Takara Bio USA, Inc.

ICELL8® Single-Cell User Manual for 3′ Differential Expression

Cat. Nos. 640005, 640143
(072619)

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I. Safety

Refer to safety sections in the operator/user manuals for all equipment used in this protocol.

Symbol	Description
	**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 D-RNase decontamination solution, water, and ethanol. Change gloves routinely.
	**WARNING:** Use of the ICELL8 3′ DE Chip with the instruments in this protocol may cause exposure to toxic or biohazardous chemicals thereby presenting a hazard. Always wear appropriate personal protective equipment (PPE), which should at a minimum include gloves, eye protection, and lab coat, when operating the instruments.
	**Note and heed all warning labels on the instruments used in this protocol.**

II. Introduction

Welcome to the ICELL8 Single-Cell Protocol

The ICELL8 Single-Cell System has been engineered to dramatically increase the pace of biological discovery.

With unparalleled cell isolation, cell selection, and sample throughput, you now have control of your single cell analysis, getting the results you need to further your research.

- **Power**—Isolate thousands of cells of any size and process multiple chips per day.
- **Control**—Selectively choose which cells to process using image analysis software.
- **Insight**—Process up to 8 samples per chip, experimental flexibility for greater biological insight.

This document explains in detail all of the samples, equipment, and procedural steps used in the Single-Cell Analysis system.

III. List of Components

The ICELL8 single-cell for 3′ DE workflow consists of the ICELL8 3′ DE for UMI Reagent Kit (Cat. No. 640005), the ICELL8 3′ DE Chip (Cat. No. 640143), the ICELL8 Loading Kit (Cat. No. 640109), and the ICELL8 Collection Kit (Cat. No. 640048). Each must be purchased separately:

ICELL8 3′ DE for UMI Reagent Kit (Cat. No. 640005, store at 20°C)

	Cap color	Volume
ICELL8 Fiducial Mix	White	60 µl
ICELL8 RT E5OLIGO	Yellow	8 µl
ICELL8 Amp Primer	Green	5 µl
ICELL8 Nextera® Primer P5	Aqua	10 µl
Second Diluent (100X)	Orange	24 µl

ICELL8 3′ DE Chip (Cat. No. 640143, store at room temperature)

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

ICELL8 Collection Kit (Cat. No. 640048, store at room temperature)

Component	Quantity per kit
Collection Fixture	1
Collection Tube	2
Collection Film	1

This protocol requires use of an ICELL8 Single-Cell System (Cat. No. 640000). Details on the components included in this product is available for download at takarabio.com.

IV. Additional Materials Required

The following reagents and materials are required but not supplied with the ICELL8 single-cell for 3′ DE workflow products or the ICELL8 Single-Cell System:

- Personal protective equipment (PPE; e.g., powder-free gloves, safety glasses, lab coat, sleeve protector)
- Nuclease-decontamination solution
- Centrifuges and rotors for conical tubes and plates (e.g., Eppendorf 5810R with swinging plate buckets, ≥2,600*g*, room temp and 4°C operation; Kubota 3740 with rotor SF-240 for cell prep)
- MSND 384-well Plates and Seals, 20 pack (Takara Bio, Cat. No. 640018, 640037, or 640192)
- Conical tubes: 15 ml and 50 ml
- Nuclease-free: 0.2-ml PCR tubes and nonstick 1.5-ml microcentrifuge tubes
- Single-channel pipettes: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Serological pipettes and controller
- Two pairs of tweezers for handling chips during imaging
- Minicentrifuges for 1.5-ml tubes and 0.2-ml tubes or strips
- Magnetic separator (e.g., Magnetic Separator - PCR Strip, Takara Bio, Cat. No. 635011)
- Vortex mixer
- Exhaust hood system with UV

For ICELL8 Single-Cell System general operation:

- Deionized water (for water reservoir and humidifier)
- Freshly mixed 0.2% sodium hypochlorite solution
- Nuclease-free water

For staining and dispensing cells:

- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4; Thermo Fisher Scientific, Cat. No. 10010-023)
- ReadyProbes Cell Viability Imaging Kit, Blue/Red (contains Hoechst 33342 and propidium iodide; Thermo Fisher Scientific, Cat. No. R37610)
- Murine RNase Inhibitor (40,000 U/ml; New England BioLabs, Cat. No. M0314L)
- Molecular-biology-grade/PCR-grade/nuclease-free water

For RT and amplification product purification:

- dNTP (New England BioLabs, Cat. No. N0447S or N0447L)
- Maxima H Minus RT (200 U/µl) with 5X RT buffer (Thermo Fisher Scientific, Cat. No. EP0753)
- DNA Clean & Concentrator-5 (Zymo Research, Cat. No. D4004)

For exonuclease treatment:

- Exonuclease I with 10X Reaction Buffer (New England BioLabs, Cat. No. M0293L)

For cDNA amplification:

- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Fluorometer (Thermo Fisher Scientific)
- Advantage® 2 PCR Kit (Takara Bio, Cat. No. 639206)
- Molecular-biology-grade/PCR-grade/nuclease-free water

For cDNA and sequencing library purification:

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

- 100% ethanol: prepared fresh from anhydrous ethanol for each experiment
- LightCycler 480 or similar for KAPA Quant
- LightCycler 480 multiwell plate and seal for KAPAQuant
- KAPA Library Quantification Kit (for Illumina® platforms; KAPA Biosystems, Cat. No. KK4854)

For sequencing library preparation:

- 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)
- Thermal cycler with block for 0.2-ml tubes

For cell counting:

- Recommended: MOXI Z Mini Automated Cell Counter Kit (ORFLO, Cat. No. MXZ001) with MOXI Z Cell Count Cassettes, Type M (25 pack; ORFLO, Cat. No. MXC001) or MOXI Z Cell Count Cassettes, Type S (25 pack; ORFLO, Cat. No. MXC002)

NOTES:

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

V. General Considerations

A. Workflow Diagram

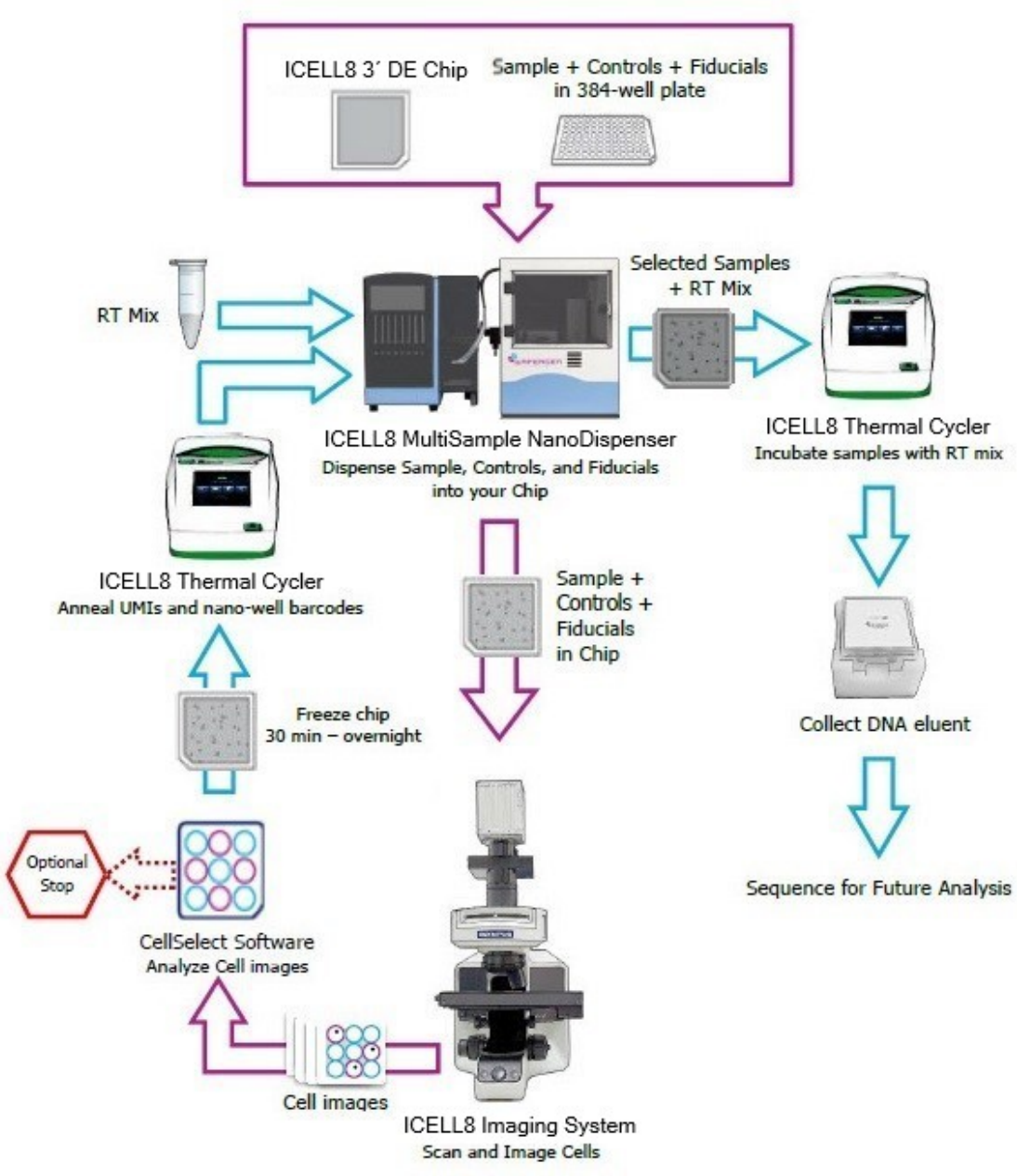


Figure 1. Single-cell workflow diagram

B. Protocol Notes

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 D-RNase 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 through exposed parts from your hands and arms.
- Use D-RNase free, molecular biology or PCR grade reagents to set up all molecular biology reactions.
- Use deionized water for the MSND pressure bottle and humidifier; clean these reservoirs routinely. Use D-RNase free molecular biology grade water for the MSND wash bottle.
- UV treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use. DO NOT UV preprinted chips.

Preprinted chips:

- Each ICELL8 3′ DE Chip is engraved with a unique five-digit number (Figure 3). You can use this number to link your chip images and other experimental record files
- Unique Molecular Identifiers (UMIs) and nanowell barcodes were preprinted into each chip; the printing lot number is stamped onto the chip (Figure 3). A total of 5,184 unique nanowell barcodes are available; each nanowell barcode was printed once into a chip.

Figure 2. ICELL8 3′ DE Chip Packaging. The chip is sealed along with a silica packet. Be careful not to cut the silica packet when opening the pouch.

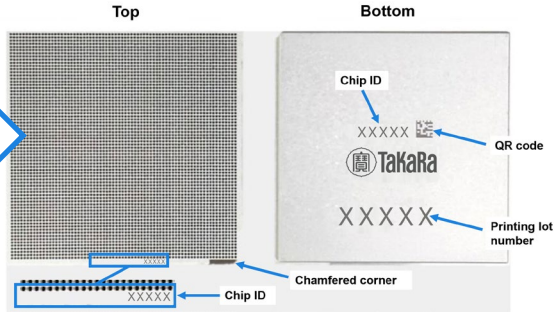


Figure 3. Preprinted Chip. (Top): Note the chamfered (notched) corner at the bottom right. “TaKaRa” and Chip ID are on the chip border, near chamfered corner.

(Bottom): Engraved is the five-digit Chip ID (also called “Chip Number”) that is unique to each chip, and the UMIs and nanowell barcodes printing lot number. Next to the Chip ID is a QR code of the Chip ID that can be scanned by a barcode reader and entered into the software.

Multisample Nanondispenser (MSND) Application Notes:

- Before operating the instrument, check water level in the pressure reservoir. Refill if the weighing scale below the pressure reservoir shows ≤4 kg.
- Add deionized water to the reservoir.
    a. Close the helium in-line and open the vent stopcock.

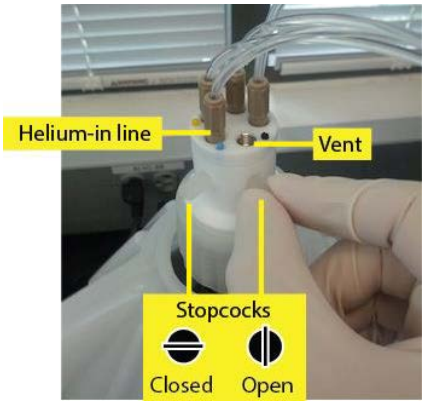


Figure 4a. Close helium line; open vent

b. Fill with deionized water to the fill line (top of the protective cover rim). Approximately ~4 kg on the weighing scale.

Figure 4b. Fill pressure reservoir

c. Reattach the cap, and de-gas for ~20 minutes. Make sure that the cap is properly threaded and securely fastened to prevent the helium from leaking out.

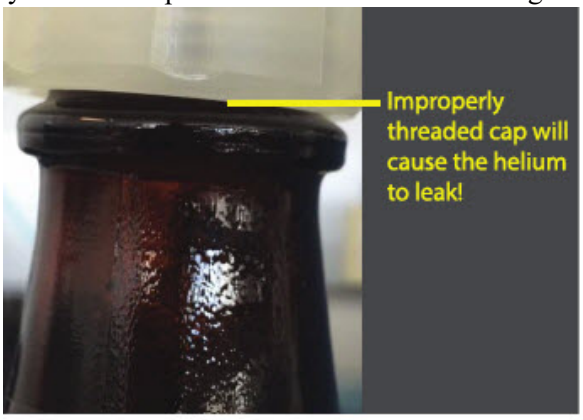


Figure 4c. Attach cap

d. Close the system by turning the stopcock on the helium in-line and close the vent stopcock.

- Check water level in the humidifier and fill if needed. Clean the humidifier regularly.
- Check the pressure on the helium tank. The regulator should be set to a supply input of >500psi (3.5 MPa) and an output of 30 to 40 psi. If the tank pressure drops <500 psi, replace with a full tank.
- Check water level in the wash bottle. If it is <25% full, replace with a new bottle of D-RNase free, molecular biology grade water.
- Check the waste container. If full, dispose the liquid waste appropriately and replace with an empty container.
- All dispense steps in the MSND Stage Module should be performed with the 384-well source plate oriented with the A1 position on the top, right corner on the 384-well Plate Nest, and the chip oriented with the chamfered (notched) corner positioned at the lower right on the Dispensing Platform (Figure 5 to Figure 7).

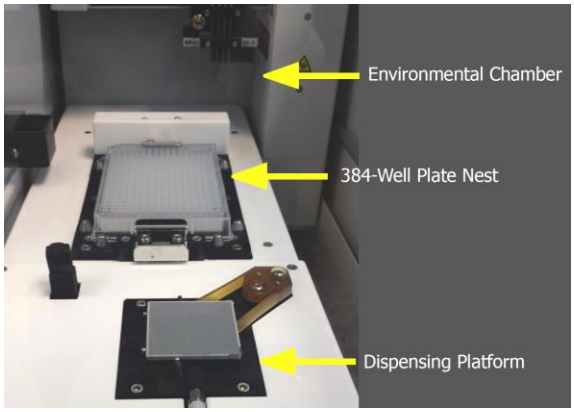


Figure 5. MSND Stage Module

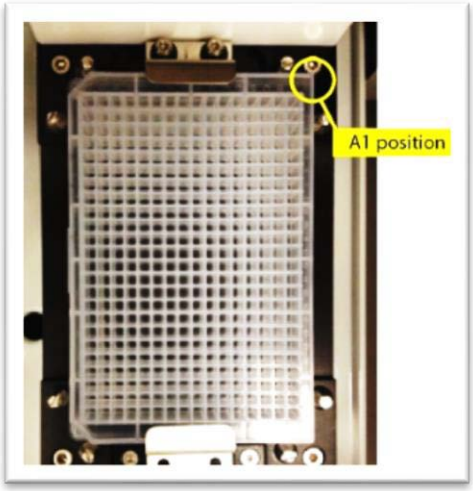


Figure 6. 384-well source plate in the Plate Nest.

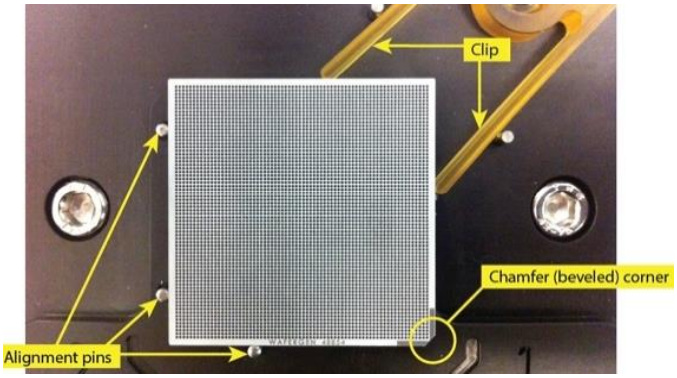


Figure 7. Chip seated in the Dispensing Platform. Stretch the arms of the clip apart and angle the chip onto the Dispensing Platform with the chamfered (notched) corner of the chip facing the lower right. The edges of the chip should be pressed against the three alignment pins.

IMPORTANT: Make sure that the entire chip lies perfectly flat against the dispensing platform and sits square on dispense platform. Make sure the alignment pins do not improperly catch on the sidewalls of the chip.

VI. Procedure

A. Protocol: Stain cells with Hoechst 33342 and Propidium Iodide

Prerequisite:

- Several milliliters of healthy cell culture suspension in a 15–ml conical tube
- Maintain cell density between 1 x 10^5 and 7.5 x 10^6 cells/ml

Before you start:

- Pre-warm 1X PBS (no Ca^{2+} or Mg^{2+}, pH 7.4), Second Diluent 2X, and D-RNase free, molecular-biology grade water to 37°C.
- Set a centrifuge and rotor that will accommodate 15 ml conical tubes to room temperature.
- Dilute Positive Control RNA to 10 ng/µl

NOTE: Positive Control RNA should be kept on ice.

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. Please refer to published standard protocols.
- 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.

Procedure:

1. Determine the initial cell density with the Moxi Z Mini Automated Cell Counter using the Type M cassette. 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 and MPI.

2. Pellet cells by centrifugation at 100 x *g* for 5 minutes at room temperature.

   RCF= 1.12 x radius of the rotor x $(rpm/1000)^2$

   NOTE: Centrifugation speed and time may need to be modified for different cell types.

3. Carefully decant the supernatant. Use a Kimwipe to catch residual outflowing liquid. Resuspend the cell pellet in 5 mL of pre-warmed 1X PBS (no Ca^{2+} or Mg^{2+}, pH 7.4). Use a disposable sterile serological pipette to slowly pipette up and down three times. Do not vortex. Return the 1X PBS to the 37°C incubator when not in use.

4. Pellet cells by centrifugation at 100 x *g* for 5 minutes at room temperature.

5. Carefully decant the supernatant. Use a Kimwipe to catch residual outflowing liquid. Repeat steps 3, 4, and 5 for 2 more times, for a total of 3 times to wash cells.

6. Use the initial cell density measured in Step 1 as a guide, resuspend the washed cells in pre-warmed 1X PBS to achieve a cell concentration between 1.3 x 10^5 cells/ml and 5 x 10^5 cells/ml (before cell staining).

7. Stain cells with Hoechst 33342 and Propidium Iodide. Add 1 drop (~40 µl) of each dye per ml of washed cells. Incubate the cell-stain suspension at 37°C for 20 minutes. **Mix the cell suspension gently by pipetting up and down several times to have homogenous cell suspension. Take the aliquot from the center of tube containing homogenous cell suspension.**

8. Count the stained cells with the MoxiTM Z Mini Automated Cell Counter using the Type M cassette. Take two cell concentration readings for each stained cell sample. Average the results. If you have less than 1.2 x 105 cells/ml, re-pellet the stained cell suspension and resuspend in a lesser volume of 1X PBS to achieve desired concentration.

B. Protocol: Dilute cells

Refer to the *ICELL8 MultiSample NanoDispenser User Manual* for instrument setup and operation.

Before you start:

- Initialize the MSND and perform **Daily warm up** on the *Set up* window. Then go to the *Advanced Setting* window and perform **Wash Prime** followed by **Tip Clean**. Read the *ICELL8 MultiSample NanoDispenser User Manual* before use.
- Start the Imager System: Turn on the Optiscan II controller, Lambda controller, burner, computer, and camera. There is a warm up period for the light source for ~5 minutes. Read the *ICELL8 Imaging System User Manual* before use.
- Pre-freeze the empty Chip Holder (Figure 8) at –80°C.

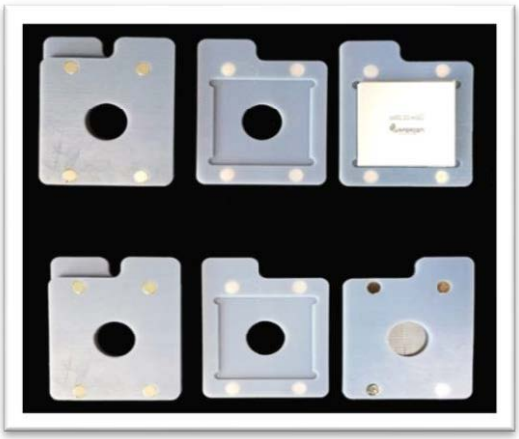


Figure 8. Chip Holder. The engraved chip number can be seen through the center opening

Procedure:

1. Vortex the Second Diluent 2X well. Add Murine RNase inhibitor to pre-warmed Second Diluent 2X just before adding to stained cells according to the following table, to make Second Diluent 2X Master Mix (SD 2X master mix), mix well. The Second Diluent 2X Master Mix contains 0.35X PBS.

Table I. Preparation of Second Diluent 2X Master Mix

Component	Concentration	Volume to add (µl)	Concentration in Master Mix
RNase Inhibitor, Murine	40 U/µl	12	2X (0.8 U/µl)
Second Diluent 2X (with 0.35X PBS)	2.04X	588	2X
Total		600	

2. Aliquot 75 µl of Second Diluent 2X Master Mix in a 1.5 ml tube and keep on ice for making positive and negative control.

3. Prepare positive control mix according to the following table, in a 1.5 ml micro-centrifuge tube using the Second Diluent 2X Master Mix (Step 1). Mix well (but do not vortex). The Positive Control ends up as 12 pg per 50 nl dispense (equivalent to the total RNA content from 1 cell). Keep the positive control mix on ice.

Table II. Positive Control Mix

Component	Concentration	Volume to add (µl)	Concentration in the 384-well source plate
Second diluent 2X master mix	2X	25	1X
D-RNase free water	NA	15	NA
1X PBS	1X	8.8	0.35
Positive Control	Diluted to 10 ng/µl	1.2	12 pg/50 nl
Total		50.0	

4. Prepare negative control mix according to the following table, in a 1.5 ml micro-centrifuge tube using the Second Diluent 2X Master Mix (Step 1.). Mix well (but do not vortex).

Table III. Negative Control Mix

Component	Concentration	Volume to add (µl)	Concentration in the 384-well source plate
Second diluent 2X master mix	2X	25	1X
D-RNase free water	NA	16.2	NA
1X PBS	1X	8.8	0.35
Total		50.0	

5. Dilute cells according to the following table. Add water, fresh 1X PBS and Second Diluent 2X Master Mix (from Table 5) into a 1.5 ml micro centrifuge tube. Vortex to mix. Mix the stained cell suspension gently by pipetting up and down several times to have homogenous cell suspension. Take the aliquot from the center of the tube containing homogenous, stained cell-PBS suspension and slowly add to 1.5 mL tube. Invert several times to mix. Do not vortex. The final PBS concentration in the 384-well source plate from the stained cell-1X PBS suspension (from step V. B. 7.) with added fresh 1X PBS should be 0.35X; adjust the volume of 1X PBS accordingly. The final cell concentration should be 1 cell per 50 nl Table VIII).

   Example: If the stained cell concentration is 2 x 10^5 cells/ml (=0.2 cell/nl), add 325 µl of water, 500 µl of Second Diluent 2X Master Mix, 75 µl of fresh 1X PBS, and 100 µl of stained cell-1X PBS suspension.

Table IV. Stained cell suspension. Added together, the total PBS concentration should end up as 0.35X (*).

Component	Concentration	Volume to add (µl)	Concentration in the 384-well source plate
Second Diluent 2X Master Mix	2X	500	1X
Pre-warmed D-RNase free water*	NA	325	NA
Pre-warmed 1X PBS*	1X	75	0.35
Stained cells-1X PBS suspension*	0.2 cells/nl	100	0.02 cells/nl
Total		1000	

NOTE: Dilute stained cells-1X PBS suspension to achieve the concentration of 1 cell/50 n in the 384-well source plate. Re-pellet stained cells and resuspend in less volume of 1X PBS if the stained cells concentration is less than 1,200,000 cells/ml. The combined volume of stained cells and 1X PBS should not exceed 17% of the reaction volume.

*Added together, the total PBS concentration should end up as 0.35X.

6. Mix the diluted stained cell suspension gently by pipetting up and down several times to have homogenous cell suspension. **Make sure to take aliquot from the center of tube containing diluted stained cell suspension.**

7. Add the mixed diluted cell suspension and other reagents to a 384-well source plate. Make sure not to introduce bubbles while adding reagents to 384-well source plate (use reverse pipetting). Refer to the source plate layout in Figure 9.

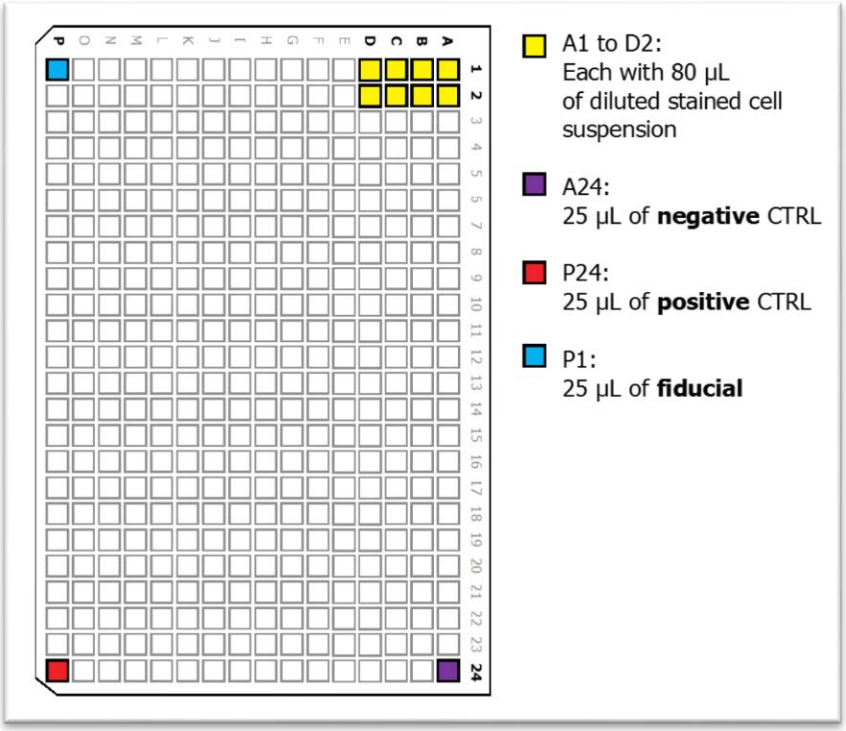


Figure 9. Add cells and other reagents to the colored source wells.

8. Seal the 384-well source plate with the ThermalSeal RTS 384-well Plate Seal. Do not centrifuge.

9. Use a chip, preprinted with UMIs and nanowell barcodes (Figure 3) for dispensing. **Remove cover film from preprinted chip.** Place the chip with the chamfered (notched) corner at the lower right corner on the Dispensing Platform in the MSND (Figure 7). The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat against the Dispensing Platform** (Figure 7).

10. Place the 384-well source plate into the MSND. Position the A1 corner at the top right of the 384-well Plate Nest (Figure 6). The beveled corners of the 384-well plate should be on the left side.

11. On the MSND Software screen, click the *Single Cell* tab. Enter or scan the Chip ID into the text field. Click [Dispense cells] (Figure 10) and click [OK] after carefully going through all questions prompted. The MSND will start dispensing 50 nl of cell suspension, control, and fiducial to the appropriate nanowells.

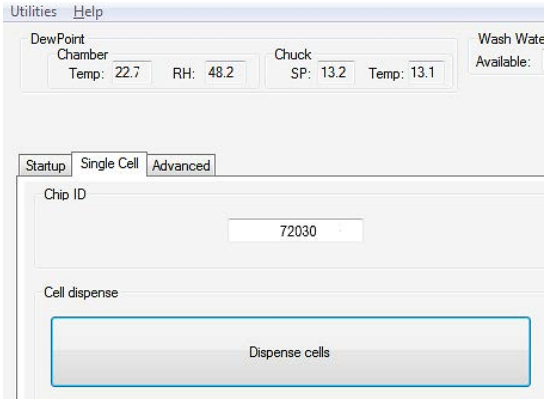


Figure 10. Click the [Dispense cells] button

12. After dispense is completed, remove the chip from the Dispensing Platform and gently blot with the Blotting paper and a Blotter (Figure 11).

Figure 11. Blot with blotting paper and blotter

13. Remove liner from one side of Optical Imaging Film and apply sticky side of Optical Imaging Film to the blotted chip (Figure 13).

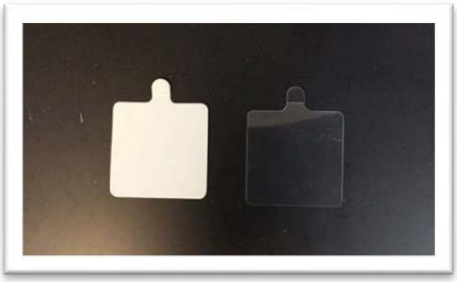


Figure 12. TE Sealing Film (white background) and Optical Imaging Film (transparent background). The Imaging Film has three layers; the intermediate layer is double-sided.

14. Use a Film Applicator to make sure chip is securely sealed to avoid well-to-well contamination (Figure 13). **Note the orientations of the chamfered corner and the Imaging Film tab**.

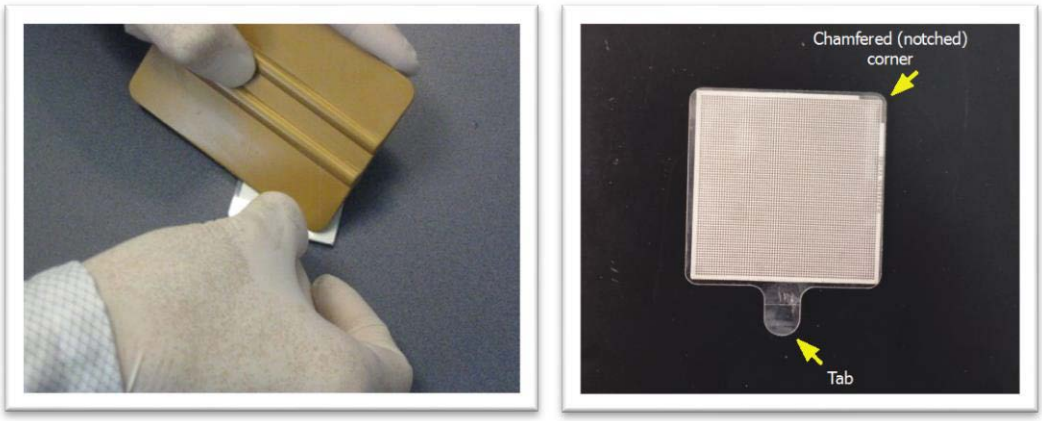


Figure 13. Seal the blotted chip with a Film Applicator. The tab of the Optical Imaging Film should be oriented downward relative to the chamfered corner of the chip.

15. Centrifuge the sealed chip at 300 x *g* for 5 minutes at 22°C with full acceleration and full brake (Figure 14). If you have one chip, balance the centrifuge with the supplied Balance Chip or a blank chip.

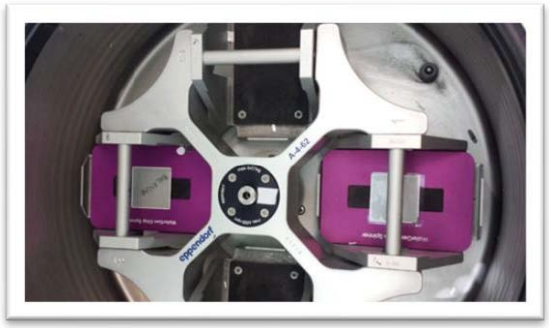


Figure 14. Centrifuge Chip Spinner

C. Protocol: Image cells

Refer to the *ICELL8 Imaging System User Manual* for instrument setup and operation.

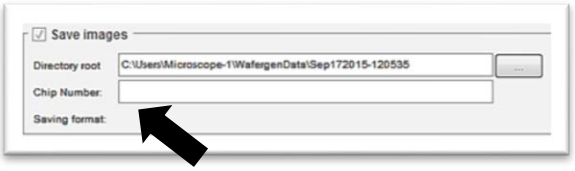
Notes:

- The light source has a warm up period of ~5 minutes. Green light should be visible indicating the burner is ready.
- The bulb has a life span of ~300 hours. Please contact Takara Bio's Field Application Scientist for assistance with changing the bulb. After a new bulb is installed, let it run for 1 hour before normal operation.
- After the burner is turned on, please allow at least 30 minutes before turning it off.
- Wait for at least 30 minutes before turning on a previously turned-off burner.
- Do not turn on and off the burner more than 4 times a day; it should be left on if you plan to use it frequently throughout the day.
- Allow the Hamachi camera to warm up for fifteen seconds (green light on and has stopped blinking) before starting the Micro-Manager software (else "Hardware Configuration Error").
- Micro-Manager software application should remain closed until all other pieces of hardware are turned on.

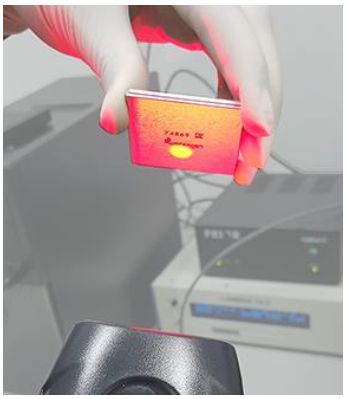
Procedure:

1. Start the Micro-Manager Software (Vale Lab, UCSF). Ensure that the manual shutter is in the Open position.
2. Click [Live] to open a window showing live image from the camera.
3. Click [Multi-dimensional Acquisition]. Make sure that the "Multiple XY" box is checked.
4. Click [Load Chip Type] and load the file "72.72.pos" configuration file if it is not pre-loaded.
5. Position the cursor so that it resides in the Chip Number field (Figure 15a). Using the barcode scanner, read the two-dimensional barcode on the bottom of the chip WITHOUT completely inverting the chip (Figure 15b)

   WARNING: Be sure to avert eyes from the scanner light.



a. Place cursor in Chip Number field



b. Scan barcode

Figure 15. Scanning barcode

6. Enter a short experimental name and the chip number in the file name prefix. The image files will be saved under this named folder.
7. Place the chip on the microscope Holding Platform with the chamfered (notched) corner facing the upper right corner (Figure 16). Ensure the chip is perfectly flat and seated square relative to the pins.

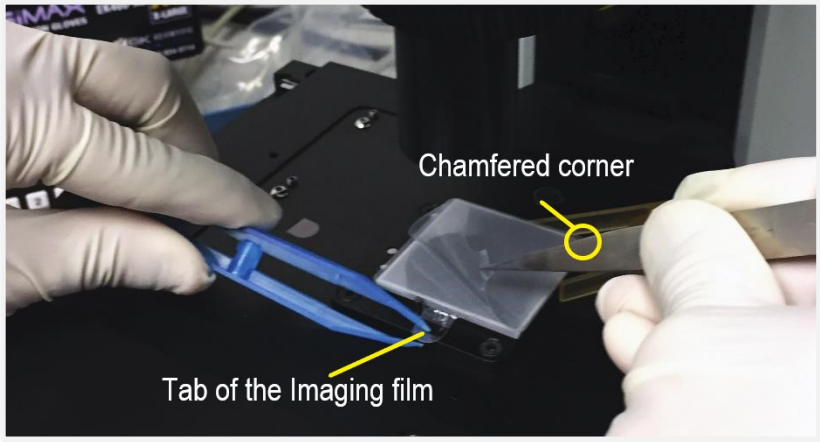


Figure 16. Seat chip on the microscope holding platform

8. 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 Optical Imaging Film using tweezers (Figure 16). Save the release liner. The chip will remain sealed by the double-sided intermediate film layer.

9. Select the row "Pos0" (Figure 17) in *Stage Position List* window, then click [Go to].

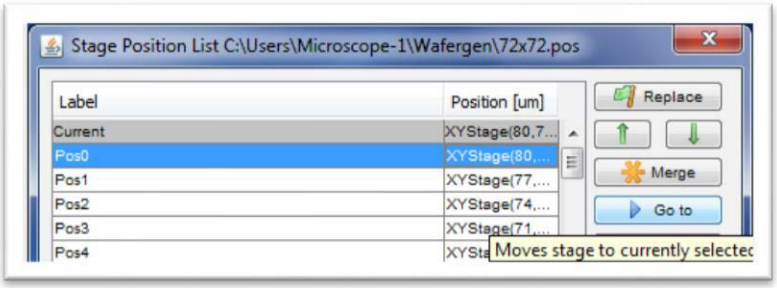


Figure 17. Stage Position Window

10. Find and focus on single cells: (refer to *ICELL8 Imaging System User Manual* for more details)

    a) Select the Illumination Channel to "DAPI" filter set in the *Configuration Setting* panel in the main window to examine Hoechst-stained cells.

    b) Adjust the "Camera Depth" to between 12 to14 bits using the pull-down menu (this is not pre-configured).

    c) Adjust the contrast of the chip image by selecting "Autostretch" and "Ignore (1–3%)" (Figure 18). If that does not achieve optimal contrast, drag the ends of the line closer to the range of the histogram peaks and Zoom (-out) when satisfied with the focus in the Z-plane.

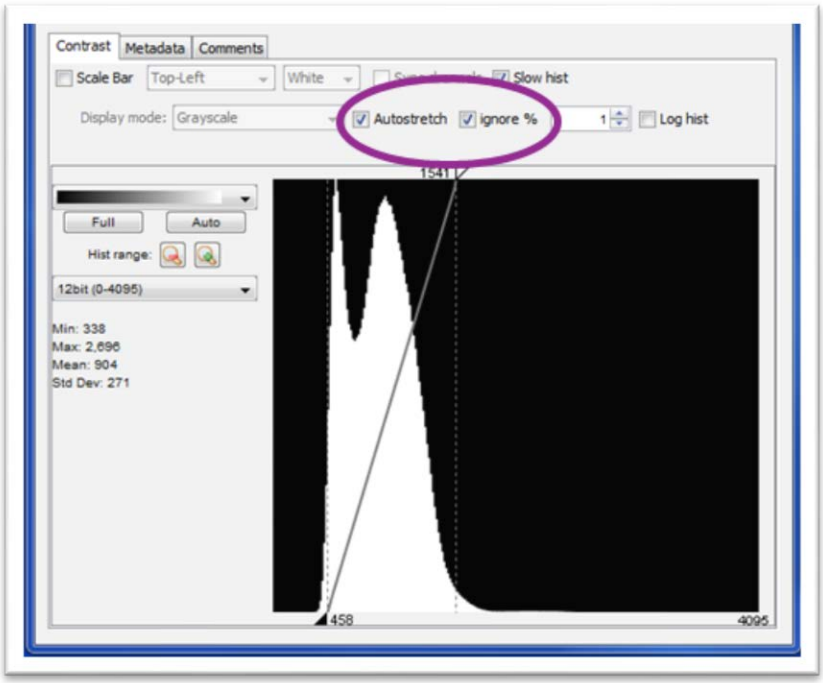


Figure 18. Adjust camera depth and contrast

d) Use the microscope manual focus to achieve the optimal Z-plane focal depth to visualize a field of single cell, preferably ones not in the center of the well.

e) Use the Zoom (-In) function in the software tool bar as needed. Adjust the focus by zooming in and out until cells appear at their smallest size. Optimal focal point may be between two cells on the Z-plane (Figure 19).

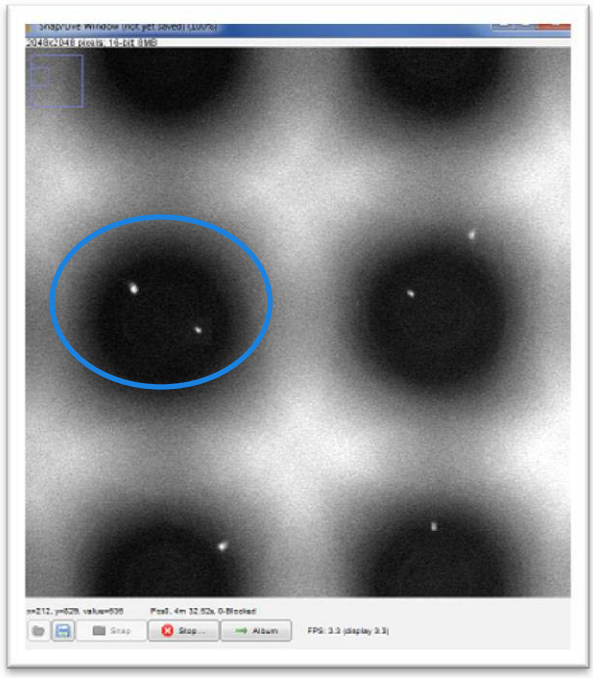


Figure 19. Adjust focus on single cells

11. Select the illumination channel “Texas Red” to examine the fiducial-containing channel. Confirm that five fiducial-containing wells are present, forming an “X” shape (Figure 20) at Pos0.

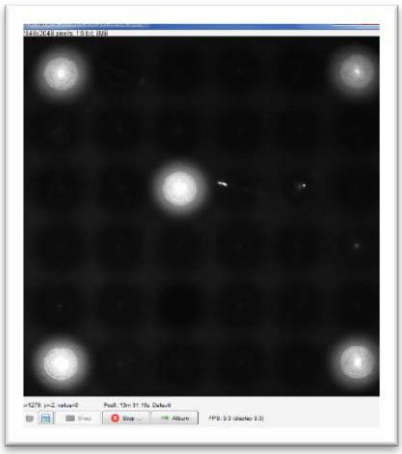


Figure 20. Brightly lit nanowells containing the fiducial mix in the first Multi-well Image of the chip from “Pos0”. There are four Multi-well Images in the entire chip with fiducial-containing nanowells. Each Multi-well Image displays 36 nanowells (a 6 x 6 grid).

12. Select “Pos143” in the Stage Position List window, confirm that all 36 nanowells are properly captured in the image, and that there are 4 fiducial-containing nanowells in the shape of a square as shown in Figure 21.

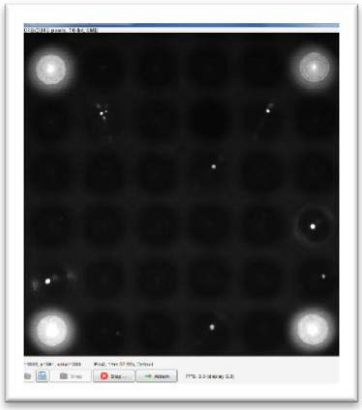


Figure 21. Brightly lit nanowells containing the fiducial mix at the last Multi-well Image from “Pos143”.

13. Select “DAPI” in the *Illumination* window for a second time.

14. Examine “Pos143” to confirm that you are satisfied with the focus. If not, check that the chip resides perfectly flat on the microscope’s Chip Holder.

15. Close the *Stage Position List* window.

16. Under the “Channels” table, select “DAPI (exposure: 200)” and “Texas Red (exposure: 300)”. “Texas Red” has the similar excitation and emission wavelengths as the Propidium Iodide.

17. Click [Acquire] and accept the prompt to create a new folder. The camera will take TIFF images of 6x6 nanowell sections (36 wells) with the “DAPI” and “Texas Red” filters. The entire image folder should contain 288 TIFF files from 144 nanowell sections (“Pos0” to “Pos143”); each nanowell section has two sets of exposures (“DAPI” and “Texas Red”).

18. After imaging is complete, the software prompts to convert file, Click [Yes]. This will launch the CellSelect Software.

19. Turn off the burner, Lambda controller, OptiScan II controller, and camera when the imaging is completed.

20. Re-apply the peeled release liner onto the top side of the double-sided intermediate film.

21. Place the imaged chip into the pre-chilled (–80°C) empty Chip Holder (Figure 8). Make sure that the Imaging Film is intact. Freeze cells at –80°C for a minimum of 30 minutes before proceeding to anneal the UMIs and nanowell barcodes (Section IV.E.). Make sure that the chip holder is closed evenly, indicating a proper magnetic seal.

D. Protocol: Analyze nanowells with CellSelect Software

Refer to the *CellSelect Software User Manual* for detailed instructions.

Load Images:

1. After imaging the cells with the Micro-Manager software, click [Yes] when prompted with the question "Run CellSelect with images from: D:\Wafergen\Wafergendata For Chip: <Chip Number>?"

NOTE: If you plan to analyze the software later, click No and close the Micro-Manager software. You can restart the CellSelect software by clicking the icon 

and importing the chip folder from the File menu.

2. Check the Chip number when prompted for Chip ID.

Figure 22. Enter Chip ID

3. Add description if needed when prompted for description.

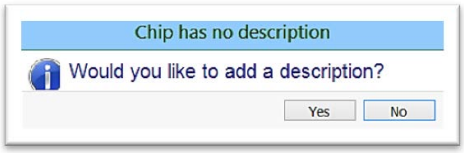


Figure 23. Add Chip Description

4. Load Settings as show below.

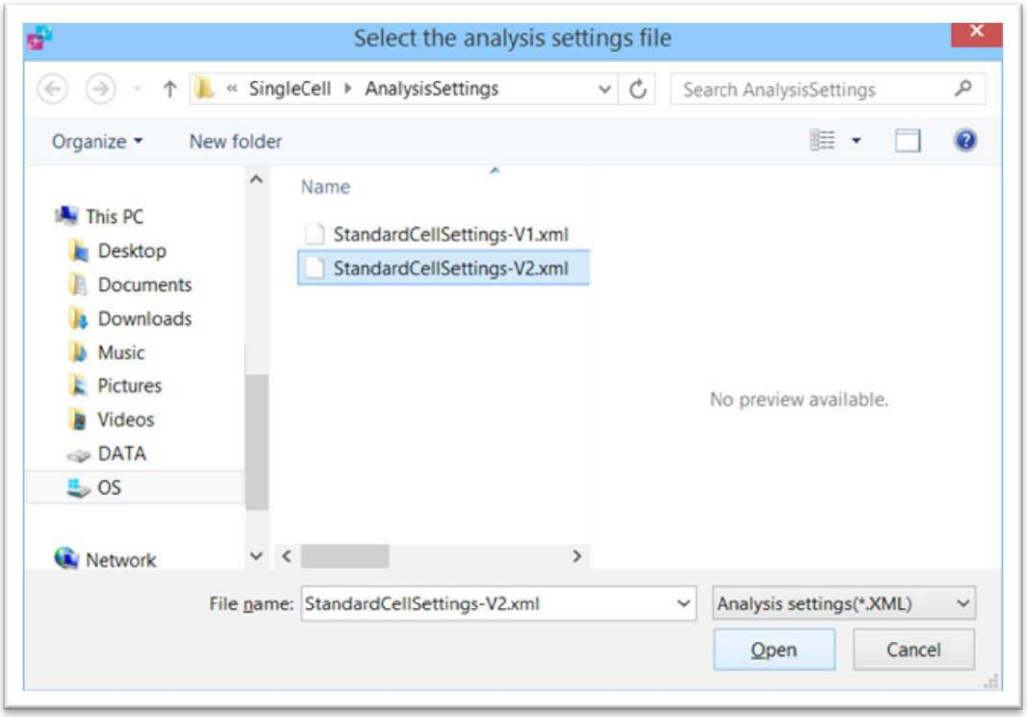


Figure 24. Load Settings

Define Fiducials:

1. Click [Define Fiducials]. The software detects fiducials and you will be prompted with 4 consecutive Fiducial images.

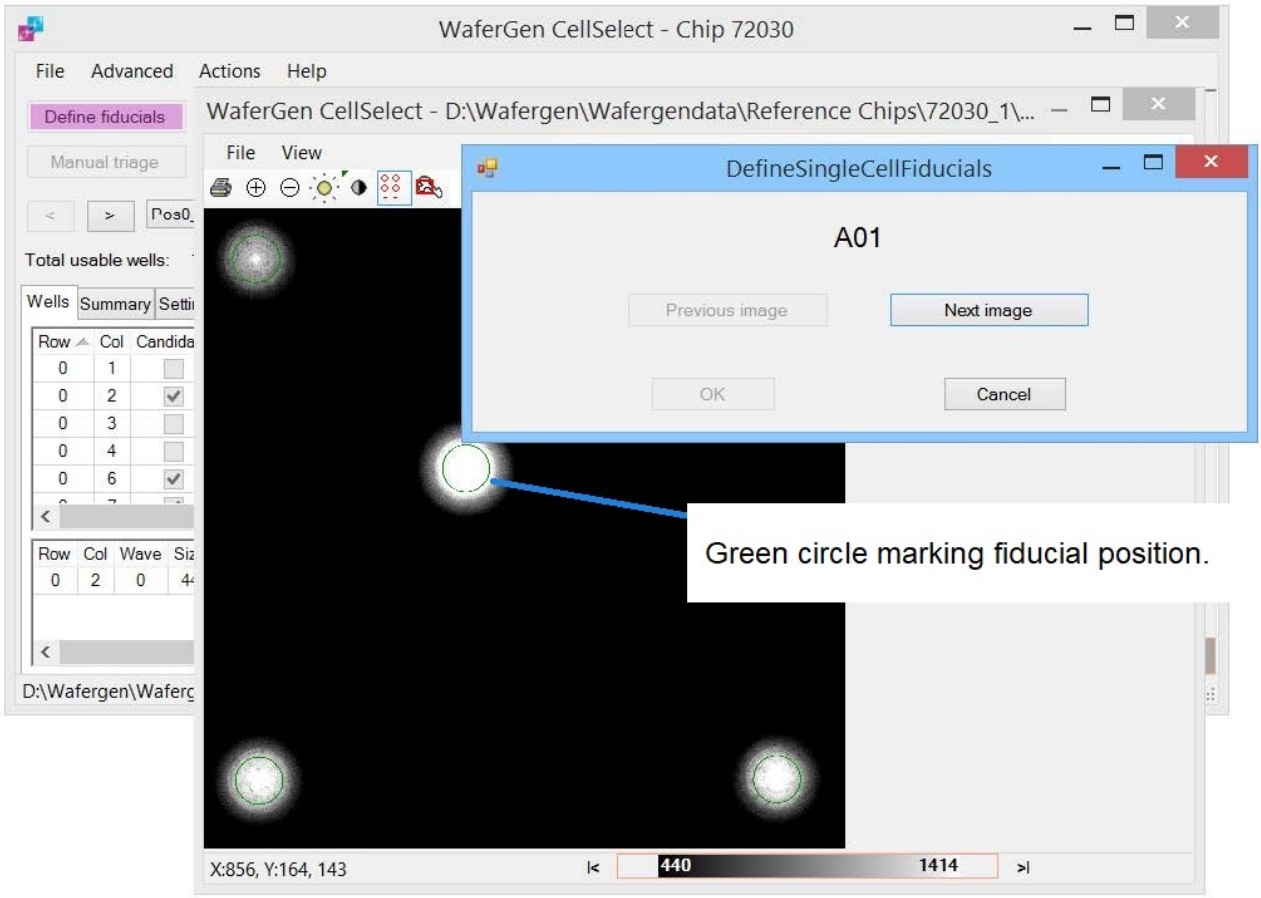


Figure 25. Define Fiducials

The first fiducial image shows 5 green circles and the other three fiducial images show 4 green circles that highlight the centers of the nanowells.

a) Inspect the fiducials in each fiducial image for proper position.

b) Move the *DefineSingleCellFiducials* box to the side to view the fiducials image.

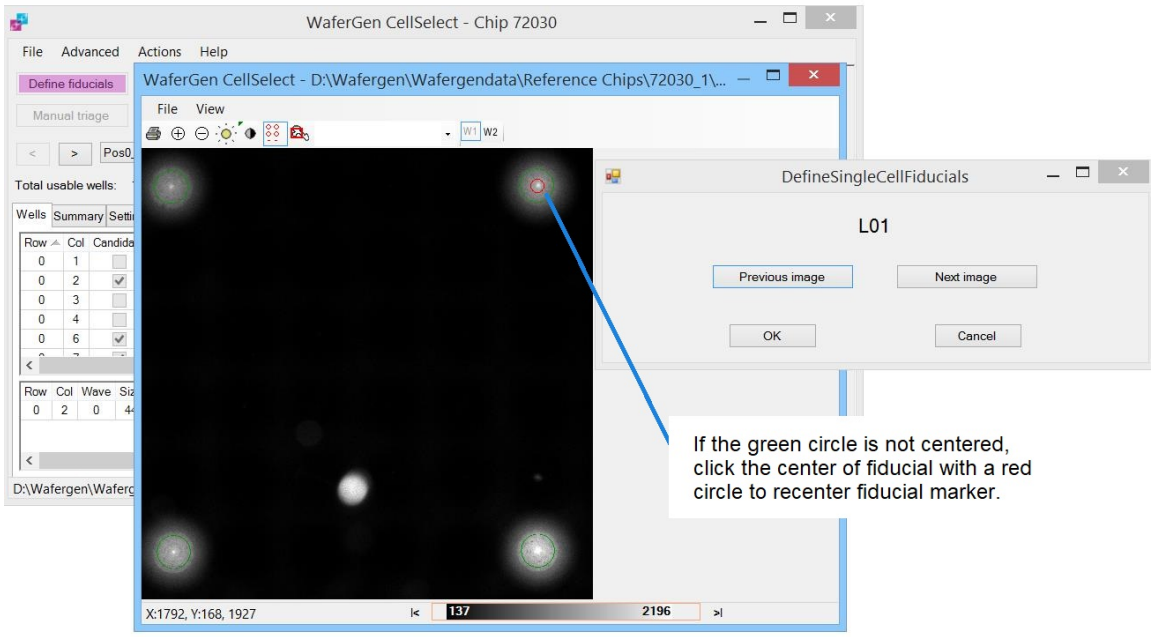


Figure 26. Inspect and Adjust Fiducials

c) If necessary, resize the fiducial image to see the 6 x 6 grid (36 nanowells). Use the tools in the toolbar to check and correct fiducials: zoom, brighten, contrast, well overlay, W (wavelengths) 1 or 2.

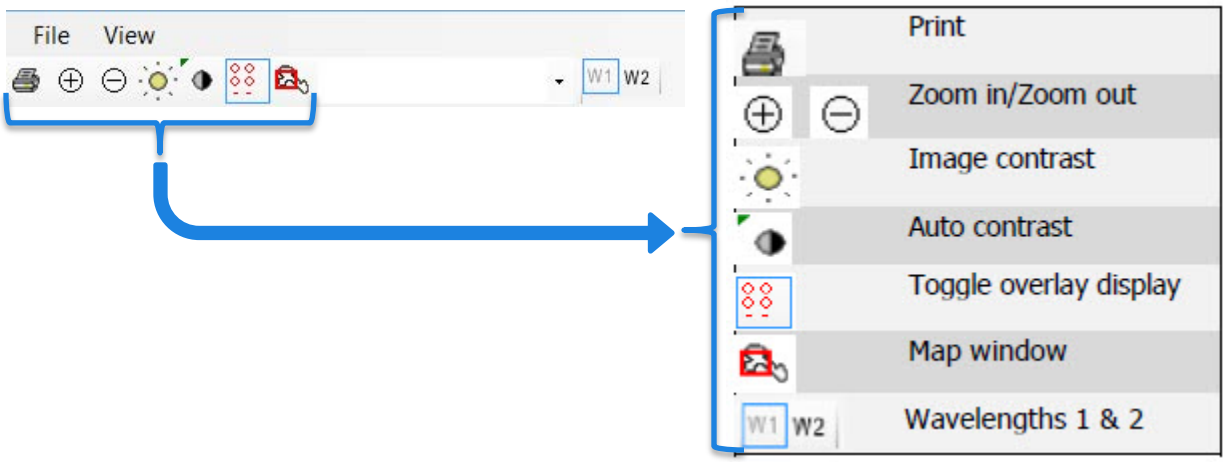


Figure 27. Image Viewer Toolbar

d) If the green circles are not centered properly, click on the nanowell centers (Figure 26) with the red circle.

e) Click through all 4 fiducial images, and then close the last fiducial image.

Load Barcode File:

1. Click [Load barcode file] under the *File* tab (Figure 28).

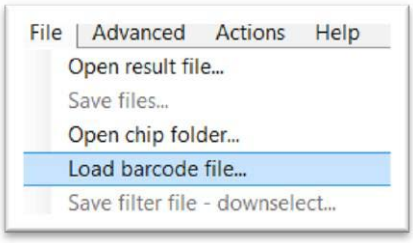


Figure 28. Load the barcode file for preprinted chip.

2. Select the pre-loaded file `5184 BarcodeSequences 21Aug2015.gal` (Figure 29). This GAL (GenePixArray List) file contains nanowell barcode sequence for each preprinted nanowell location.

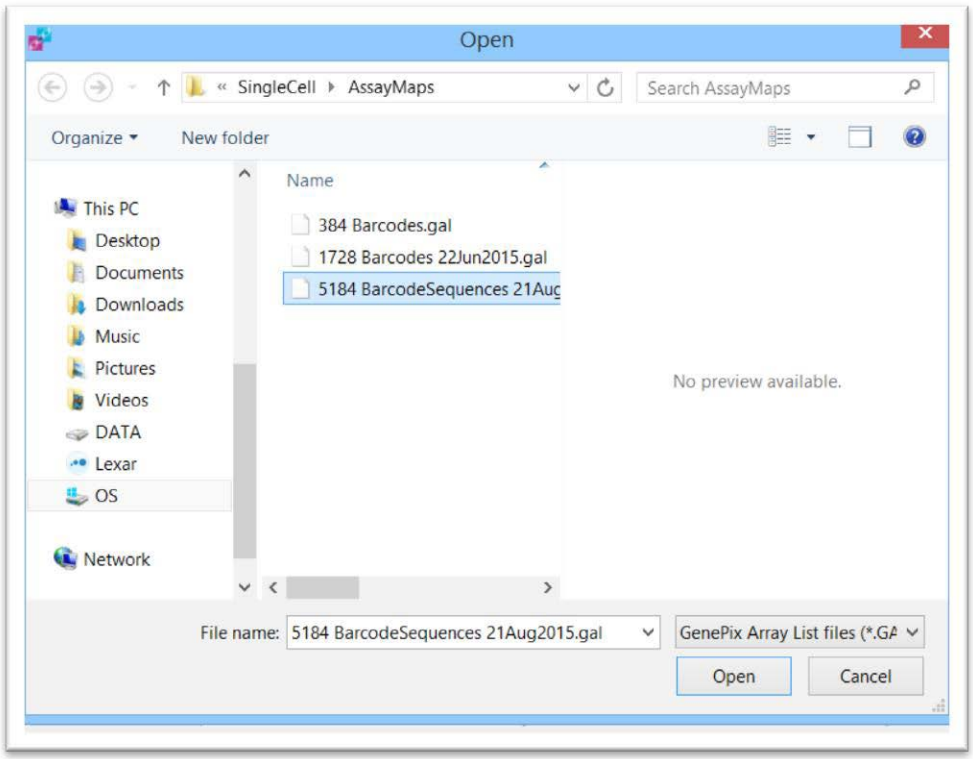


Figure 29. Select the barcode file for preprinted chip.

Process Images:

1. Click [Process Images]. The software will automatically identify and select all nanowells that contain live, single cells and controls based on the parameters defined in *Settings* (Figure 30).

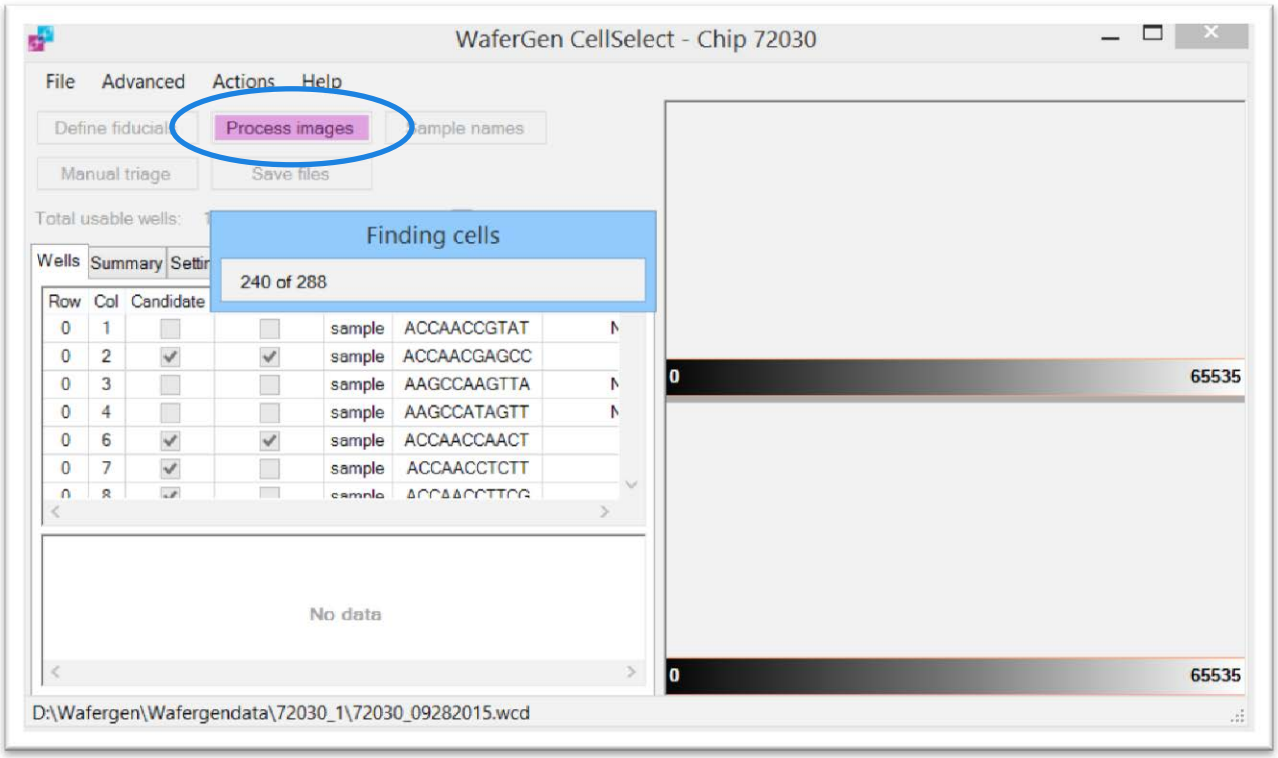
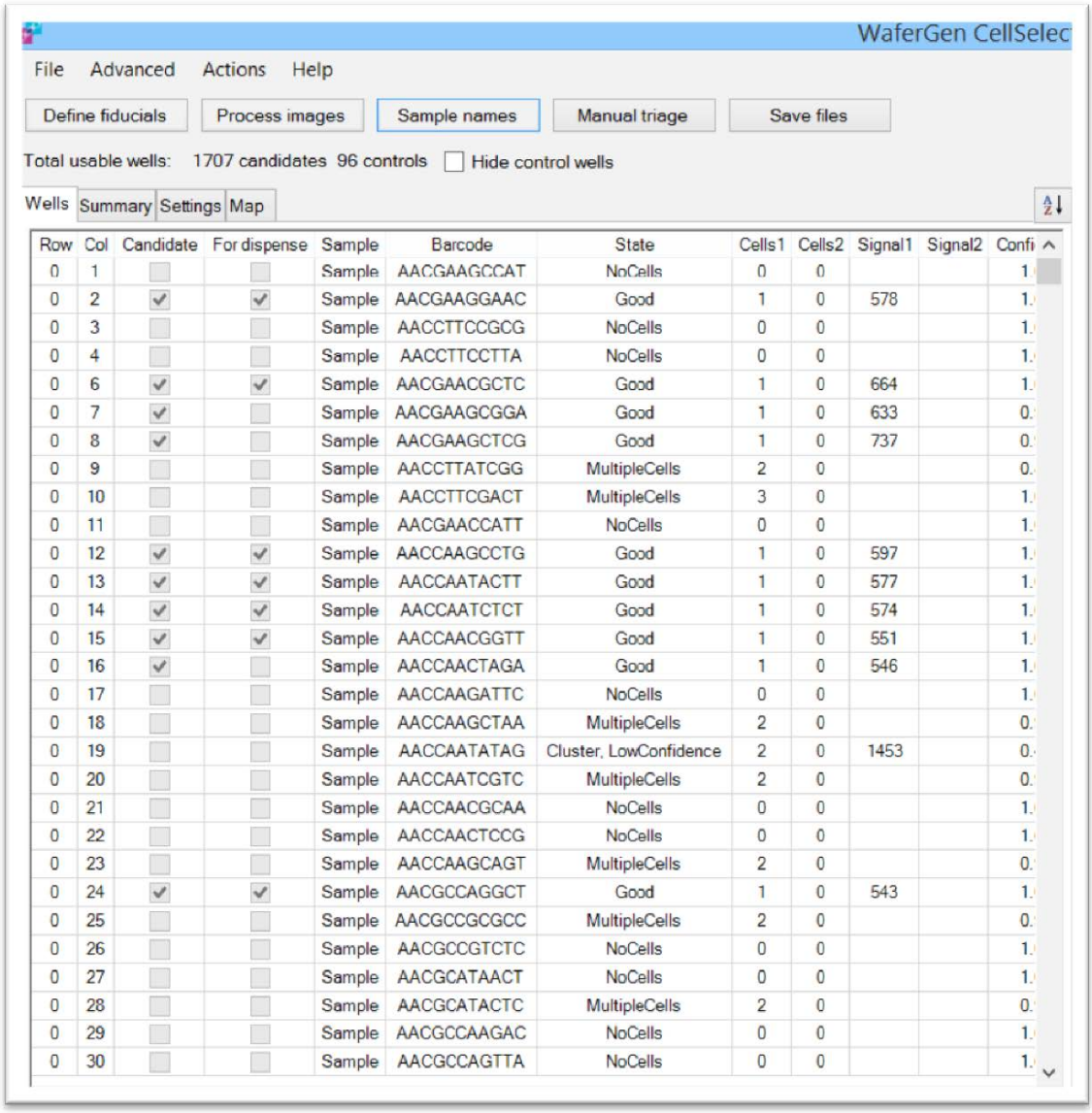


Figure 30. The software finds cells based on the parameters defined in "Settings".

2. When prompted with "Save Results", click [Yes] and give a file name prefixed with Chip number. The software will do the following:

   - Select wells among all those that are checked under "Candidate" according to the best parameters defined under *Settings*. See Figure 30.
   - Save the result file. The result file will contain the selected nanowells and setting parameter sunder a new file name.
   - Automatically generate the Filter file for RT dispense. The selected wells in the "Filter File" will be displayed in the *For Dispense* tab.

   Each preprinted chip contains a total of 5,184 unique nanowell barcodes. You can load the results along with the settings from the saved WCD file.



Row	Col	Candidate	For dispense	Sample	Barcode	State	Cells1	Cells2	Signal1	Signal2	Confi
0	1	☐	☐	Sample	AACGAAGCCAT	NoCells	0	0			1
0	2	☑	☑	Sample	AACGAAGGAAC	Good	1	0	578		1.
0	3	☐	☐	Sample	AACCTTCCGCG	NoCells	0	0			1.
0	4	☐	☐	Sample	AACCTTCCTTA	NoCells	0	0			1.
0	6	☑	☑	Sample	AACGAACGCTC	Good	1	0	664		1.
0	7	☑	☐	Sample	AACGAAGCGGA	Good	1	0	633		0.
0	8	☑	☐	Sample	AACGAAGCTCG	Good	1	0	737		0.
0	9	☐	☐	Sample	AACCTTATCGG	MultipleCells	2	0			0.
0	10	☐	☐	Sample	AACCTTCGACT	MultipleCells	3	0			1.
0	11	☐	☐	Sample	AACGAACCATT	NoCells	0	0			1.
0	12	☑	☑	Sample	AACCAAGCCTG	Good	1	0	597		1.
0	13	☑	☑	Sample	AACCAATACTT	Good	1	0	577		1.
0	14	☑	☑	Sample	AACCAATCTCT	Good	1	0	574		1.
0	15	☑	☑	Sample	AACCAACGGTT	Good	1	0	551		1.
0	16	☑	☐	Sample	AACCAACTAGA	Good	1	0	546		1.
0	17	☐	☐	Sample	AACCAAGATTC	NoCells	0	0			1.
0	18	☐	☐	Sample	AACCAAGCTAA	MultipleCells	2	0			0.
0	19	☐	☐	Sample	AACCAATATAG	Cluster, LowConfidence	2	0	1453		0.
0	20	☐	☐	Sample	AACCAATCGTC	MultipleCells	2	0			0.
0	21	☐	☐	Sample	AACCAACGCAA	NoCells	0	0			1.
0	22	☐	☐	Sample	AACCAACTCCG	NoCells	0	0			1.
0	23	☐	☐	Sample	AACCAAGCAGT	MultipleCells	2	0			0.
0	24	☑	☑	Sample	AACGCCAGGCT	Good	1	0	543		1.
0	25	☐	☐	Sample	AACGCCGCGCC	MultipleCells	2	0			0.
0	26	☐	☐	Sample	AACGCCGTCTC	NoCells	0	0			1.
0	27	☐	☐	Sample	AACGCATAACT	NoCells	0	0			1.
0	28	☐	☐	Sample	AACGCATACTC	MultipleCells	2	0			0.
0	29	☐	☐	Sample	AACGCCAAGAC	NoCells	0	0			1.
0	30	☐	☐	Sample	AACGCCAGTTA	NoCells	0	0			1.

Figure 31. After reading the image stack.

The software uses the following legend when examining nanowells:

- Green – a cell
- Yellow – ignored
- Blue – a reflection

See the *CellSelect Software User Manual* for more information.

Change Sample Names:

1. Click [Sample names] and enter the sample description: one sample name for all samples (Figure 32), or sample names based on source plate configuration layout in Section VI.B.7.

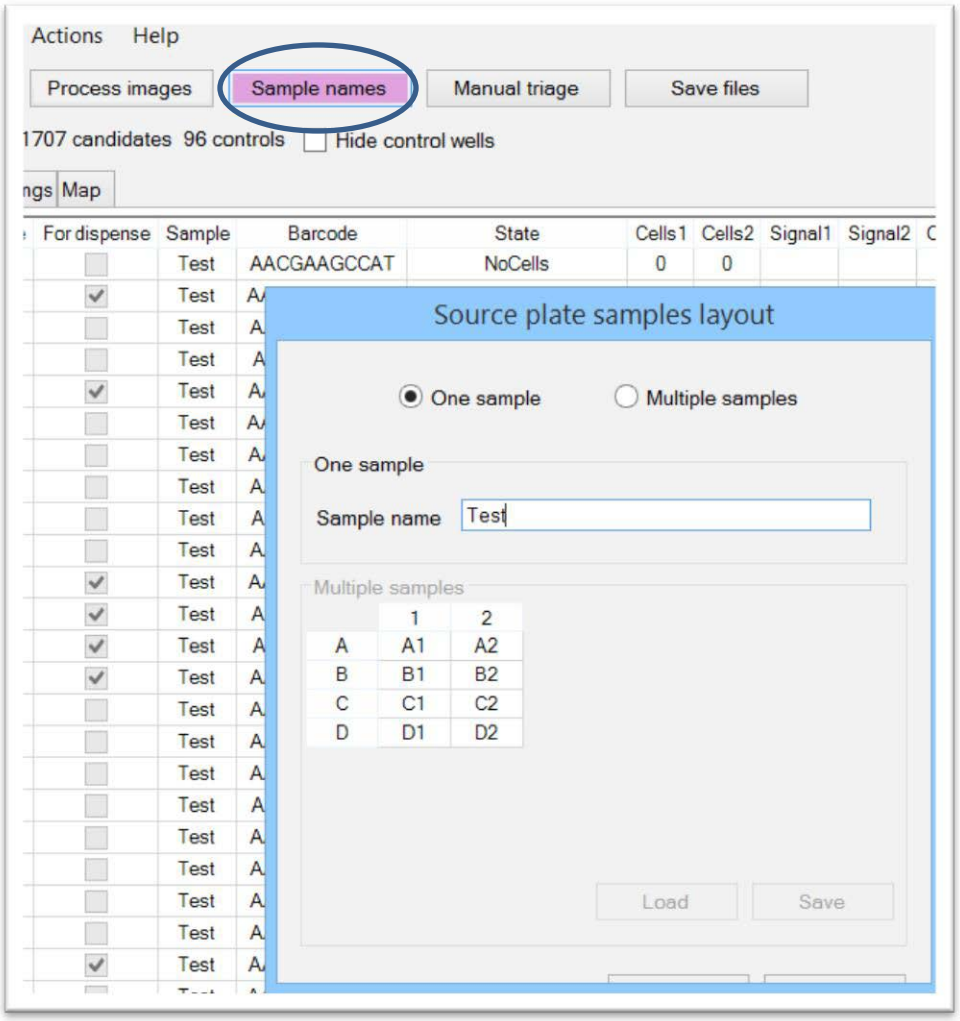


Figure 32. Changing Sample Names. For the One Sample option, "Test" is repeated down the Sample column. Control and fiducial wells are identified automatically.

Review Images:

1. Click on the *Wells* tab and sort the nanowell statistics in the "Candidate" column by clicking the Candidate heading (Figure 33), which sorts and consolidates all selected nanowells to the top.

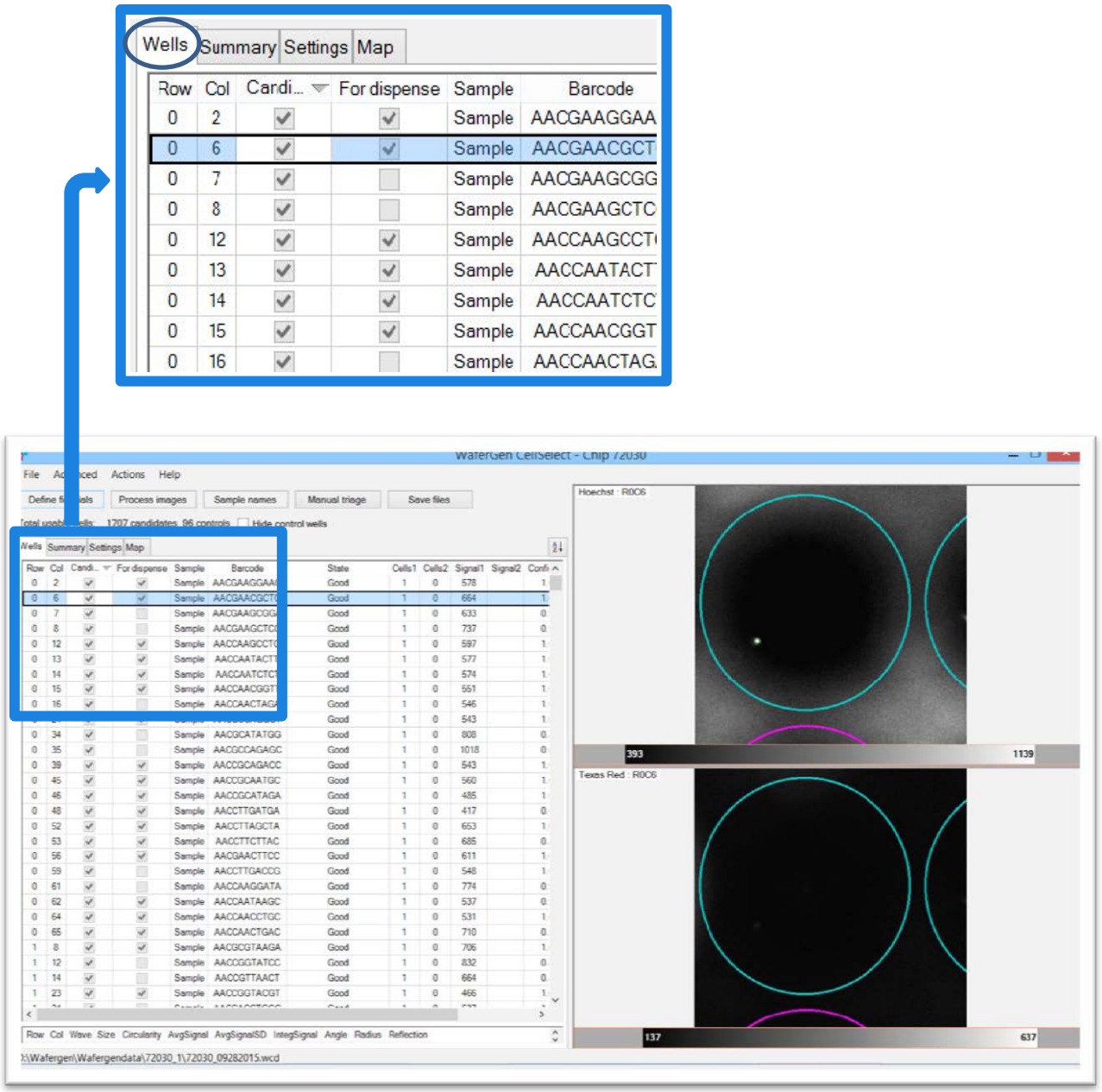


Figure 33. "Candidate" column is sorted to consolidate all selected nanowells to the top of the table

2. If desired, manually examine the selected nanowells. Click the *Advanced* tab and [Image viewer]. The Multi-well Image highlighting the selected nanowell in the results table will appear. Arrange windows according to Figure 34. When you click on any row in the "Candidate" column of the results table, the software will navigate to the individual nanowell images and the corresponding Multi-well Image. Click the [Manual Triage] button to examine each nanowell and deselect those that you would like to exclude.

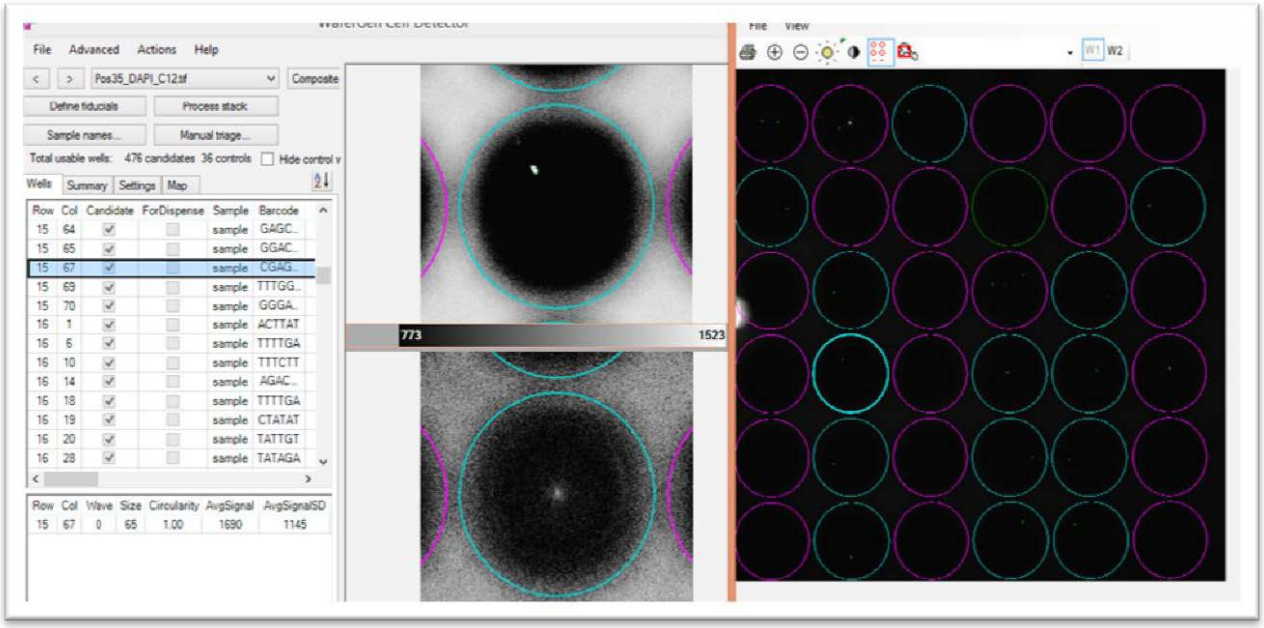


Figure 34. Arrangement of windows on the CellSelect screen. The image on the furthest right shows the 36 nanowell from a Multi-well Image. The horizontally split images in the center show a single cell in a nanowell stained by Hoechst 33342 (top) and the absence of Propidium Iodide stain in the corresponding 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 not from a dead cell because it does not match the location of the Hoechst stained cell.

Save files:

1. Click the *File* tab and select [Save Files] to save the manually triaged file. Go to the *Summary* Tab to see the number of wells selected from each individual sample for RT dispense.

2. If you would like to down-select nanowells, select [Save Filter File – downselect].

3. Enter the desired number of nanowells for the cell- and control-containing nanowells. If there are fewer wells checked under the "Candidate" column than what you specified, the software will automatically select up to the maximum possible numbers.

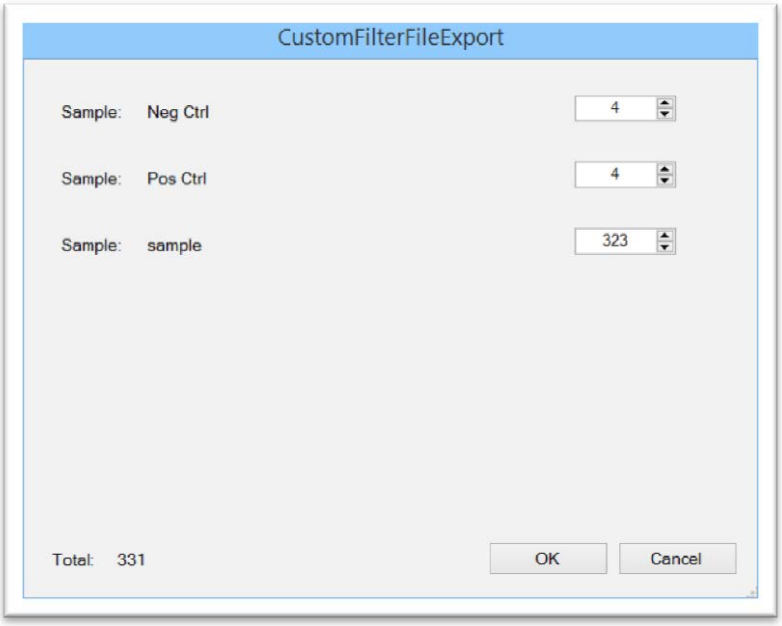


Figure 35. Save filter file by down selecting nanowells.

4. Save the filter file after downselecting the number of wells. A 72 x 72 grid of the entire chip will be saved as a CSV file automatically named as `<Chip ID>.CSV`, where "1" marks included nanowells for future dispense and "0" marks those that are not.

5. Copy the filter file (.csv file) and bring it with you to the MSND. It will be used for dispensing the RT reaction mix.

E. Protocol: Anneal UMIs and nanowell barcodes

Procedure:

1. Remove the Chip Holder with chip from the freezer. Take the chip out of Chip Holder and thaw the Chip at room temperature for 10 minutes to lyse cells. Use a Kimwipe to dry any liquid on the surface. Centrifuge the chip at 3,220 x *g* (minimum 2,600 x *g*) for 3 minutes at 4° C.

2. Place the chip in the Chip Cycler equipped with a Chip Adapter and an Insulation Foam. Place the provided blotting paper on top of the chip. This will prevent the chip from adhering to the Insulation Foam (Figure 36).

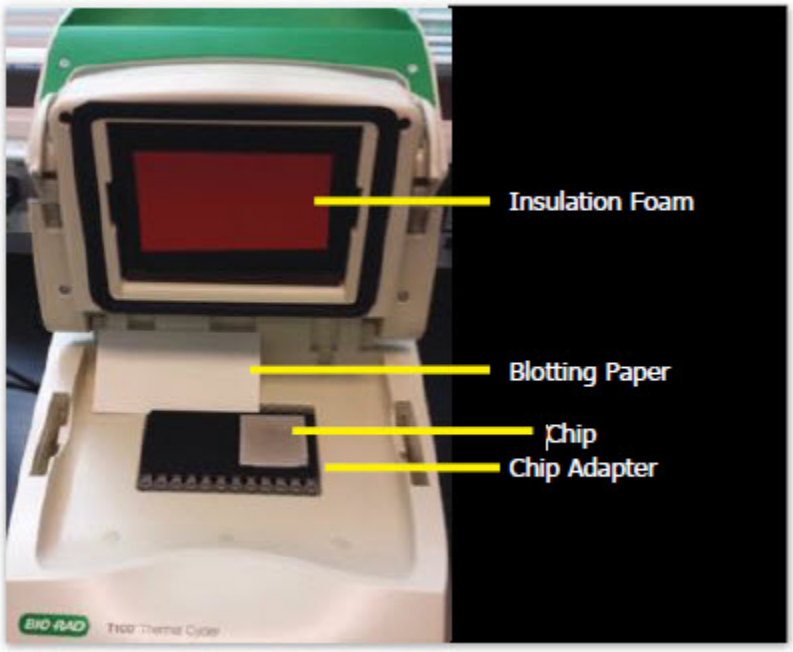


Figure 36. The Chip Cycler is equipped with a Chip Adapter and an Insulation Foam.

3. Run the thermal cycling program in Table IX to anneal the barcode oligos to poly(A)$^+$ RNA. This program, named 'ANNEALBC' was pre-entered in the Chip Cycler control software, under the folder "VAL-CHIP".

Table V. Thermal cycling program for UMIs and nanowell barcodes annealing in the chip.

Temperature	Cycling time	Number of cycles
72˚ C	3 minutes	1
4˚ C	Forever	Hold

4. Remove the chip from the Chip Cycler and centrifuge at 3,220 x *g* (minimum 2,600 x *g*) for 3 minutes at 4°C. Keep the chip at 4°C or on a pre-chilled Cold Block until you are ready for next step (Figure 37).

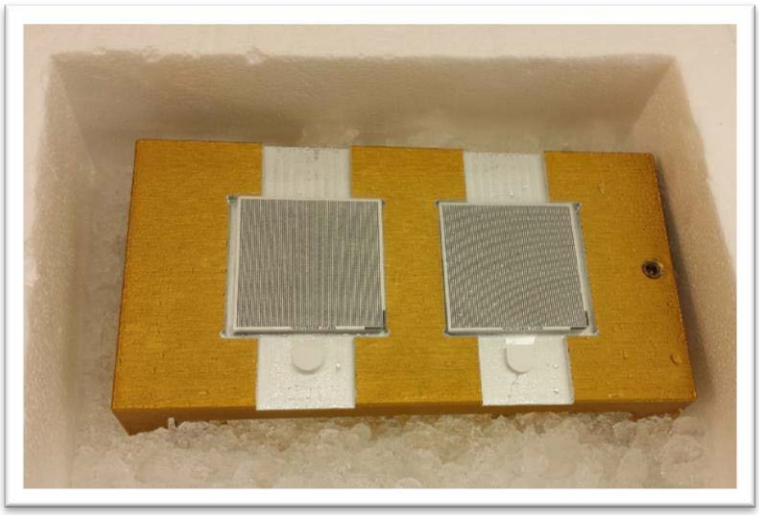


Figure 37. Place the chip on a cold block on ice.

F. Protocol: Synthesize first and second strands with template switching

Procedure:

1. Prepare RT Reaction mix according to Table X.

Table VI. Recipe for RT Reaction Mix. 50 nl of the reaction mix will be added to each nanowell. Each nanowell already contains 50 nl of cell– or control– second diluent mix (Table V).

Temperature	Supplied concentration	Volume to add per chip (µl)	Concentration at RT reaction
Maxima H Minus RT buffer	5X	88.0	1X
dNTPs	10 mM	44.0	1 mM
RT E5OLIGO	100 µM	4.4	1 µM
D-RNase free water	NA	57.2	NA
Maxima H Minus Reverse Transcriptase	200 U/µl	26.4	12 U/µl
Total		220.0	

2. Pipette 50 µl of RT Reaction Mix into the colored 384-plate source wells (Figure 38).

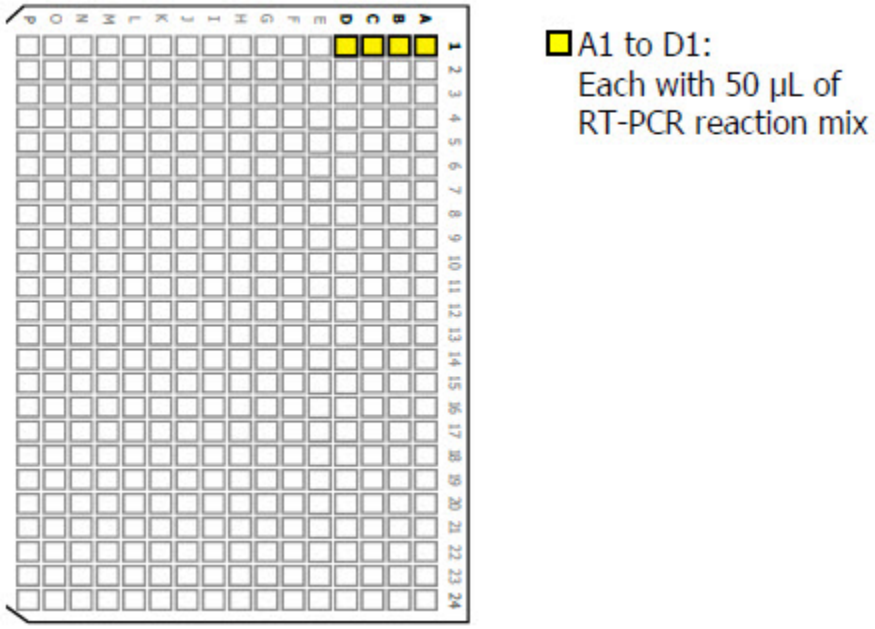


Figure 38. Aliquot 50 µl of RT Reaction Mix to the colored source wells.

3. Seal the 384-well source plate with a ThermalSeal RTS 384-well Plate Seal.

4. Centrifuge the source plate at 3,220 x *g* (minimum 2,600 x *g*) for 3 minutes at 4°C.

5. Remove the seal and place the 384-well source plate in the MSND (Figure 6).

6. Place the chip in the Dispensing Platform and make sure that the chamfered corner is facing the bottom right (Figure 7). On the MSND Software screen, under the *Single Cell* tab, type or scan the chip number in the Chip ID field.

7. Under the *Single Cell* tab, click the browse button next to the "Filter file" field to load the filter file `<Chip Number>.CSV` file (Figure 39).

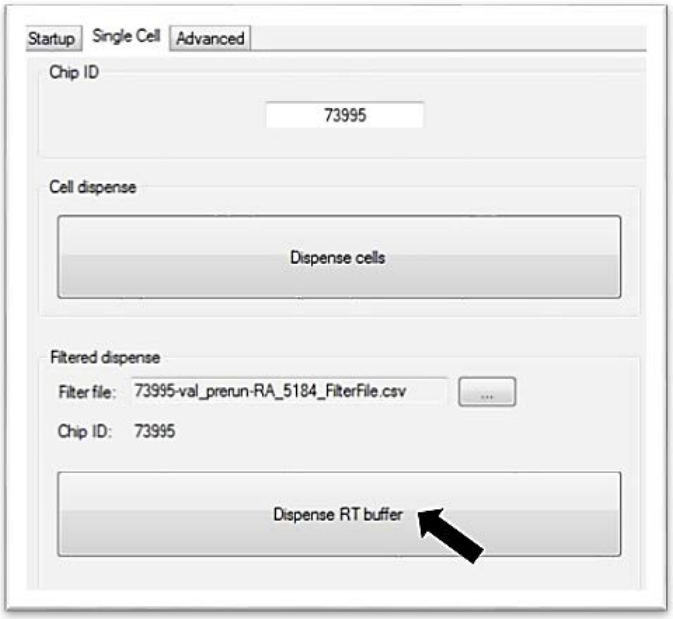


Figure 39. Load the `PredispensedFilter.CSV` file into the MSND Software.

8. Click [Dispense RT buffer] and carefully go through all questions prompted and then Click [OK]. The MSND will dispense 50 nl of RT reaction mix into each nanowell.

9. After dispensing is completed, remove the chip from the Dispensing Platform and gently blot with the blotting paper and a Blotter (Figure 11).

10. Seal the blotted chip with a TE Sealing Film (Figures 12) using a Film Applicator.

11. Centrifuge the sealed chip at 3,220 x *g* (minimum 2,600 x *g*) for 3 minutes at 4°C.

12. Run the Thermal Cycling Program in Table 11 in the Chip Cycler. This program, named 'RT-PCR' was pre-entered in the Chip Cycler control software, under the folder `VAL-CHIP\`. It is important to incubate the reaction for a full 1 hour and 30 minutes to facilitate full-length cDNA synthesis.

Table VII. Thermal cycling program for first and second strand syntheses.

Temperature	Cycling time	Number of cycles
42° C	1 hour 30 minutes	1
4° C	Forever	Hold

13. After the thermal cycling program is completed, remove the chip from the thermal cycler and centrifuge at 3,220 x *g* (minimum 2,600 x *g*) for 3 minutes at 4°C. Keep the chip on a pre-chilled Cold Block.

G. Protocol: Collect DNA

Procedure:

1. Open the supplied ICELL8 Collection Kit. Label the 600 µl capacity Collection Tube with the engraved chip number and attach the tube to the Collection Fixture (Figure 40).

Figure 40. Attach the Collection Tube to a Collection Fixture.

2. Carefully peel off the TE Sealing Film from the chip (Figure 41).

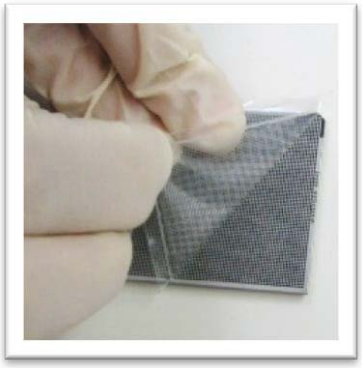


Figure 41. Carefully peel off the TE Sealing Film. Start from one corner and apply even pressure to pull.

3. With the nanowells facing down, place the chip into an assembled Collection Module (Collection Tube and Collection Fixture) (Figure 42). Surface tension will hold the liquid in the nanowells. Place the chip with the nanowells facing down into Collection Module.

Figure 42. Place chip into Collection Module

4. Seal the chip and the top of the collection module with a supplied Collection Film.

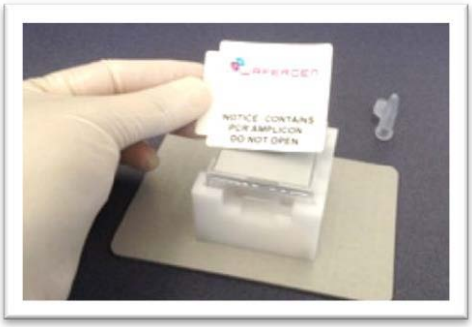


Figure 43. Secure with Collection Film.

5. With a Balance or blank chip, assemble another Collection Module. Centrifuge the balanced modules at 3,220 x *g* (minimum 2,600 x *g*) for 10 minutes at 4°C.

6. Carefully remove the collection tube that has the cDNA (Figure 44). Attach the supplied second Collection Tube to the Collection Fixture and seal the entire module. Discard the module in a biohazard waste bin.

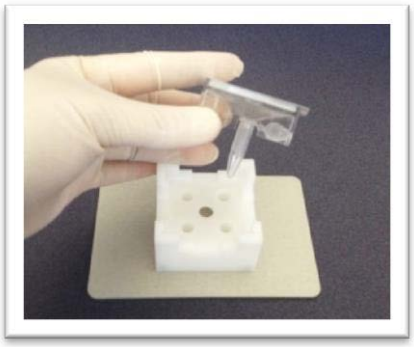


Figure 44. Remove the Collection Tube that has the eluent.

H. Protocol: Concentrate cDNA products

Before you start:

In the DNA Clean & Concentrator-5 kit (Zymo Research), make sure that ethanol was added to make the wash buffer prior to the first use.

1. Transfer the cDNA eluent to a 1.5 ml D-RNase free microcentrifuge tube.

2. Measure the extracted volume with a pipette tip. Use the DNA Clean & Concentrator-5 kit (Zymo Research) to purify the cDNA product by following the manufacturer's protocol:
   - Add 7 volumes of DNA binding buffer to 1 volume of the cDNA product.
   - Transfer the mixture (~700 µl at a time) to a Zymo-Spin Column affixed with a Collection Tube.
   - Centrifuge at maximum speed (≥10,000 x *g*) for 30 seconds. Discard the flow-through. Repeat until all mixture is collected in the same collection tube.
   - Add 200 µl DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat the wash step, for a total of 2 washes.
   - Spin the column at maximum speed (≥10,000 x *g*) for 1 min to dry the column matrix.
   - Add 20 µl of D-RNase free water directly to the column matrix and incubate at room temperature for 1 minute. Transfer the column to a 1.5 ml D-RNase free microcentrifuge tube and centrifuge at maximum speed (≥ 10,000 x *g*) for 30 seconds to elute the cDNA. You will end up with ~18 µl of eluent.

Treat with Exonuclease I:

1. Transfer exactly 17 µl of the cDNA eluent to a 0.2 ml D-RNase free PCR tube.
2. Combine the Exonuclease I reaction components (Table XII).

Table VIII. Reaction mix for the Exonuclease I reaction.

Component	Supplied concentration	Volume to add per tube (µl)	Final concentration at incubation
Concentrated cDNA	NA	17	NA
Exonuclease Buffer	10X	2	1X
Exonuclease I	20 U/µl	1	1 U/µl
Total		20	

3. Place the PCR tube in a conventional thermal cycler.
4. Incubate the reaction with the thermal cycling profile in Table XIII.

Table IX. Thermal cycling program for the Exonuclease I reaction.

Temperature	Cycling time	Number of cycles
37 ° C	30 minutes	1
80 ° C	20 minutes	1
4° C	Forever	Hold

I. Protocol: Amplify full-length cDNA

Procedure:

1. Prepare the PCR reaction master mix according to Table XIV in a 0.2 ml D-RNase free PCR tube using Advantage 2 PCR kit.

Table X. Reaction mix for amplifying full-length cDNA

Component	Supplied concentration	Volume to add per tube (µl)	Concentration at PCR reaction
Exonuclease I treated cDNA	NA	20	NA
10X Advantage 2 PCR Buffer	10X	5	1X
s50X dNTP Mix	50X	1	1X
Amp Primer	10 µM		200 nM
50X Advantage 2 Polymerase Mix	50X		1X
D-RNAse free water	NA	22	NA
Total		50	

2. Place the tube containing the reaction mix in a conventional Thermal Cycler and run the thermal cycling program in Table XV.

Table XI. Thermal cycling program for full-length cDNA amplification.

Temperature (°C)	Cycling time	Number of cycles
95	1 minute	1
95	15 seconds	18
65	30 seconds	
68	6 minutes	
72	10 minutes	1
4	Forever	Hold

NOTE: Here is a safe point to stop. Store the amplified full-length cDNA at –20°C or leave it in the Thermal Cycler until you are ready for next step.

J. Protocol: Purify the full-length ds cDNA

Before you start:

- Equilibrate the DNA Beads to room temperature prior to use.
- Prepare fresh 70% vol/vol ethanol. Use an anhydrous ethanol stock.

Procedure:

1. Purify the amplified full-length cDNA product with room temperature DNA Beads.
2. Add 0.6X volume of well-vortexed DNA Beads. For example, to a 50 µl of cDNA suspension, add 30 µl of DNA Beads.
3. Vortex to mix. Spin down briefly to collect all liquid.
4. Incubate the mixture at room temperature for 5 minutes. Place the reaction on a magnetic stand for 2 minutes to separate beads from the solution.
5. Carefully remove the supernatant with a pipette. Discard the supernatant.
6. Wash the pellet with 200 µl of 70% vol/vol ethanol. Incubate the reaction on the magnetic stand at room temperature for 1 minute. Carefully remove and discard the supernatant. Repeat one more time to wash, for a total of 2 washes.
7. Air-dry the washed beads at room temperature for ~5 minutes. Visually examine the pellet to make sure that it is dry. Do not over-dry the pellet.
8. Add 12 µl of D-RNase free water. Pipette up and down to mix. Make sure that all beads are suspended.
9. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 minutes and then incubate on a magnetic stand for 1 minute.
10. Carefully transfer the supernatant with a pipette. Save the supernatant.

K. Protocol: Quantify the full-length ds cDNA

Procedure:

1. Examine the purified ds cDNA length distribution. Dilute the purified cDNA product 1:10 times. Use 1 µl of the purified product and diluted purified product to run on an Agilent's High Sensitivity DNA chip (Figure 45).

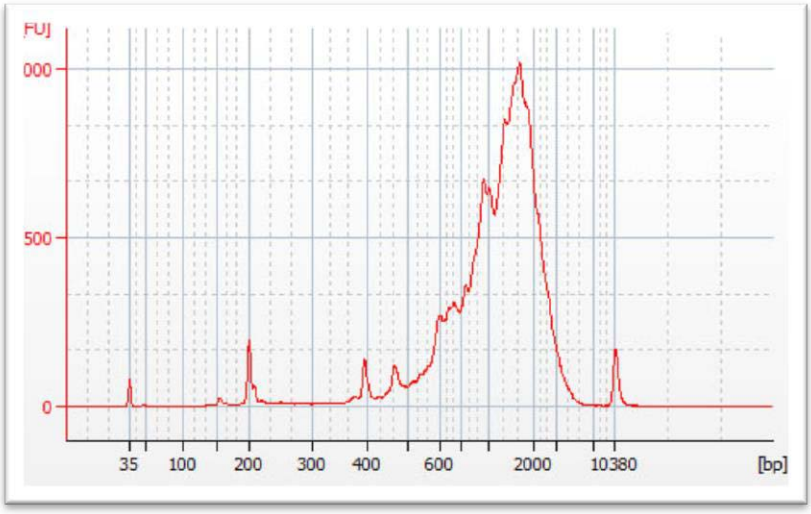


Figure 45. A typical Bioanalyzer trace of the purified full-length ds cDNA.

2. Quantify the purified ds cDNA product with the Qubit dsDNA HS Fluorometric Assay (Life Technologies) according to manufacturer's instructions. Use 2-3 µl of diluted purified cDNA product for Qubit quantification.

L. Protocol: Prepare sequencing library with Nextera XT (Illumina)

Before you start:

- Remove the ATM (Amplicon Tagment Mix) and TD (Tagment DNA Buffer) reagents from the Nextera XT DNA Library Preparation Kit to thaw on ice.
- Thaw the ds cDNA if previously frozen. Dilute the ds cDNA to 0.2 ng/µl. Use 1 ng of the purified ds cDNA for library preparation. You will end up with ~20 nM of final purified library in a total of ~10 µl volume. Setup 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 seen, vortex to resuspend the particulates.

Note:

- The Nextera XT DNA Library Preparation Kit and Index Kit(s) are not supplied.

Procedure:

1. Set up the tagmentation, neutralization, and PCR reactions according to manufacturer's recommendations.

2. Assemble the reagent components (Table XVI) in a 0.2 ml D-RNase free tube.

Table XII. Nextera XT tagmentation reaction mix

Component	Concentration	Volume to add per tube (µl)
TD	NA	10
Purified and diluted ds cDNA	0.2 ng/µl	5
ATM	NA	5
Total		20

3. Centrifuge briefly to collect all liquid. Incubate the reaction in a conventional Thermal Cycler with the cycling program in Table XVII.

Table XIII. Thermal cycling program for tagmentation.

Temperature	Cycling time	Number of cycles
55° C	5 minutes	1
10° C	Forever	Hold

4. Immediately after cooling, add 5 µl of NT buffer to the reaction mix. Mix by pipetting up and down 5 times.

5. Centrifuge briefly. Incubate at room temperature for 5 minutes.

6. 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. The i5 index primer (white cap) supplied in the Nextera XT Index Kit will **NOT** be used.

7. Thaw the supplied Nextera Primer P5.

8. Assemble the amplification reaction according to Table XVIII in a 0.2 ml D-RNase free PCR tube.

Table XIV. Reagent components for the Nextera XT library amplification reaction

Component	Concentration	Volume to add per tube (µl)
TD	NA	10
Purified and diluted ds cDNA	0.2 ng/µl	5
ATM	NA	5
Total		20

9. Vortex to mix. Centrifuge briefly to collect all liquid.

10. Incubate the reaction with the thermal cycling program in Table XIX.

Table XV. Thermal cycling program to amplify the Nextera XT library.

Temperature (°C)	Cycling time	Number of cycles
72	3 minutes	1
95	30 seconds	1
95	10 seconds	12
55	30 seconds	
72	30 seconds	
72	5 minutes	1
10	Forever	Hold

M. Protocol: Purify sequencing library and size select

Procedure:

1. Purify the amplified library with the supplied DNA Beads. Equilibrate the DNA Beads suspension to room temperature prior to use.
2. Add 1X beads. For example, add 50 µl of beads to 50 µl of the amplified library product.
3. Wash and air-dry the bead pellet (**Section IV.J.** Steps 6-9). Add 51 µl of D-RNase free water to the dried bead pellet for elution. You will end up with ~50 µl.
4. Add 0.5X beads (25 µl) to the 1X bead-purified eluent (the 50 µl eluent obtained from the previous step). Vortex to mix. Spin down briefly to collect all liquid.
5. Incubate the mixture at room temperature for 5 minutes. Place the reaction on the magnetic stand for 2 minutes to separate beads from the solution.
6. Carefully transfer the supernatant with a pipette. **Save the supernatant. Discard the beads.**
7. Add 0.2X DNA Beads suspension (10 µl) to the supernatant. Wash and air-dry the bead pellet (**Section IV.J.** Steps 6-9). Add 11 µl of D-RNase free water to the air-dried beads for elution. You will end up with ~10 µl of >15 nM final library.

N. Protocol: Assess sequencing library quality and quantity

Procedure:

1. Examine the purified final library length distribution. Run 1 µl of the purified product on an Agilent's High Sensitivity DNA chip (Figure 46).

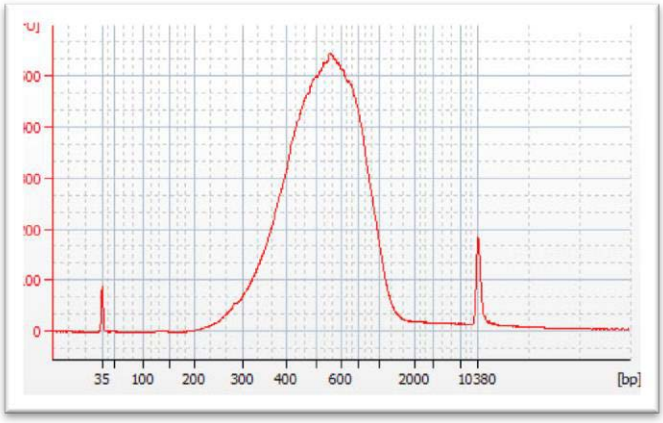


Figure 46. A typical Bioanalyzer trace of the purified Nextera XT library.

2. Quantify the diluted library with the KAPA Library Quantification kit for Illumina (KAPA Biosystems).

3. Store the sequencing library at –20°C.

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