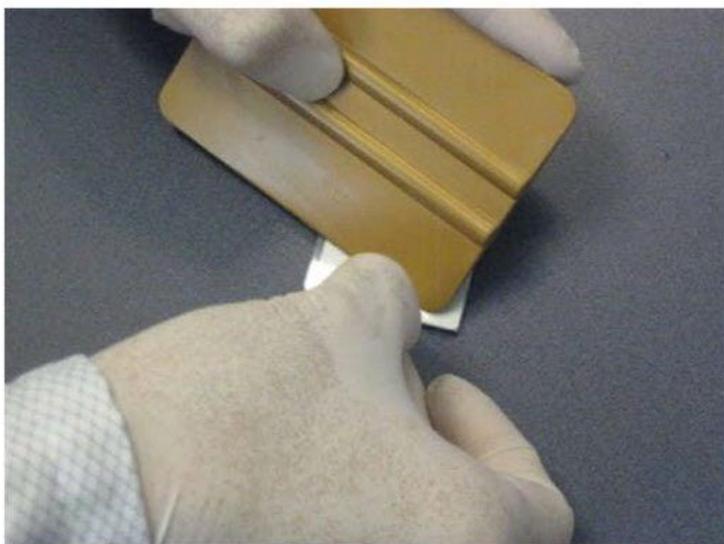


Figure 14. Sealing the blotted chip. Seal the blotted chip using a film applicator (left). The tab of the imaging film should be oriented downward relative to the chamfered corner of the chip (right).

18. Place the sealed chip on a centrifuge spin plate and centrifuge the sealed chip at 300g for 5 min at 22°C with full acceleration and full brake (Figure 15, 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 15. Centrifuge Chip Spinner.

C. Protocol: Image Cells

In this protocol, images of all 5,184 nanowells of the ICELL8 TCR Chip are acquired. Refer to the ICELL8 Imaging System User Manual for detailed information about instrument setup and operation.

ICELL8 Imaging System notes

- The light source requires a warm up period of ~5 min. A blue light should be visible indicating that the burner is ready.
 - The bulb has a life span of ~2,000 hr. Please contact Takara Bio Field Support for assistance with changing the bulb. After a new bulb is installed, let it run for 1 hr before normal operation.
 - After the burner is turned on, please allow at least 30 min before turning it off.
 - Wait for at least 30 min before turning on a previously turned-off burner.
 - Do not turn the burner on and off more than 4 times a day; it should be left on if you plan to use it frequently throughout the day.
 - Allow the imaging system's camera to warm up for 15 sec (green light is on and has stopped blinking) before starting the Micro-Manager software.
- NOTE:** Failure to allow the camera to warm up before starting the software could result in a hardware configuration error. If the error occurs, close the software and restart the camera.
- Micro-Manager software application should remain closed until all other pieces of hardware are turned on.

Procedure:

Start software and input chip information

1. Start the Micro-Manager software. Ensure that the manual shutter is in the Open position.
2. Click **Live** in the *Main* window to open a window showing a live image from the camera.
3. Click **Multi-D Acq.** to open the *Multi-Dimensional Acquisition* window.

4. Make sure that the [Multiple positions (XY)] box is checked.
5. Click **Load Chip type** and load the configuration file 72.72.pos (if it is not preloaded).
6. Position the cursor so that it resides in the “Chip Number” field (indicated by the arrow in Figure 16, Panel A, below). Input the chip ID or use the barcode scanner to scan the two-dimensional barcode on the back of the chip **WITHOUT** completely inverting the chip (Figure 16, Panel B, below).



CAUTION: Be sure to avert your eyes from the scanner light emitted by the Barcode Reader.

A

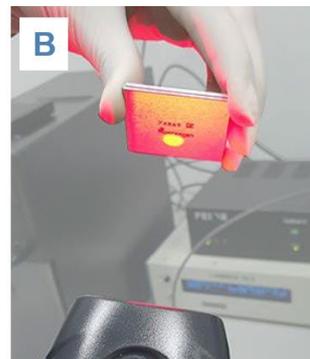
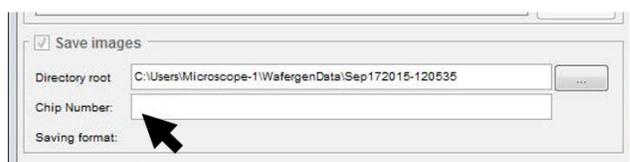


Figure 16. Inputting the Chip ID. Place the cursor in the “Chip Number” field (**Panel A**), and either input the Chip ID manually, or scan the QR code on the bottom of the chip (**Panel B**).

7. Enter a short experimental name and the chip number in the file name prefix (`..\WafergenData\<>experiment name_chip number<>`). The image files will be saved under this named folder.

Position chip on microscope

8. Place the chip on the microscope holding platform with the chamfered (notched) corner facing the upper right corner (Figure 17, below). **Ensure that the chip is sitting flat on the chuck from all sides and is in the correct orientation.**

NOTE: The chip should be centrifuged prior to imaging.

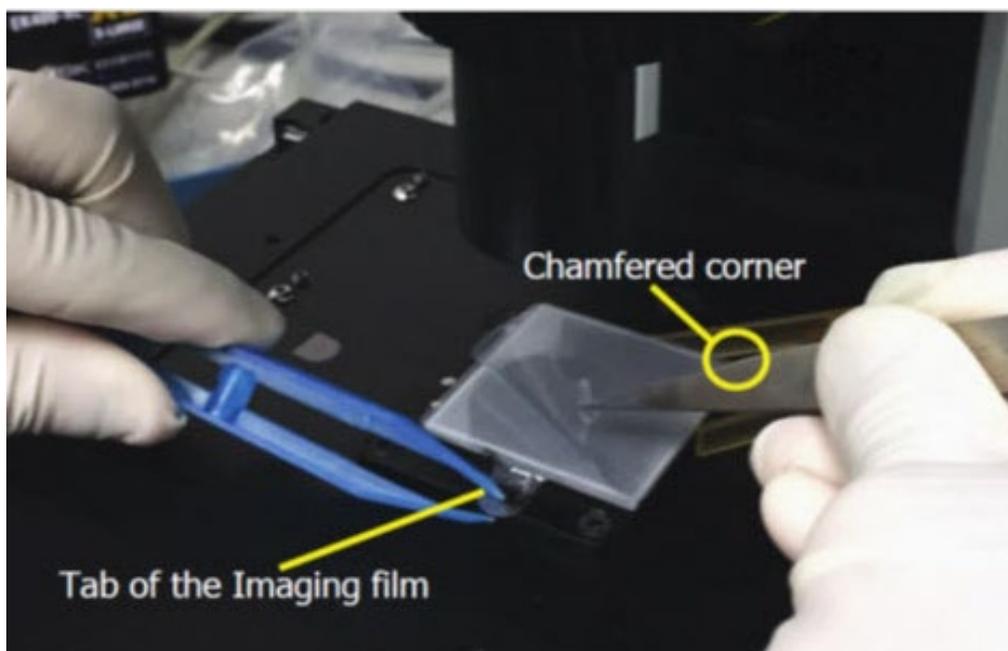


Figure 17. Seating the chip on the microscope holding platform.

9. Ensure the chip is perfectly flat and seated square relative to the pins before removing the second release liner. Peel off the top layer release liner of the imaging film using tweezers (Figure 17, above). **Save the release liner.** The chip will remain sealed by the double-sided intermediate film layer.
10. Move the objective over the chip. Ensure that the objective is vertical and centered (you should hear/feel a slight click).
11. Select the row “Pos0” in the *Stage Position List* window (Figure 18, below), and then click the [Go to] button.

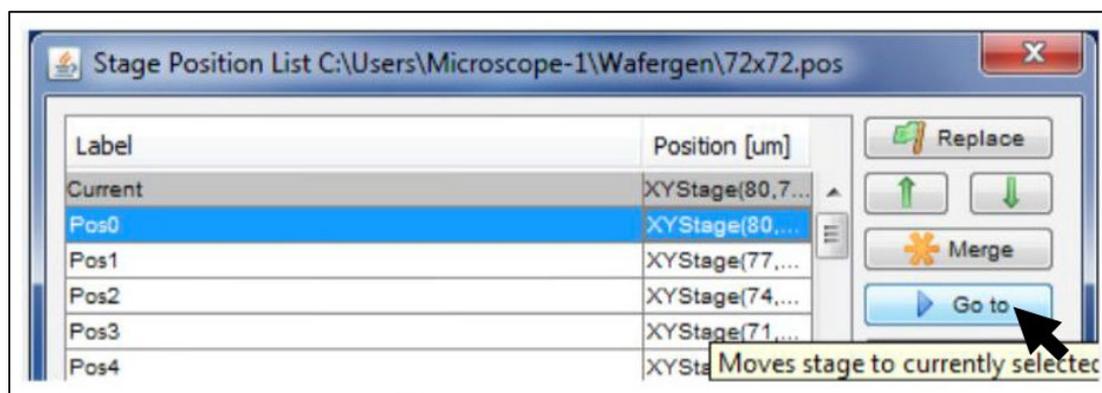


Figure 18. Moving the motorized stage to Position 0.

12. In the *Main* window, under “Configuration settings”, select **3-Texas Red** as the illumination channel for examination of fiducial-containing nanowells (Figure 19, below).

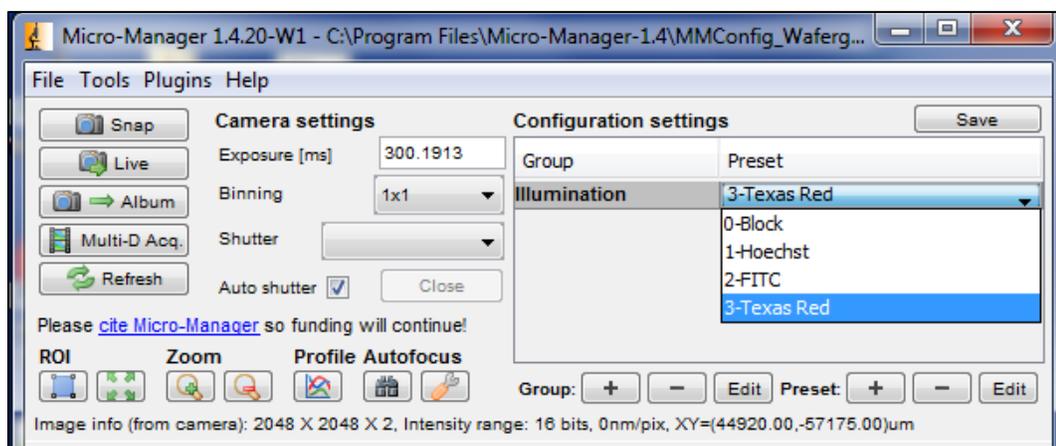


Figure 19. Setting the illumination channel to Texas Red.

13. Confirm that five fiducial-containing wells are present, forming an “X” shape at Pos0 (Figure 20, below). There are four fields of view across an entire chip that include fiducial-containing nanowells (Pos0, Pos11, Pos132, and Pos143). Each field of view includes 36 nanowells (a 6 x 6 grid).

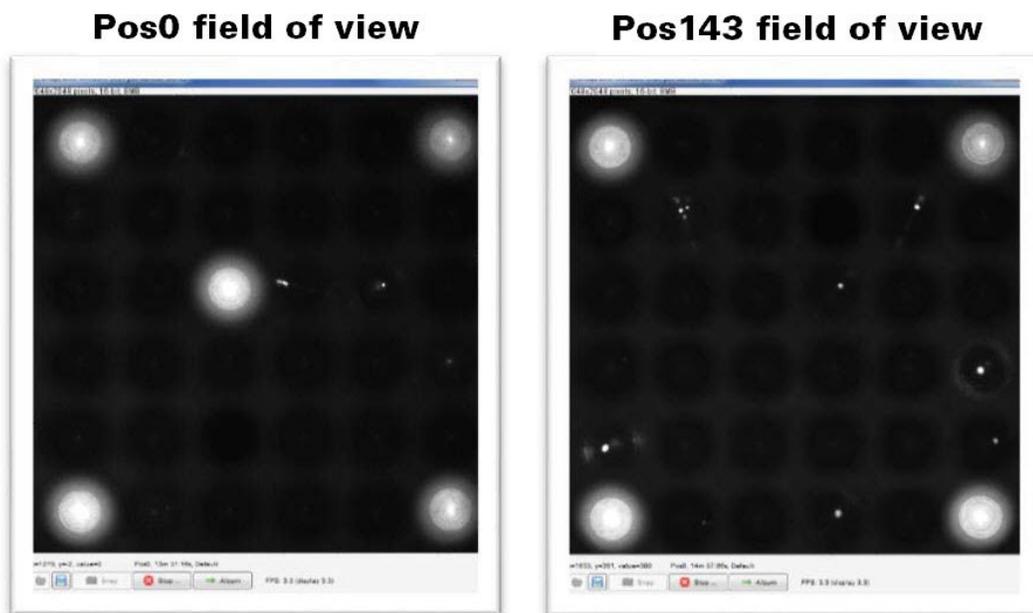


Figure 20. Multiwell images including fiducial-containing nanowells.

14. Select “Pos143” in the *Stage Position List* window and confirm that all 36 nanowells are properly captured in the image. There will be four fiducial-containing nanowells in the shape of a square, as shown in Figure 20 (above). Pos143 is located at the lower right corner of the stage fixture.

Optimize image focus

15. Adjust image contrast and focus settings for optimal visualization of single cells (refer to the ICCELL8 Imaging System User Manual for more details):
 - a. In the *Main* window, under “Configuration settings”, select **1-Hoechst** as the illumination channel for examination of Hoechst-stained cells (Figure 19, above).
 - b. Use the microscope’s manual focus to achieve the optimal Z-plane focal depth to visualize a field of single cells.

- c. Use the zoom function to zoom in on a well containing one or a few cells that are off center (i.e., not in the center of a well; Figure 21, below).
- d. Adjust the fine contrast knob back and forth to find the optimal focal point for a cell (smallest, sharpest view) or the optimal focal point between two cells on the Z-plane (blue box in Figure 21, below).
- e. Zoom out to view all 36 nanowells at Pos143 and confirm that the focus is optimal.

NOTE: If the cells are truly out of focus, ensure that the chip is perfectly flat on the Dispense Platform. Close the *Stage Position List* window.

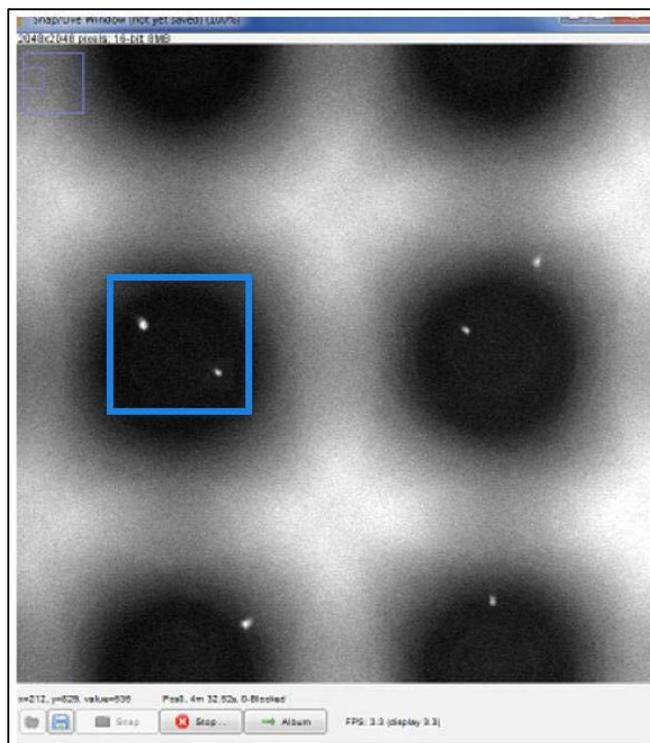


Figure 21. Adjusting the focus on single cells.

Acquire images

16. In the “Channels” table of the *Multi-Dimensional Acquisition* window, the following two preconfigured channels should be preselected: **Hoechst** (exposure: 200) and **Texas Red** (exposure: 300). The Hoechst channel enables visualization of Hoechst staining, while the Texas Red channel is used for visualization of propidium iodide staining.
17. Click **Acquire!** and accept the prompt to create a new folder based on the current date. The camera will acquire TIFF images from 144 fields of view (Pos0–Pos143) using Hoechst and Texas Red filters, respectively, with each field of view including 36 nanowells in a 6 x 6 grid. Following acquisition, the entire image folder should contain a total of 288 TIFF files comprising the 144 fields of view in both the Hoechst and Texas Red channels.
 - a. In the *Main* window, select the *Contrast* tab and confirm that the “Camera Depth” menu is set to **12 bit (0-4095)** for Hoechst and **10 bit (0-1023)** bit for Texas Red in the (purple box in Figure 22, below).
 - b. Next, confirm that the [Autostretch] and [Ignore] boxes are selected and that the percentage value is in the range of **1–3%** for the Ignore option (blue box in Figure 22, below). If this does not

provide optimal contrast, drag the ends of the diagonal line (see arrows in Figure 22, below) closer to the range of the histogram peaks and zoom out when satisfied with the focus in the Z-plane.

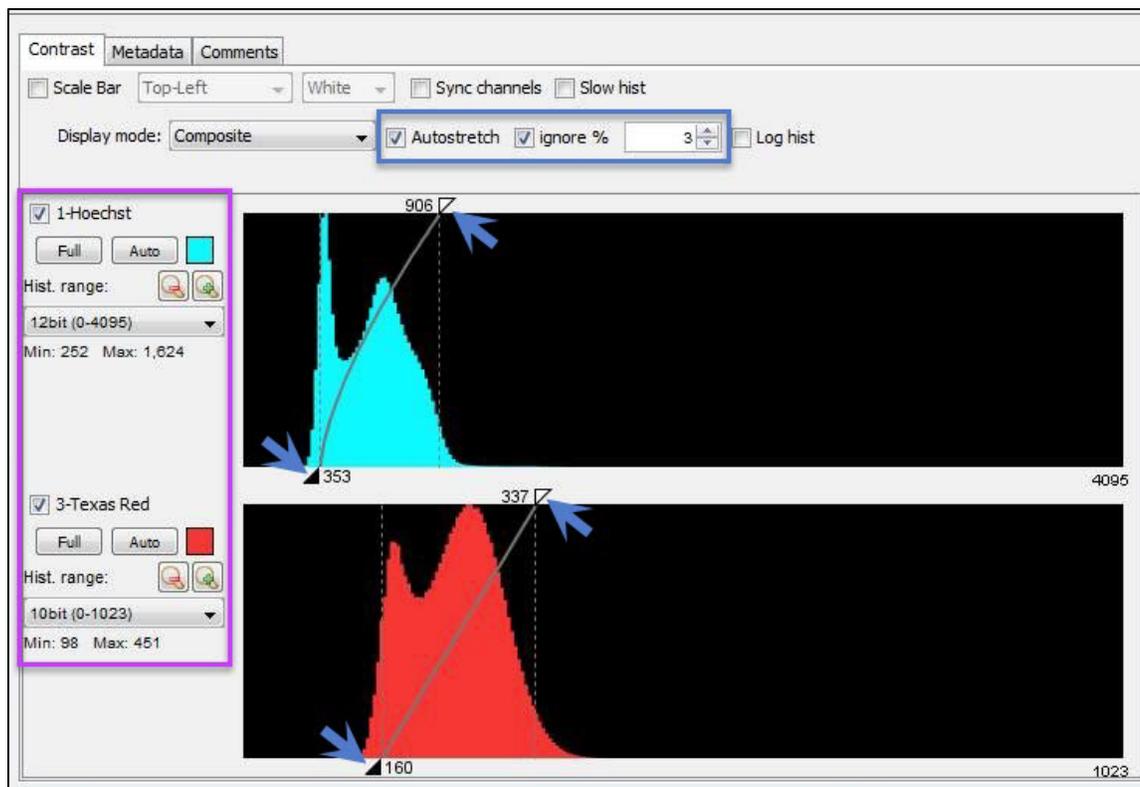


Figure 22. Adjusting camera depth and image contrast.

18. After imaging is complete, the software will provide the prompt “Run CellSelect with images from: C:\Wafergen\WafergenData For Chip: <Chip ID>?”. If proceeding to the next protocol (Section V.D, below), click **Yes**. This will launch the CellSelect Software. If you plan to analyze the images later, click **No** and close the Micro-Manager software.

NOTE: The directory will be different if users select to save the data elsewhere.

19. Reapply the peeled release liner onto the top side of the imaging film on the chip.
20. Place the imaged chip into an empty Chip Holder that has been prechilled at -80°C (Figure 9, Section V.B). Make sure that the imaging film is well sealed over the chip. The Chip Holder should click closed and should close evenly, indicating a proper magnetic seal.
21. Freeze the chip dispensed with cells at -80°C for a minimum of 30 min before proceeding to RT-PCR (Section V.E, below).
22. Turn off the controllers for the microscope imaging filters and stage, the camera, and the fluorescence light source when the imaging is completed.

SAFE STOPPING POINT: The chip can be frozen in the Chip Holder at -80°C overnight.

D. Protocol: Analyze Nanowells with CellSelect Software

In this protocol, CellSelect Software is used to analyze the images of the ICELL8 TCR Chip in order to identify nanowells containing viable single cells that are suitable for further processing and analysis via RT-PCR. Refer to the CellSelect Software User Manual for detailed information about using the software.

Load images, settings, and barcodes

1. If continuing from the previous protocol (Section V.C, above) skip to Step 2. Otherwise, start the CellSelect Software by clicking the **CellSelect** icon below and import the chip folder by clicking the **File > Open chip folder** menu option.



2. Click **YES** when prompted to convert the detected Micro-Manager image stack (Figure 23, below).



Figure 23. Converting image stack.

NOTE: This prompt will only display the first time that chip images are loaded into the CellSelect Software.

3. Confirm the Chip ID, load the settings file `StandardCellSettings-V5.1.xml`, select the preloaded GenePix Array List (.gal) file `1728x3_Quadded_10_17_2017_Rev1.gal`, and enter a chip comment, if helpful, as shown in Figure 24 below. This file contains nanowells barcode sequence for each preprinted nanowell location.

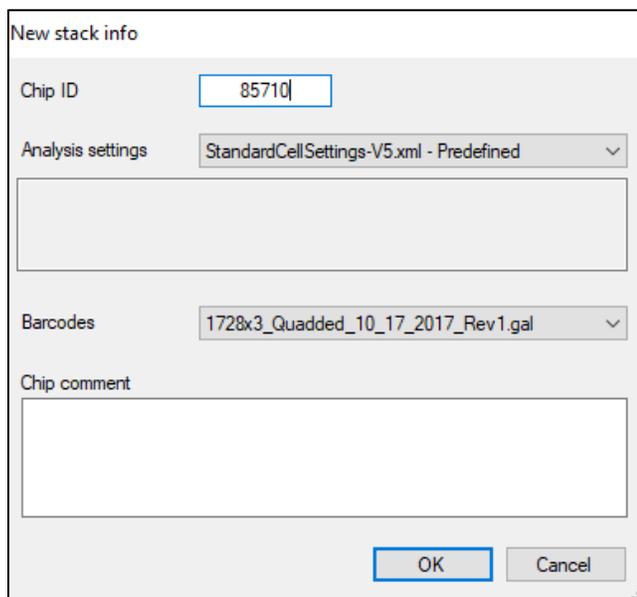


Figure 24. Loading the settings file.

Define fiducials

- The software will automatically detect fiducials and prompts you with four consecutive fiducial images. The [Define fiducials] box in the top-left portion in the *Main* window will be highlighted as this process occurs (see Figure 25, below). The first fiducial image (Pos0) shows five green circles, while the other three fiducial images (Pos11, Pos132, Pos143) show four green circles. The green circles highlight the centers of the nanowells.

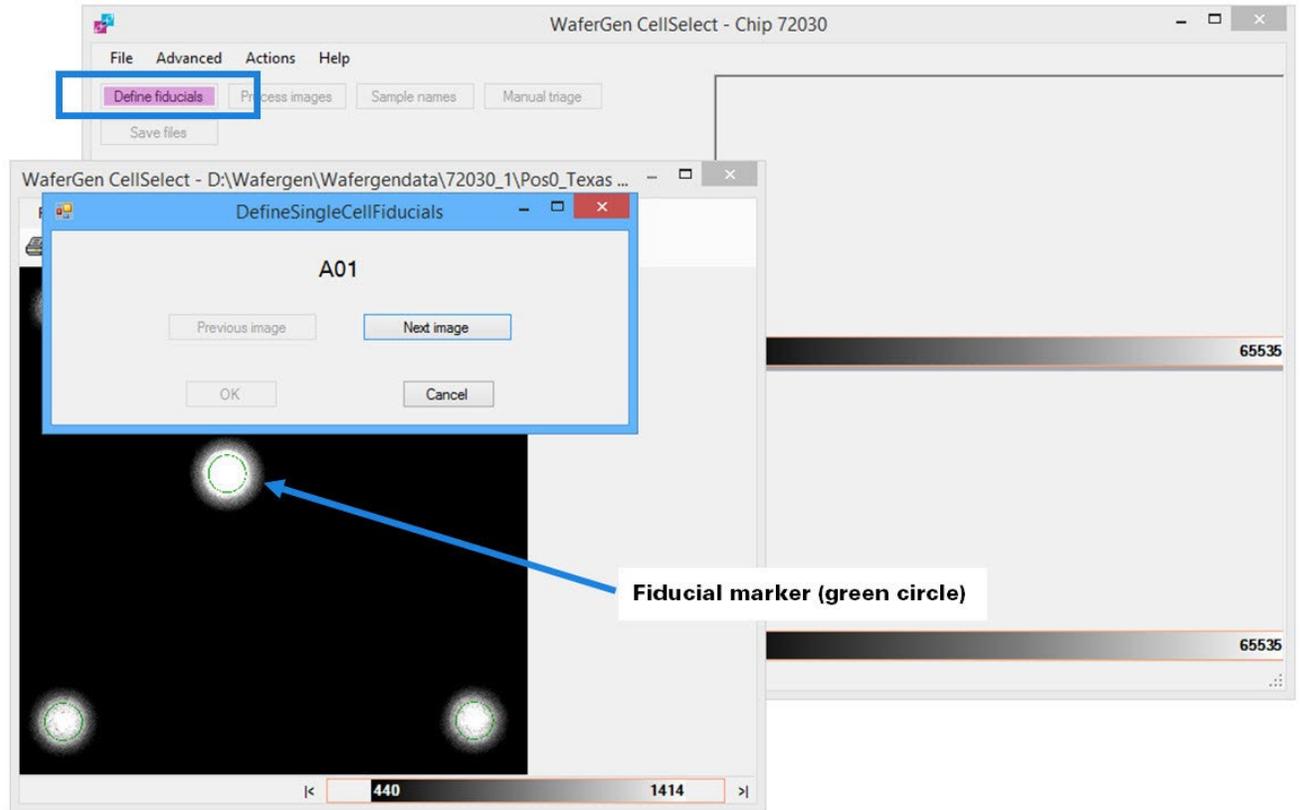


Figure 25. Defining fiducials.

5. Inspect the fiducials in each fiducial image for proper positioning.
 - a. Click and drag the *DefineSingleCellFiducials* prompt window to the side to view the fiducials image (see Figure 26, below).

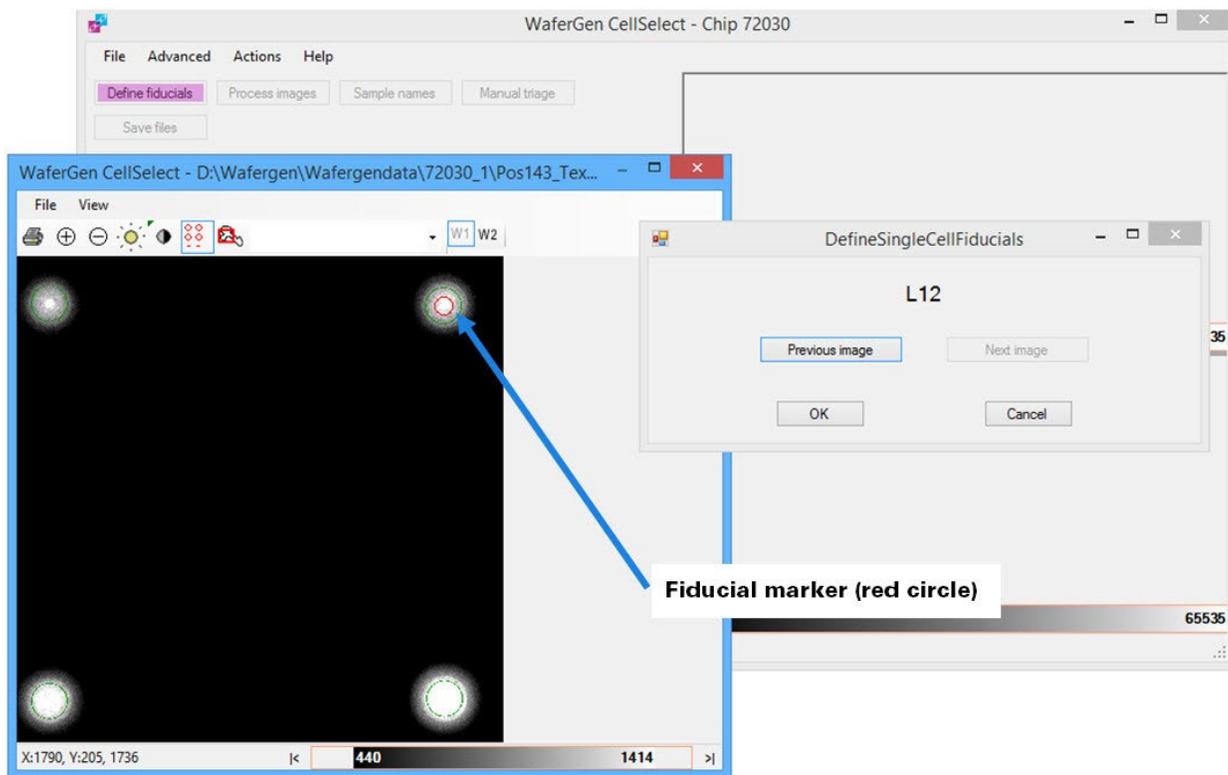


Figure 26. Inspecting and adjusting fiducials.

- b. If the green circles are not centered properly, click on the nanowell centers with the mouse pointer (changes to red circle; see Figure 26, above).
- c. If necessary, resize the fiducial image to view a 6 x 6 grid (36 nanowells). Use the following tools in the *Image Viewer* toolbar to check and correct fiducials: **Zoom in/Zoom out**, **Image contrast**, **Auto contrast**, **Well overlay**, **Wavelength 1 or 2 (W1, W2)**. Figure 27, below, provides a legend for the *Image Viewer* toolbar icons.



Figure 27. *Image Viewer* toolbar icons.

- d. Click through all four fiducial images and confirm that the chip was oriented correctly for dispensing samples. The image for the Pos0 field of view should show five fiducial-containing nanowells, while the images for the Pos11, Pos132, and Pos143 fields of view should each show four fiducial-containing nanowells. If the layout of fiducial-containing nanowells is correct, click **OK** and close the last fiducial image. If the layout of fiducial-containing nanowells is incorrect, contact Technical Support (refer to the end of manual for contact information) before dispensing RT-PCR mix.

Process images

6. Click the [Process Images] box (Figure 28, below). The software will analyze the two sets of the 144 multi-well images taken using Hoechst and Texas Red filters, respectively, and automatically identify and select all nanowells that contain viable, single cells (i.e., “candidates”) and controls based on the parameters defined in the *Settings* tab.

NOTE: For each image, the following criteria must be satisfied for the software to identify a cell as being a candidate for downstream analysis:

- The cell must appear in the Hoechst channel and NOT in the Texas Red channel.
- No other cells are visible in the well.
- The cell must satisfy the size/shape parameters specified in the *Settings* tab.

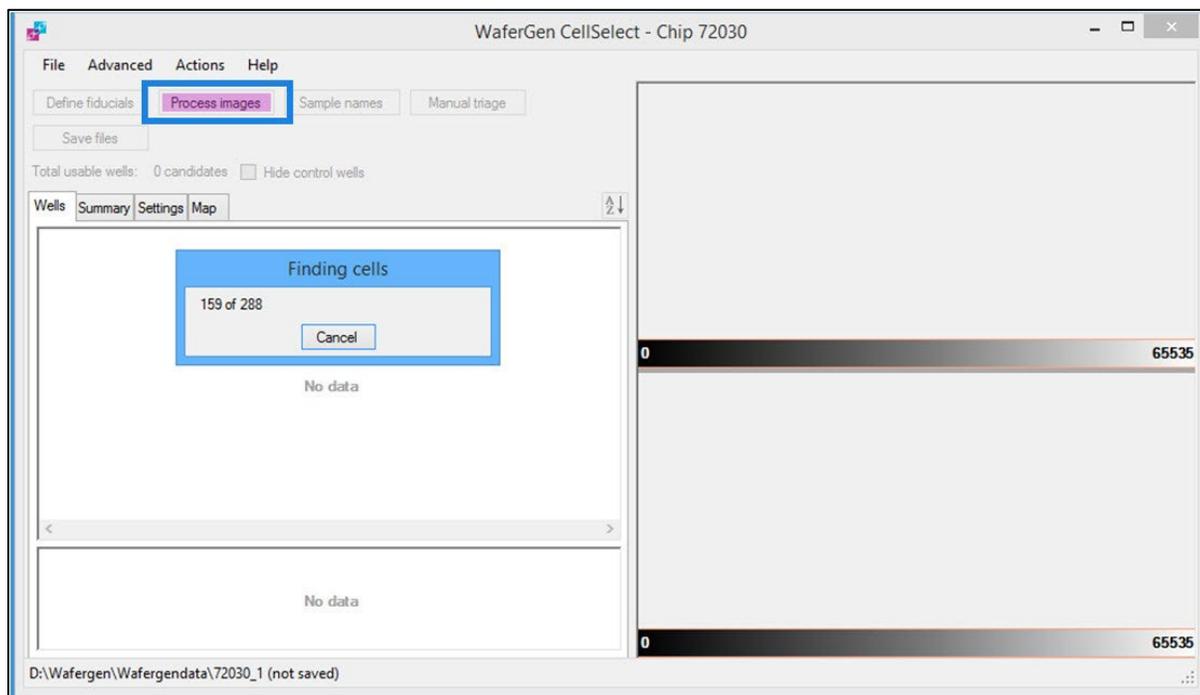


Figure 28. Having the software find cells based on parameters defined in *Settings* tab.

7. When prompted, click **Yes** to save results. You will then be prompted to downselect the number of wells (Figure 29, below).

NOTE: If by mistake at the *Save Results* window, **No** is clicked the Downselection can also be performed by clicking the **Actions > Downselect** menu item (Figure 30, below). The Downselection must be actively performed even if choosing the maximum number of single-cell samples.

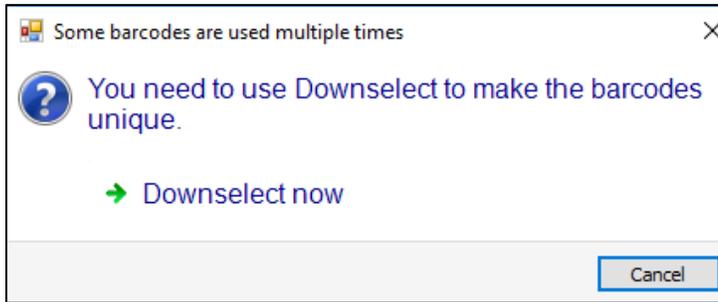


Figure 29. Downselect prompt.

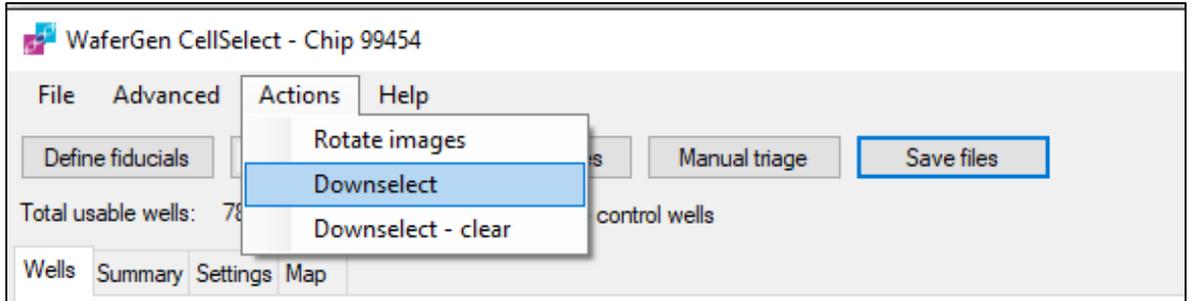


Figure 30. Manual downselect.

8. The CellSelect Software will prompt you to choose the number of wells for the Sample, Negative Control (Neg Cntrl), and Positive Control (Pos Cntrl) categories (Figure 31, below).

NOTE: The number of wells for each category should be less than maximum number available. In Figure 31’s example, a total of 1,100 samples were chosen out of the 1,316 maximum available wells.

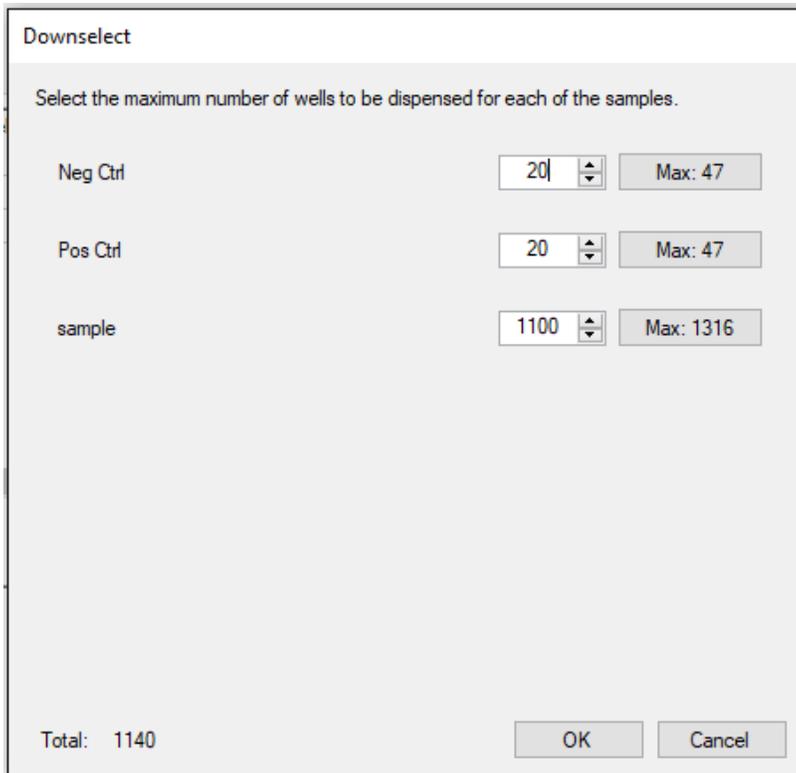


Figure 31. Downselecting sample, negative control, and positive control nanowells for the filter file.

9. When prompted, click **Yes** to save results
10. Go to the *Summary* tab (Figure 32, below) which will show the number for requested wells and the number available for dispensing.

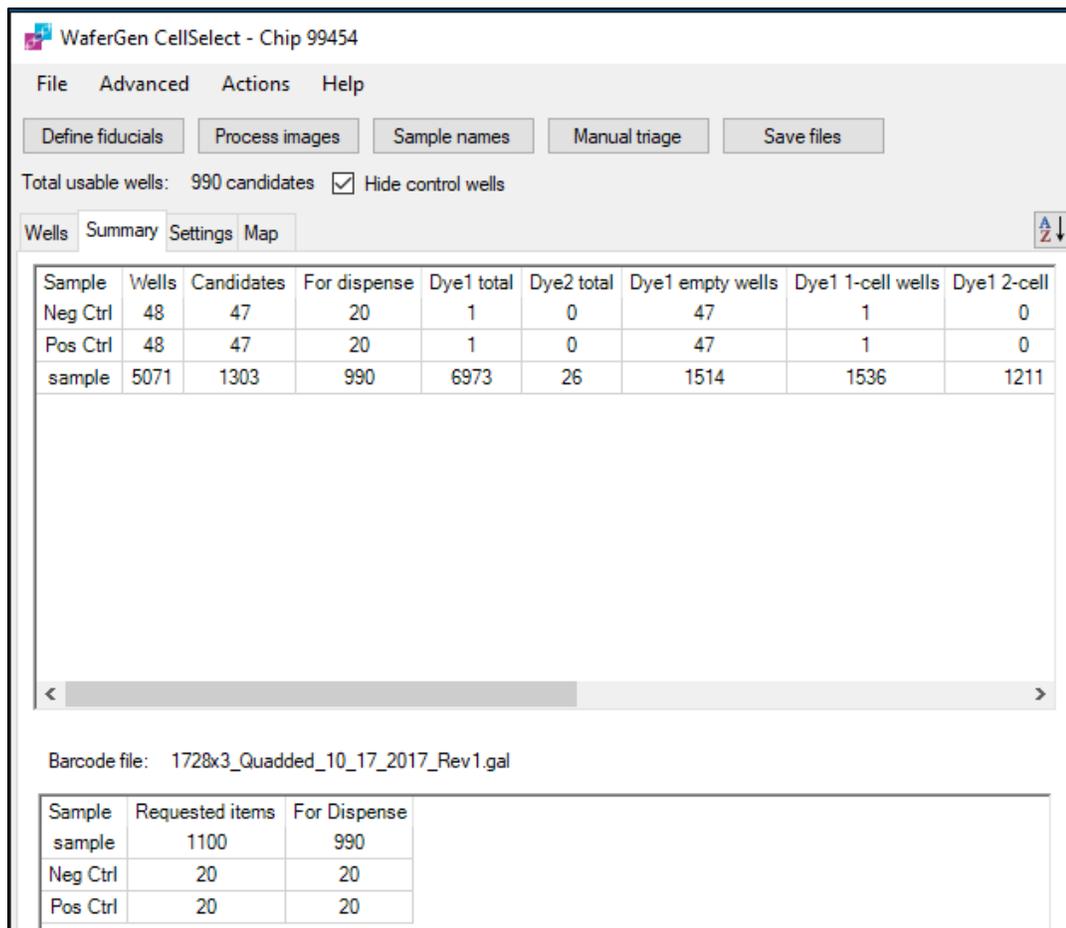


Figure 32. Number of wells available for dispensing.

11. Each preprinted chip contains a total of 1,728 unique nanowell barcodes. Each barcode is printed three times on the chip. The software is trained to include each barcode once while creating a filter file. You can load the results along with the settings from the saved WCD file (Figure 33, below). The software uses the following legend when examining nanowells:
 - **Green** = a cell
 - **Yellow** = ignored
 - **Blue** = a reflection

Refer to the CellSelect Software User Manual for more information.

WaferGen CellSelect - Chip 99454

File Advanced Actions Help

Define fiducials Process images Sample names Manual triage Save files

Total usable wells: 990 candidates 40 controls Hide control wells

Wells Summary Settings Map

Row	Col	Candidate	For dispense	Sample	Barcode	State	Cells1	Cells2	Signal1
0	1	no	no	sample	CTCCGCAACC	MultipleCells	4	0	
0	2	no	no	sample	AAGAACTGCC	NoCells	0	0	
0	3	no	no	sample	TCTCTTAGCC	NoCells	0	0	
0	4	no	no	sample	CTCATGATCC	MultipleCells	5	0	
0	6	no	no	sample	AACCGCTTCC	MultipleCells	2	0	
0	7	no	no	sample	ATCAAGAGCC	NoCells	0	0	
0	8	no	no	sample	AGGACGTACC	MultipleCells	2	0	
0	9	yes	yes	sample	GACGCTAACC	Good	1	0	162
0	10	no	no	sample	GGCTCCAGCC	LowConfidence	1	0	388
0	11	no	no	sample	ACTGCTATCC	MultipleCells	2	0	
0	12	yes	yes	sample	GTTACCTTCC	Good	1	0	414
0	13	no	no	sample	AGCCGCAGCC	MultipleCells	3	0	
0	14	yes	no	sample	CATACCAACC	Good	1	0	144
0	15	no	no	sample	GAGATAATCC	MultipleCells	2	0	
0	16	no	no	sample	GATGGCTACC	MultipleCells	3	0	
0	17	no	no	sample	TGCGCCTACC	MultipleCells	5	0	
0	18	yes	yes	sample	CGCTCTTGCC	Good	1	0	225
0	19	no	no	sample	CTACGAAGCC	MultipleCells	4	0	
0	20	yes	yes	sample	TGCCATTGCC	Good	1	0	354
0	21	no	no	sample	ATCGGCTACC	MultipleCells	2	0	
0	22	no	no	sample	ACTCTCAGCC	NoCells	0	0	

Figure 33. Viewing the results list after reading the image stack.

Specify sample names

- Click the [Sample names] box and enter the sample description; enter one sample name for all samples (Figure 34, below), or multiple sample names based on the source plate configuration layout in Figure 10, Section V.B (above), and click the [OK] box (Figure 34, below).

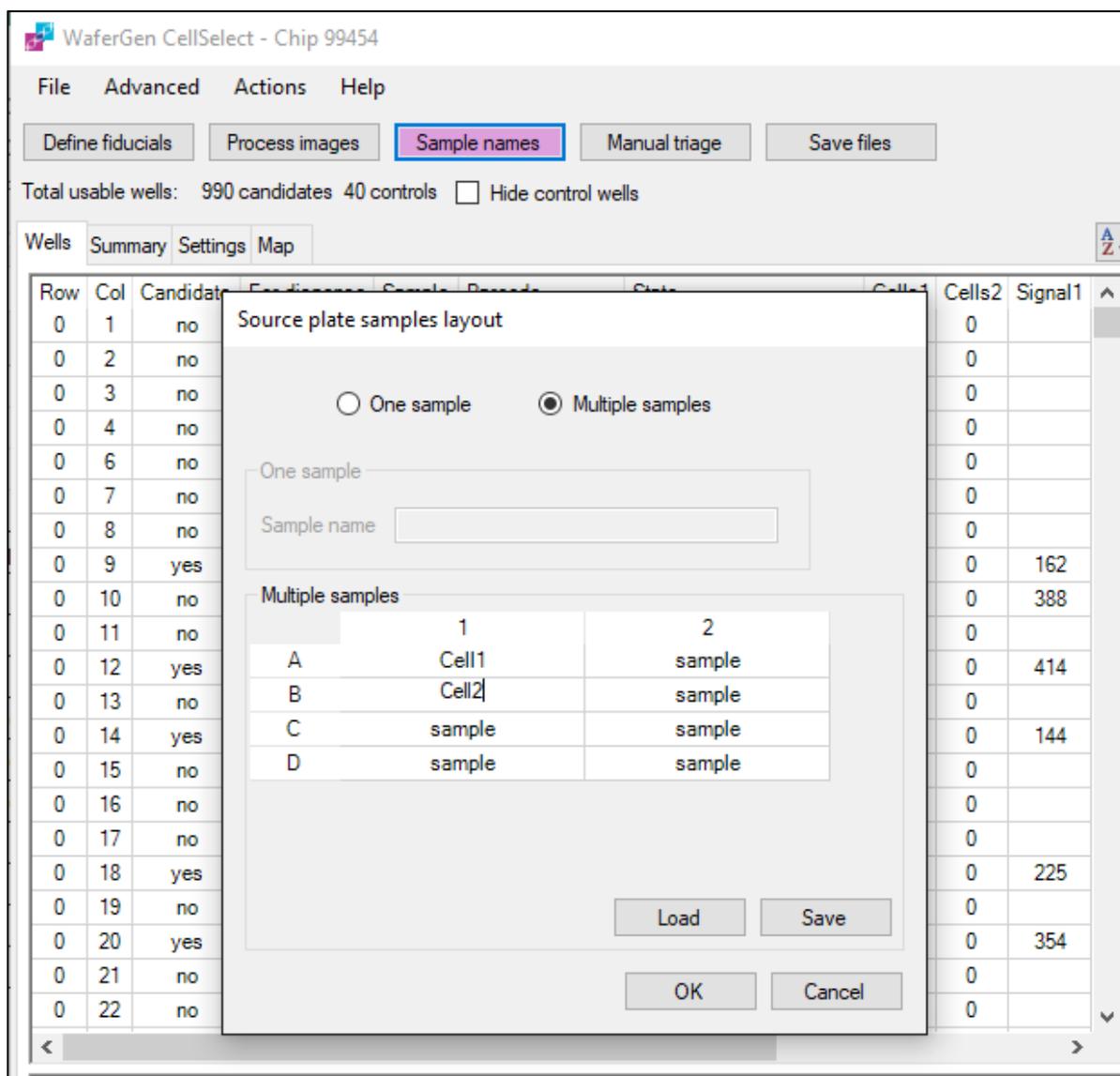


Figure 34. Specifying sample names. For the One sample option, “Test” is repeated down the “Sample” column. Control and fiducial wells are identified automatically.

Review images

- Click the *Wells* tab and sort the nanowell statistics in the “Candidate” column by clicking the row heading (see Figure 35, below). The software will sort and consolidate all selected nanowells (i.e., “candidates”) to the top of the table.

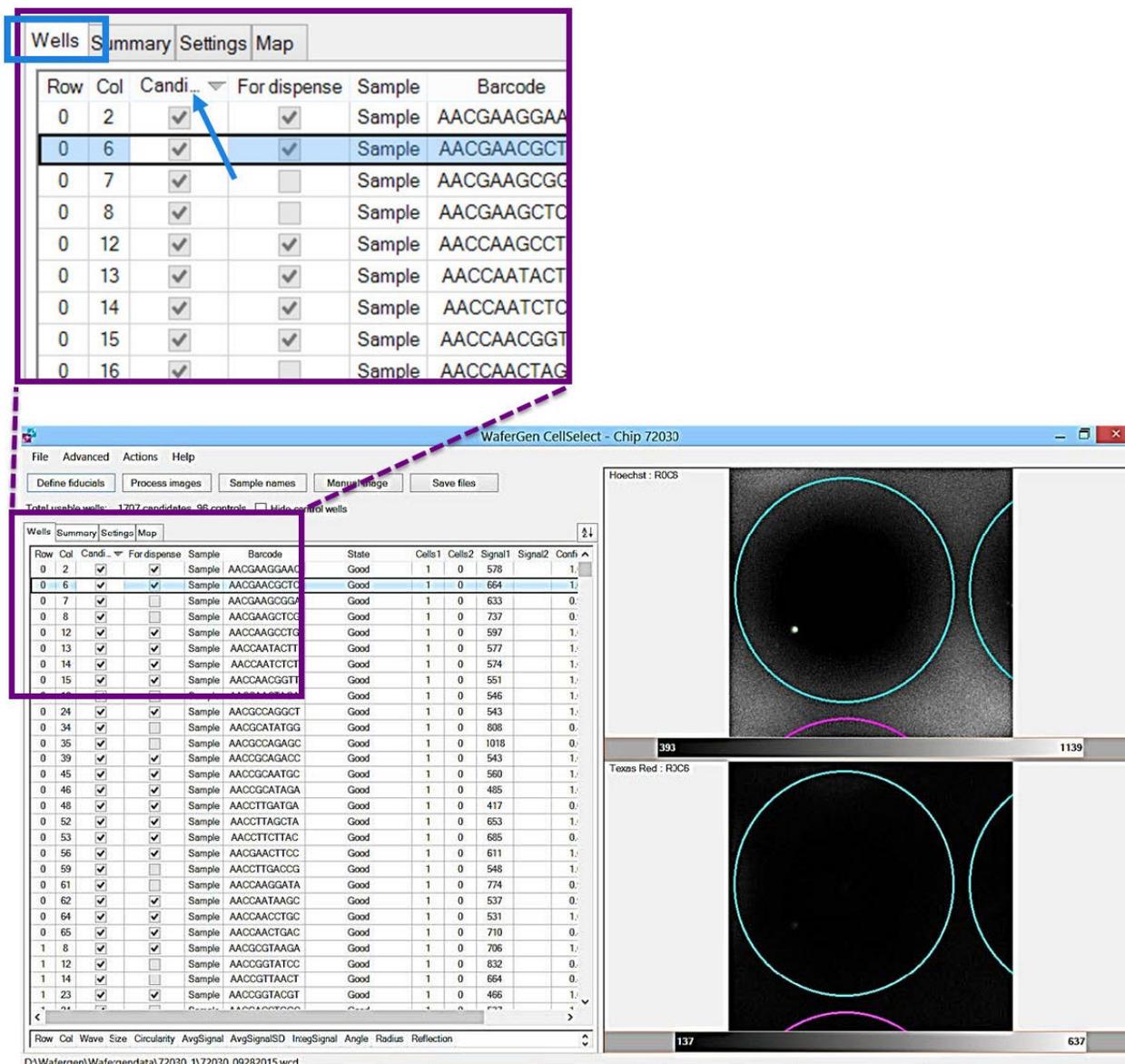


Figure 35. Sorting the “Candidate” column to consolidate all selected nanowells to the top of the table.

- If desired, manually inspect the selected nanowells. Click any row for close-up views of the selected nanowell in both the Hoechst and Texas Red channels (top and bottom single-well images, respectively, in Figure 35, above).
- Selected nanowells can also be viewed in the context of the corresponding multi-well image via the *Image Viewer* window: click on **Advanced > Image viewer** menu item (Figure 36, below). A multi-well image highlighting the nanowell selected in the results table (on the *Wells* tab) will appear.
- Arrange the windows as demonstrated in Figure 36 (below). When you click on any of the Candidate rows in the results table (on the *Wells* tab), the software will present corresponding single-well images, and display the corresponding multi-well image in the *Image Viewer* window.

17. To manually exclude one or more candidate wells, right-click the highlighted rows on the *Wells* tab and select **Exclude selected wells**. To exclude several candidate wells, consider using the Manual triage function. Refer to the corresponding section of the CellSelect Software User Manual for more information.

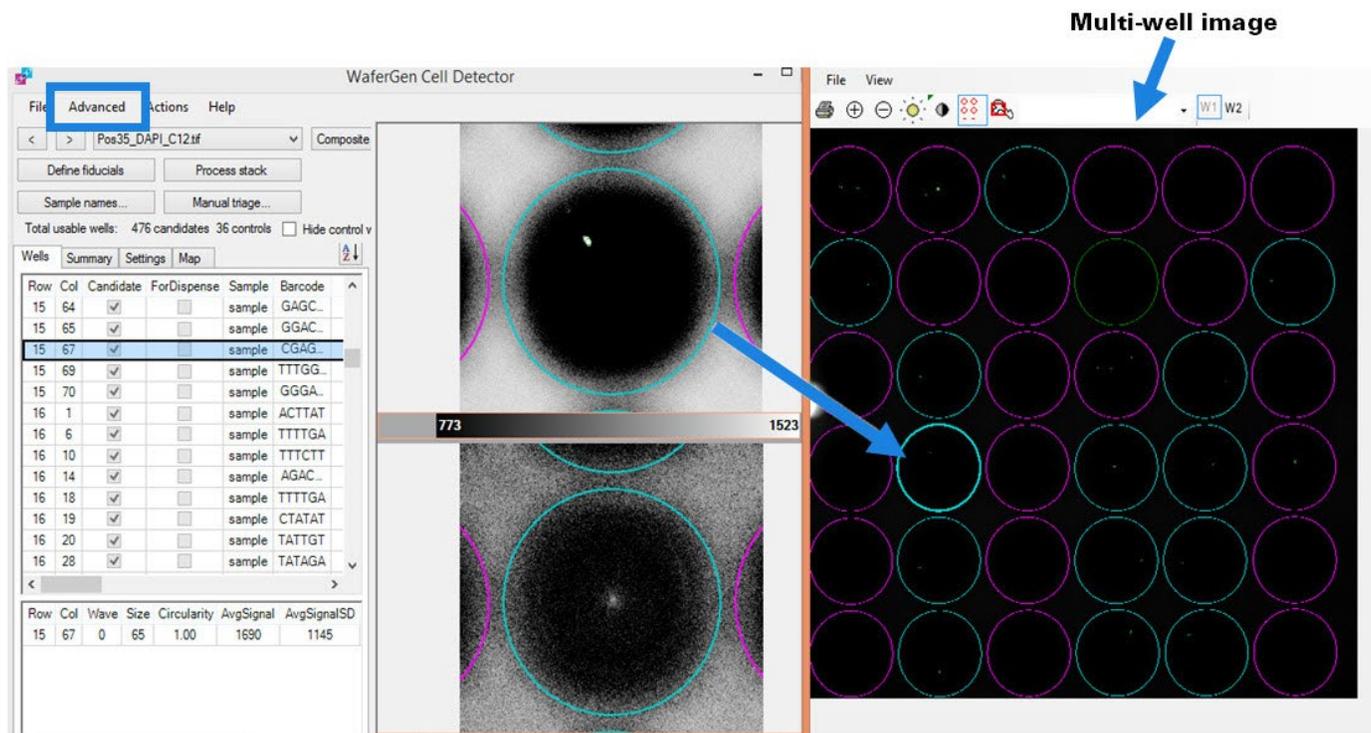


Figure 36. Arrangement of CellSelect windows. The image on the far right is the multi-well image. The horizontally split images in the center show a single cell in a nanowell stained by Hoechst 33342 (top) and the absence of propidium iodide stain in the corresponding position (dead-cell stain; bottom). In the bottom image, the green dot in the center (if seen) marks the bottom of the nanowell and is probably not from a dead cell because it does not match the location of the Hoechst-stained cell.

18. Click the **Save Files** button when prompted and input a file name prefixed with the chip number (<Chip ID>_<Date>.wcd). The software will then do the following:
- Select wells among all those that are checked under the “Candidate” column according to the best parameters defined in the *Settings* tab.
 - Save the result file under the specified name (<Chip ID>_<Date>.wcd). The result file will contain the selected nanowells and setting parameters.
 - Automatically generate the filter file for dispensing RT reagents (<Chip ID>_FilterFile.csv). The selected wells in the filter file will be displayed in the *For Dispense* tab.

Additional downselection and saving files after manual triaging of the images

19. If any manual triaging was performed, click the **File > Save Files** menu item to save any changes you have made to the results file. We strongly recommend using a new file name to avoid overwriting the original results file. The file is saved with the .wcd file extension and stored in the C:\Users\ICELL8\WafergenData folder.

NOTE: If the image files were saved in a folder or directory other than the default (C:\Users\ICELL8\WafergenData), the CellSelect software will save to the same directory used where the images were saved.

20. Select the *Summary* tab to view the number of wells selected for dispensing RT-PCR reagents in the next protocol.
21. Go to the *Downselect* window and adjust the number of controls and samples to reflect the number of excluded wells selected during the manual triaging.
22. When prompted, click **Yes** to save the updated filter file that was created during the additional downselection step. A 72 x 72 grid of the entire chip will be saved as a .csv file automatically named as <Chip ID>.csv, in which nanowells to be included in subsequent dispensing of reagents are marked with a 1, and nanowells to be excluded are marked with a 0.
23. Copy the filter file (.csv) and bring it with you to the ICELL8 MSND. It will be used for dispensing the RT-PCR reaction mix in the next protocol (Section V.E, below).

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 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:

GC Melt (white cap), cDNA Amplification dNTP Mixture (white cap), MgCl₂ (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:

- Initialize the ICELL8 MSND; perform daily warm-up via the *Startup* window, then pull up the *Advanced* window and execute the **Wash Prime** function followed by **Tip Clean**, then repeat the **Tip Clean** function 2–3 more times.
- Set the centrifuge(s) used for spinning the chip and 384-well source plate to 4°C.
- This protocol requires an MSND 384-Well Source Plate (with seal), Blotting Paper, TE Sealing Film, 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.
- Remove the chip holder containing the ICELL8 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. Use a Kimwipe to dry any liquid on the chip surface, and then centrifuge the chip at 3,220g (minimum 2,600g) for 3 min at 4°C. Keep the chip on ice until ready for dispensing of RT-PCR mix.

Procedure:

1. 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.

2. 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
<hr/>	
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.

- Pipette 50 μ l of the RT-PCR reaction mix into wells A1, B1, C1, and D1 of a MSND 384-Well Source Plate, as shown in Figure 37.

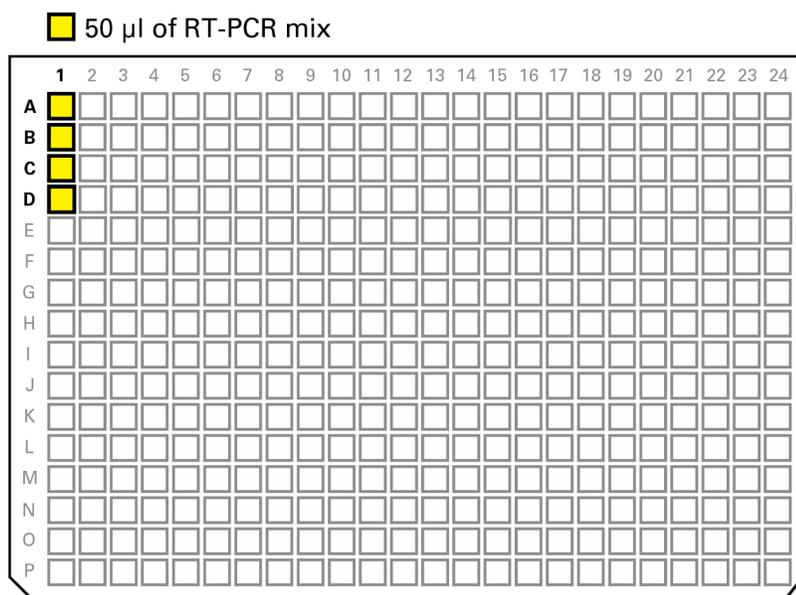


Figure 37. Setting up the source plate for dispensing RT-PCR mix.

- Seal the 384-well source plate with a 384-well plate seal (Takara Bio, Cat. No. 640018).
- Centrifuge the source plate at 3,220g (minimum 2,600g) for 3 min at 4°C.
- Place the 384-well source plate in the MSND with the A1 corner positioned at the top-right corner of the Plate Nest (see Figure 7 in Section IV.E, above). The beveled corners of the 384-well source plate should be on the left side.
- Gently remove the seal from the 384-well source plate and lock the plate into position.
- Peel off the imaging film from the chip. Place the chip on the chuck of the Dispensing Platform in the MSND with the chamfered (notched) corner of the chip at the lower-right corner of the Dispensing Platform, aligned with the chamfered corner of the chuck (see Figure 8 in Section IV.E, above).

IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip lies flat on the chuck on all sides and is in the correct orientation** (see Figure 8 in Section IV.E, above).

- In the ICELL8 MSND GUI, load the filter file generated in the previous protocol (Section V.D, above); click the *Single Cell* tab followed by the [Browse] button ([...]; Figure 38, below) next to the “Filter file” field then select the appropriate filter file (.CSV). Click within the “Chip ID” field and enter or scan the corresponding Chip ID (refer to the [ICELL8 MultiSample NanoDispenser User Manual](#) for more details).

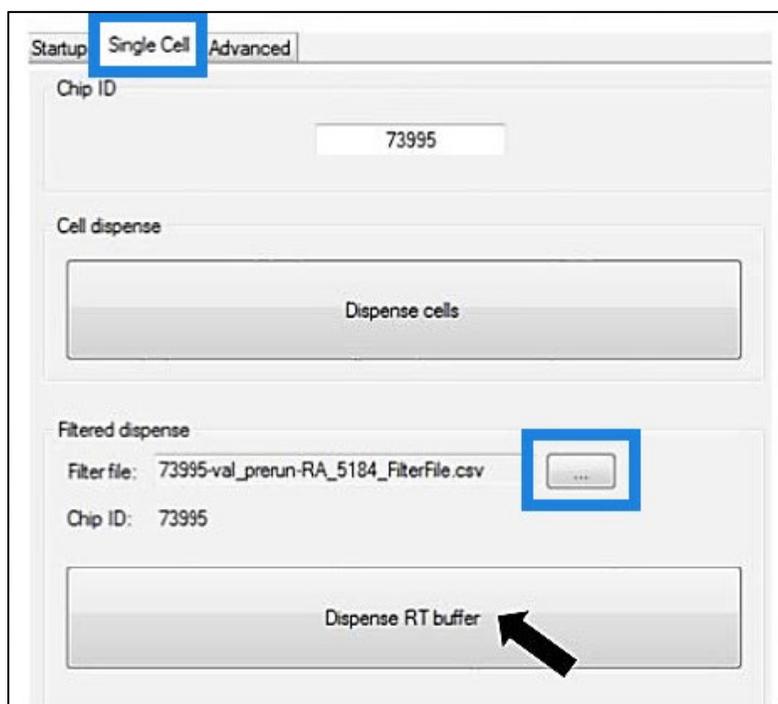


Figure 38. Loading the filter file and dispensing RT-PCR mix with the MSND software.

10. Click the [Dispense RT buffer] button, carefully go through all questions prompted, and then click **OK**. The ICELL8 MSND will dispense 50 nl of RT-PCR reaction mix into each chip nanowell.
11. After dispensing of reaction mix is completed, remove the chip from the Dispensing Platform and gently blot for 2 sec with a fresh blotting paper and a blotter (Figure 12, Section V.B).
12. Seal the chip with a sealing film using the Film Applicator. Make sure that the chip is securely sealed to avoid well-to-well contamination.
13. Centrifuge the ICELL8 TCR Chip at 3,220g for 3 min at 4°C.
14. Using the ICELL8 MSND GUI, repeat the Tip Clean procedure 3–4 more times.
15. Place the ICELL8 TCR Chip into the ICELL8 Chip Cycler to perform the RT-PCR reaction using the preinstalled 2DIS-PCR thermal profile program.

NOTE: The RT-PCR program described below is preinstalled on the ICELL8 Chip Cycler provided with the ICELL8 system, using temperatures that are calibrated individually for each machine and that may vary slightly from the temperatures indicated below. DO NOT alter the preinstalled program or substitute a different program without first consulting with a member of the Field Support Team.

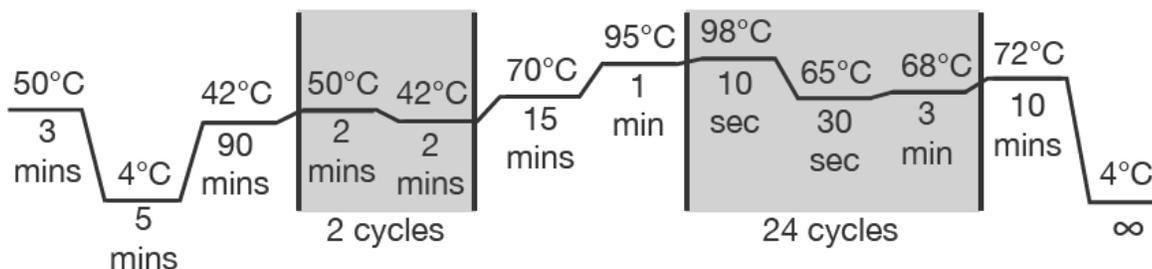


Figure 39. RT-PCR thermal profile.

SAFE STOPPING POINT: The chip can be left in the ICELL8 Chip Cycler at 4°C overnight.

F. Protocol: Full-length cDNA Extraction

1. Remove the chip from the ICELL8 Chip Cycler. Centrifuge the chip at 3,220g for 3 min at 4°C.
2. Open the Collection Kit and label a Collection Tube with the engraved chip number. Assemble the Collection Module by attaching the Collection Tube to the Collection Fixture.



Figure 40. Assembling the Collection Module.

3. Carefully peel off the sealing film from the chip (Figure 41, below).

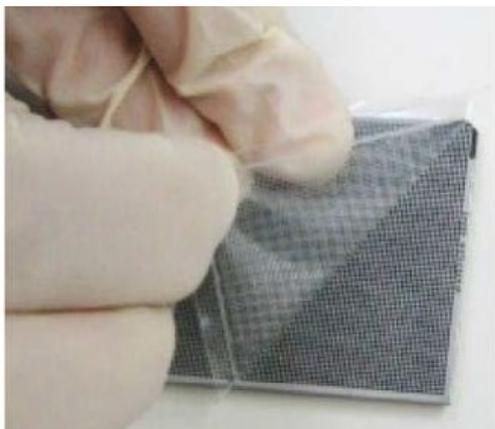


Figure 41. Removing the TE Sealing Film from the chip. Start from one corner and apply even pressure to pull.

4. With the nanowells facing down, place the chip into the assembled Collection Module (consisting of the Collection Tube plus the Collection Fixture; Figure 42, below). Surface tension will hold the liquid in the nanowells.



Figure 42. Placing the chip into the Collection Module.

5. Seal the chip and the top of the Collection Module with a supplied Collection Film (Figure 43, below).



Figure 43. Securing the Collection Module with Collection Film.

6. Using a balance or blank chip, assemble another Collection Module. Centrifuge both Collection Modules at 3,220g (minimum 2,600g) for 10 min at 4°C.
7. Carefully remove the Collection Tube containing the extracted cDNA (Figure 44, below).

IMPORTANT: Measure the volume of the collected eluent. The volume should be no less than 15% of the expected value. For example, if 500 nanowell were selected for processing, you should collect 242–284 μ l of eluent.

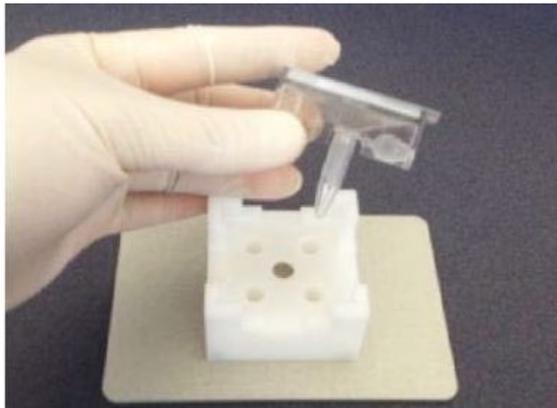


Figure 44. Removing the Collection Tube containing the cDNA eluent.

8. Attach the second supplied Collection Tube to the Collection Fixture and seal the entire module. Discard the module in a biohazard waste bin. DO NOT discard the Collection Module containing the balance or blank chip, retain it as a balance module for future extractions.

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.

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. The beads are viscous, so pipette them slowly.

- Prepare fresh 70% (v/v) ethanol from a stock of anhydrous ethanol.

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 NT1.
 - b. Place a NucleoSpin Gel and PCR Clean-up Column into a 2-ml collection tube and load up to 700 μ l of sample.
 - c. Centrifuge for 30 sec at 11,000g. 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 μ l of Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column. Centrifuge for 30 sec at 11,000g. 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 11,000g 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 μ l of water and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000g.
 - h. Repeat the elution step twice using the same microcentrifuge tube used in the previous step, add 26 μ l of DNase-RNase free water directly to the column matrix and incubate at room temperature for 1 min and centrifuge at maximum speed (\geq 10,000g) for 30 sec to elute the cDNA. You will end up with ~50 μ l of total eluent.

H. Protocol: Purify the Full-Length cDNA with AMPure XP beads

Before you start:

- Equilibrate an aliquot of AMPure XP beads to room temperature for ~30 min 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. The beads are viscous, so pipette them slowly.

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

EXAMPLE: To a 50 μ l of cDNA suspension obtained in [Section V.G](#), Step 2h, add 30 μ l of DNA 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.

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

4. Carefully remove the supernatant with a pipette. Discard the supernatant.
5. Wash the pellet with 200 μ l of 70% (v/v) ethanol. Wait for 10 sec.
6. 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.
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.

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

10. 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.
11. Add 15 μ l of Nuclease-Free Water and pipette up and down to mix. Make sure that all the beads are suspended.
12. 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.

13. Carefully transfer 14 μ l 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 μ l 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 \sim 2 ng/ μ l.
3. Use 1 μ l 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 45 (below) to verify whether each sample is suitable for further processing. Successful cDNA synthesis and purification should yield a broad peak spanning \sim 400 bp to \sim 5,000 bp.

NOTE: If the Bioanalyzer profile indicates the presence of adapters, repeat the bead purification procedure and analysis of the purified cDNA ([Section 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/ μ l.

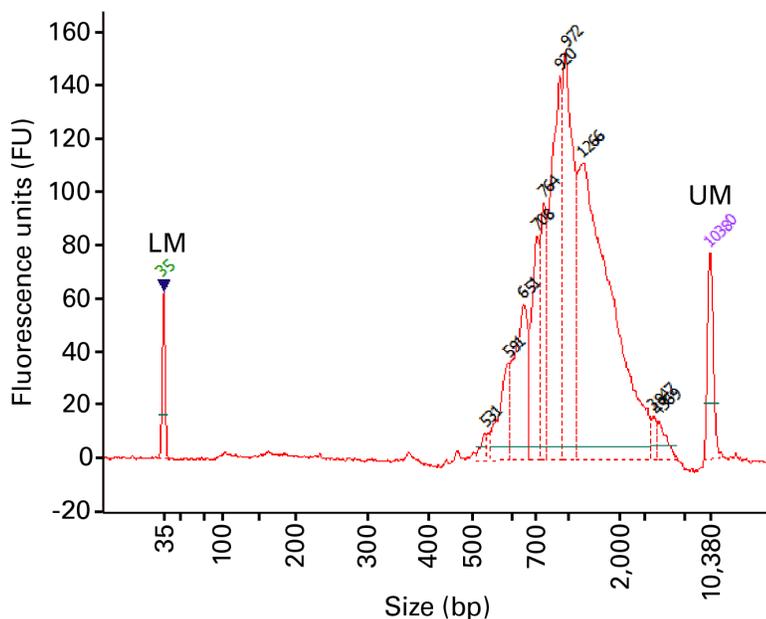


Figure 45. Typical Bioanalyzer trace for purified full-length cDNA that has been normalized to 2 ng/ μ l. Peaks labeled “LM” and “UM” correspond to DNA reference markers included in each analysis.

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 (blue cap), TCR Amplification dNTP Mixture (blue cap), Primer P5 (blue cap), TCRA Human Primer 1 (yellow cap), TCRb Human Primer 1 (yellow cap), TCR Amplification Polymerase (blue 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
<hr/>	
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: Different Forward HT indexes (F1–F10) must be used for each sample if samples are to be multiplexed on a single flow cell (See Appendix A).

For this protocol, you will need the following components:

TCR Amplification Buffer (blue cap), TCR Amplification dNTP Mixture (blue cap), Primer P5 (blue cap), ICELL8 TCR a Human Forward HT Indexes (purple caps), ICELL8 TCR b Human Forward HT Indexes (purple caps), TCR Amplification Polymerase (blue 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
<hr/>	
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.I) 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 17) 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 μ l 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/ μ l samples by further diluting the 1:5 dilution prepared in Step 1. Use 1 μ l 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 46 (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 \sim 700 bp and \sim 900 bp for samples generated from cells containing TCR libraries.

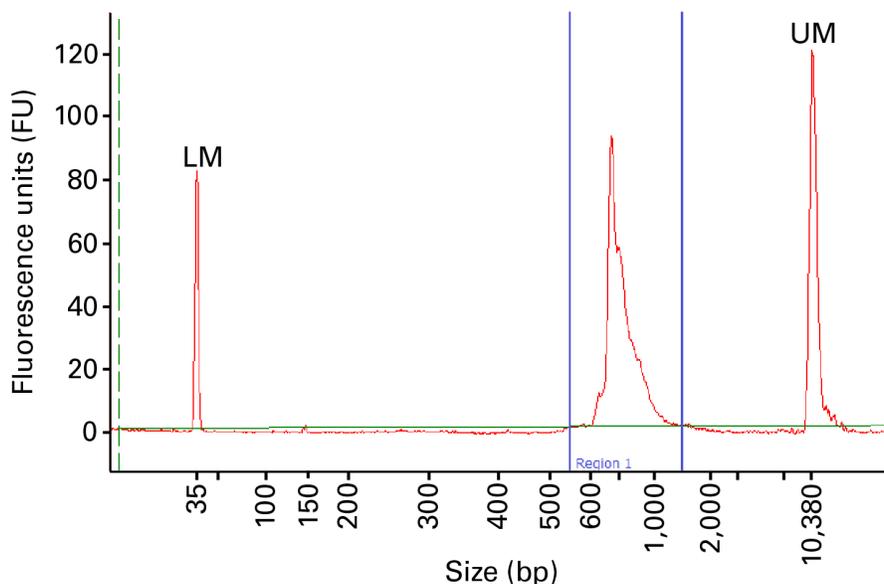


Figure 46. Example electropherogram results for TCRA and TCRb library. A library containing TCRA and TCRb sequences were generated using the ICELL8 Human scTCR a/b Profiling workflow. The library was produced using 24 cycles of amplification for the cDNA PCR, 16 amplification cycles for TCR PCR 1, and 14 amplification cycles for TCR PCR 2 (the cDNA profile for this library is shown in Figure 45). 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/ul based on a Qubit value of 1.47 ng/ul 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 46) 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 (“F1”–“F10”), 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 1728x3_Quadded_10_17_2017_Rev1.gal file provided with CellSelect Software contains nanowell barcode sequence for each preprinted nanowell location on a chip.

Appendix D: Data Analysis

Data analysis can be performed on a variety of platforms, a detailed but nonexhaustive 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 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

Please follow instructions as outlined in ICELL8 Human TCR a/b Profiling User Manual (Sections IV.E–IV.I) for cDNA synthesis, amplification, quantification, and validation.

1. 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 ICELL8 Human TCR a/b 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
10°C	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
95°C	30 sec
<u>12 cycles:</u>	
95°C	10 sec
55°C	30 sec
72°C	30 sec
72°C	5 min
10°C	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). Alternatively, 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 Profiling Reagent Kit.

Procedure:**Purify and size select sequencing library**

1. To each 50- μ l volume of PCR product from the previous protocol (Appendix E, Section B.1 above), add 50 μ l (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 μ l of 70% (v/v) ethanol. Wait for 10 seconds.
6. 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 seconds and turn the tube back to its original position. 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 51 μ l 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 minutes and then incubate on a magnetic stand for 2 minutes or longer until the solution is completely clear.
13. Carefully transfer the clear supernatant (~ 50 μ l) with a pipette to a new PCR tube. Save the supernatant (eluent).
14. To the eluent from the previous step (Step 13), add 25 μ l (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 (~ 50 μ l) to a clean PCR tube. **Save the supernatant and discard the beads.**
18. To the supernatant from the previous step (Step 17), add 10 μ l (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 μ l 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 μ l of sequencing-ready library.

Evaluate sequencing library

27. Aliquot 1 μ l 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/ μ l, and use 1 μ l 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 47, 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.

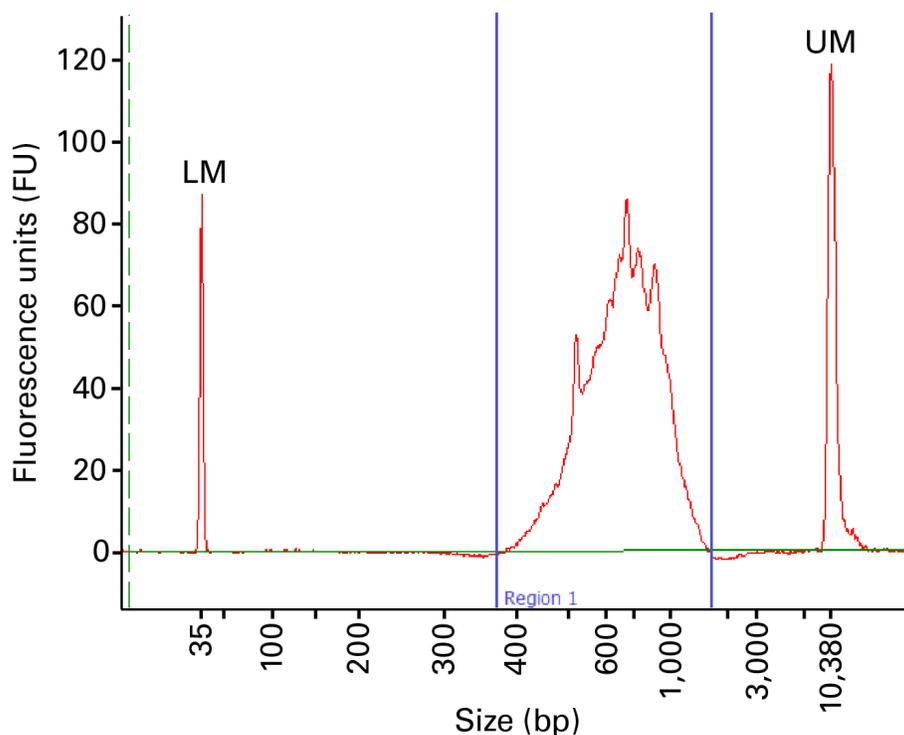


Figure 47. Typical Bioanalyzer trace for purified Nextera XT NGS library that has been normalized to 1 ng/ μ l.

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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