

User Manual

Version 1.3 – UMFC3300INT



Evercode™ Cells and Nuclei Fixation v3 with INTEGRA ASSIST PLUS

For use with
ECFC3300, ECFC3301
ECFC3303, ECFC3305
ECFC3501, ECFC3503
ECFC3505, ECFN3300
ECFN3301, ECFN3501

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U.S. Pat. No. 11,168,355

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Patents pending in the U.S. and other countries

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Overview

Workflow

The Evercode Cells and Nuclei Fixation workflow is compatible with the INTEGRA ASSIST PLUS to enable the large-scale fixation of single cell/nuclei through a robust, semi-automated process.

From a single cell/nuclei suspension, the Evercode Cell Fixation and Nuclei Fixation kits generate fixed and permeabilized cells/nuclei ready for use in all downstream Evercode assays.

Three different throughput options provide the flexibility to process anywhere between 1 to 96 samples in parallel:

- 12 Reactions workflows uses ECFC3300/ECFN3300
- 48 Reactions workflows uses half of ECFC3501/ECFN3501
- 96 Reactions workflows uses ECFC3501/ECFN3501

Fixation maintains cell structure, prevents RNA degradation, and locks the RNA inside the cells, which are crucial for downstream processing with Evercode split-pool combinatorial barcoding technology (Figures 1 and 2).

Because fixed samples are also stable for up to 6 months at -80°C , Evercode Cell/Nuclei Fixation Kit with INTEGRA ASSIST PLUS provides flexibility by separating sample collection from library preparation. It also enables samples to be stored and batched after fixation so they can be processed through library preparation together, reducing the potential of batch effects.

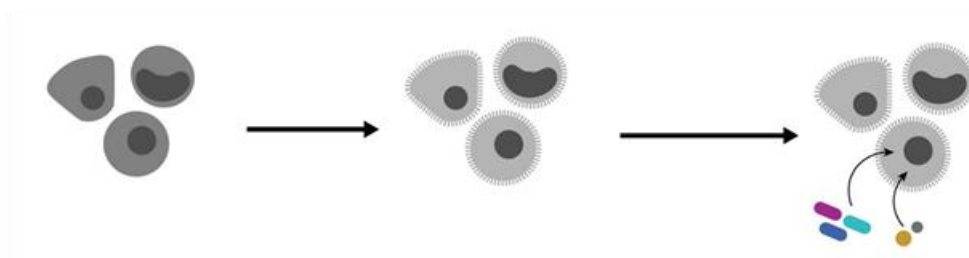


Figure 1: Evercode Cell Fixation. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

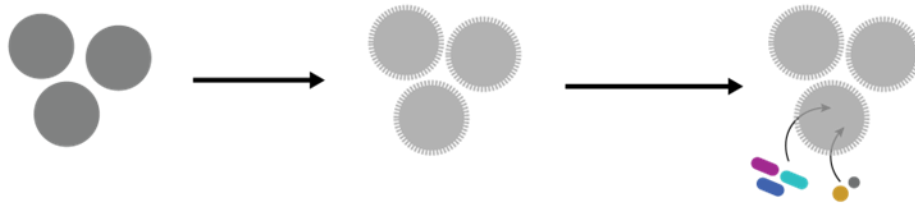


Figure 2: Evercode Nuclei Fixation. Nuclei in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

Important Guidelines

These guidelines provide additional information to obtain optimal performance beyond the detailed instructions in the protocol. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at <https://support.parsebiosciences.com/>.

Sample Input

- This protocol begins with a previously prepared single cell/nuclei suspension. We recommend suspensions with >70% viability (ideally above 90%) and <5% aggregation/debris.
- If cells/nuclei were previously frozen, ensure the suspension is completely thawed and in suspension before beginning fixation.
- We recommend minimizing the length of time samples are stored on ice prior to fixation, as it can negatively impact results.
- Between 100,000 and 1 million cells/nuclei can be fixed in a single reaction. However, we recommend using the highest number available up to 1 million total. Exceeding 1 million cells/nuclei in a single fixation will result in substantially elevated doublet rates.
- The minimum input into fixation should also be determined based on how the samples will be processed downstream. The table below provides guidance on the post-fixation concentrations needed for downstream kits. However, more or less sample input may be required depending on the exact experimental design. To accurately determine the required post-fixation cell/nuclei concentrations and volumes, reference the relevant [Sample Loading Table](#).
- Note that retention during fixation varies typically between 40-60%, and some cells/nuclei will be lost when freezing and thawing fixed samples, typically between 5-15%. The final concentration of cells/nuclei post-fixation is also influenced by the resuspension volume. These factors should all be taken into account when determining how much sample input is needed for fixation.

CELL/NUCLEI CONCENTRATIONS		
Kit	Target Post-Fixation Concentration/ μ L	Minimum Post-Thaw Concentration to Fully Load Kit/ μ L
Evercode WT	$\geq 1,000$ cells/nuclei	520 cells/nuclei
Evercode WT Mega	$\geq 3,000$ cells/nuclei	2,126 cells/nuclei

Avoiding RNase Contamination

- Standard precautions should be taken to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique.
- Although RNases are not inactivated by ethanol or isopropanol, they are inactivated by products such as RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific). These can be sprayed on benchtops and pipettes.
- Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Cell Detachment

- If using adherent cell line samples, we recommend TrypLE Express Enzyme (1X), phenol red (Thermo Fisher Scientific). Due to high RNase activity, we do not recommend dissociation with standard trypsin, which may reduce gene and transcript detection.

Plate Strainers

- An example video of using a Plate Strainer can be found on our support site. We recommend watching this video and practicing using Plate Strainers before processing your samples.
- After opening their plastic sleeves, Plate Strainers should be stored in their original plastic sleeve until use and used on the same day they were opened. We do not recommend using Plate Strainers more than one day after the plastic sleeve has been opened.
- Plate Strainers may need to be cut prior to processing samples. Plate Strainers should be cut with sterile scissors, razor blade, or scalpel that have been cleaned with RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific) immediately prior to use.
- Plate Strainers should be thoroughly adhered to 96 well plates prior to use. After being placed by hand, a plate seal applicator should be used to completely seal the strainer over each well by pressing along the outside of the wells. The plate seal applicator should be cleaned with RNaseZap RNase Decontamination Solution (Thermo Fisher Scientific) immediately prior to use. Applicators should never be pressed directly onto the mesh.

Cell/Nuclei Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when first using Evercode Fixation v3 kits.

- When first using Evercode Fixation v3 kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- After fixation, the cells/nuclei are permeabilized and should appear dead with viability stains. If using Acridine Orange/Propidium Iodide (AO/PI) stains, we suggest using the red (PI) channel to count to avoid the impact of any autofluorescence in the green (AO) channel.
- Examples of trypan blue stained fixed cells/nuclei are shown below. High quality fixed samples have single distinct cells/nuclei with <5% cell/nuclei aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed cells/nuclei, it is critical to avoid counting cell/nuclei debris to avoid overestimating the number of cells/nuclei.

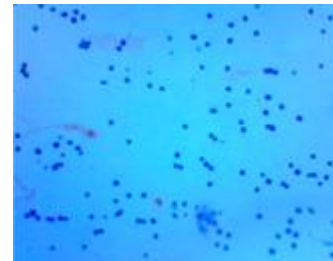
High Quality Sample



Aggregation



Debris



Example of trypan blue stained fixed cells.

Centrifugation

- A range of centrifugation speeds and durations are given in this protocol rather than a single speed. When using Evercode Fixation v3 kits for the first time or when testing a new sample type, we recommend optimizing centrifugation conditions in 1.5 mL tubes before using the plate-based workflows. See the tube-based protocol on our support site, which includes detailed optimization recommendations.
- A swinging bucket rotor should be used for all high-speed centrifugation steps in this protocol. The use of a fixed-angle rotor will lead to substantial cell/nuclei loss.

Reagent Stability

- Reagents in the Cell/Nuclei Fixation Reagents box should not be frozen and thawed more than 3 times.

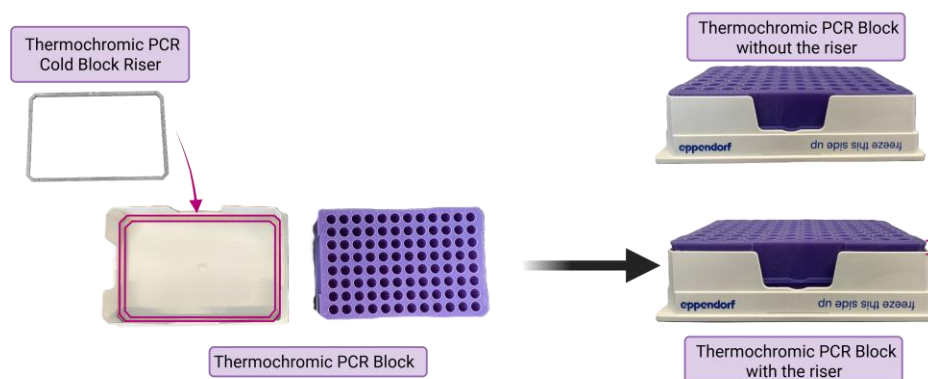
- If the kit is going to be used more than 4 times, the reagents should be aliquoted into nuclease-free 1.5 mL tubes and stored at -20°C until use. We do not recommend making single use aliquots to minimize the impact of evaporation during storage.
- Reagent master mixes should be made fresh and used the same day.

Storage of Fixed Samples

- Fixed samples can be stored at -80°C for up to 6 months. Fixed samples should not be refrozen after thawing.
- When possible, we recommend splitting samples into aliquots after fixation. Aliquots should be at least 20 µL when stored in 0.2 mL PCR tubes or tube strip(s).

Thermochromic PCR Cold Block

- The Thermochromic PCR Cold Block changes color from dark purple to bright pink (too warm) when the block temperature exceeds 7°C. If greater than 30% of the block is bright pink, replace the Thermochromic PCR Cold Block with a fully frozen dark purple block.
- To maintain optimal performance and minimize temperature fluctuations, store the Thermochromic PCR Cold Blocks in a -20°C freezer when not in use. The blocks should be stored upside down to prevent warping.
- The Thermochromic PCR Cold Block Riser needs to be installed in the Thermochromic PCR Cold Block prior to use as illustrated below. The Riser does not need to be removed when freezing.

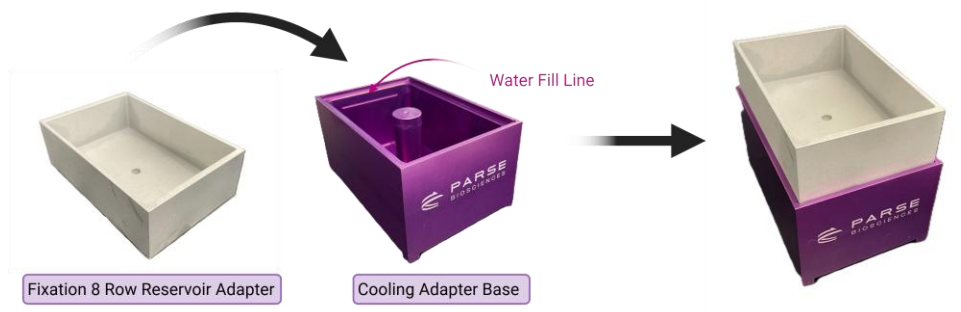


Note: Uneven freezing of the liquid inside the Thermochromic PCR Block can cause slanting of the cooling position. The Thermochromic PCR Cold Block Riser reduces the variability caused by the freezing process. If the PCR Cold Block riser is not used, there will be extra liquid left in the wells of the PCR plate during aspiration steps.

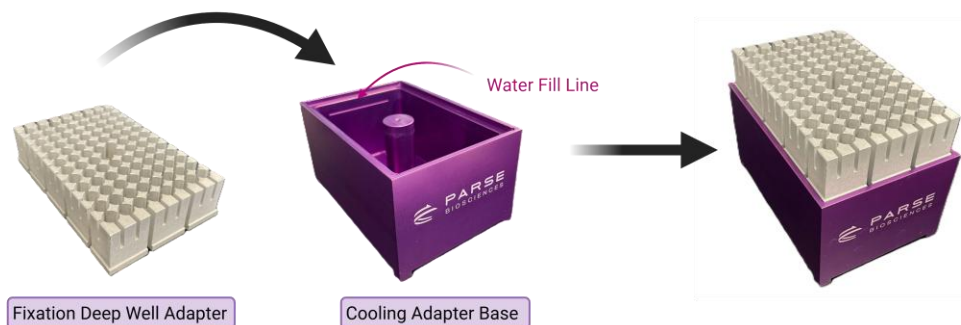
- Tip pinching may occur when using a fully frozen Thermochromic PCR Cold Block. To minimize tip pinching, a fully frozen Thermochromic PCR Cold Block should be warmed slightly by a few degrees by leaving it at room temperature for 10 minutes before using it on the INTEGRA ASSIST PLUS Deck.
- To ensure a secure positioning within the Thermochromic PCR Cold Block, apply firm pressure on the PCR plates until they are securely seated. Avoid touching the top of the open wells.

Cooling Adapter Bases

- To assemble the Cooling Adapter Bases place the Fixation 8 Row Reservoir Adapter or the Fixation Deep Well Adapter on top of the Cooling Adapter Base as shown in the figures below.



Fixation 8 Row Reservoir Adapter with Cooling Adapter Base.



Fixation Deep Well Adapter with Cooling Adapter Base.



Note: If the Cooling Adapter Base is filled with water and frozen before use, ensure that the water level does not exceed the water fill line. The water will expand when frozen and the adapter will not fit properly if the base is overfilled.

- Before use, the Cooling Adapter Base should be filled with water and frozen the night before, then thawed at room temperature for at least 10 minutes prior to use. Alternatively, it can be filled with pebble ice immediately before being placed on the Integra Deck.

INTEGRA ASSIST PLUS Pipetting Programs

- Ensure that the VIALAB program is installed on the same computer that will be used to communicate with the Pipetting Module.
- Ensure that the Evercode workflow script precheck has been confirmed prior to running the INTEGRA + Parse workflow. This can be done by running the [Evercode WT with INTEGRA ASSIST PLUS Precheck Scripts](#) available on the Customer Support Suite.
- Automated pipetting programs are set up and can be selected using the pipette's user interface. In the INTEGRA + Parse workflow, prompts are built into the programs as checkpoints. When the prompt is followed by a double dash, "--", the program will continue. If the prompt does not contain a double dash, "--", it is followed by another prompt.









Deck Loading

- To prevent malfunctions during the procedure, ensure that all unnecessary labware is cleared from the deck before initiating a new program on the INTEGRA ASSIST PLUS.


Cell Fixation Reagents

The Evercode Cell Fixation v3, 12 reactions workflow requires Cell Fixation Reagents and Cell Fixation Enhancer boxes.

Cell Fixation Reagents, 12 reactions. Store at -20°C, PN CF100

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Buffer	CF101	8 mL bottle	1
	Storage Buffer	CF102	2 mL tube	1
	Fixative Solution A	CF103	1.5 mL tube	1
	Fixative Solution B	CF104	1.5 mL tube	1
	Permeabilization Solution	CF105	1.5 mL tube	1
	Fix and Perm Stop Buffer	CF106	8 mL bottle	1
	RNase Inhibitor	CF107	1.5 mL tube	1
	DMSO	CF108	1.5 mL tube	1

Cell Prefixation Enhancer, 12 reactions. Store at 4°C, PN CF400

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	CF201	1.5 mL tube	1

30 μ M Plate Strainer*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 μ M	PS030	Plastic sleeve	1

70 μ M Plate Strainer*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 70 μ M	PS070	Plastic sleeve	1

100 μ M Plate Strainer*. Store at Room Temperature





LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 100 μ M	PS100	Plastic sleeve	1



Note: * Only one mesh size of Plate Strainer is required for the Evercode Cell Fixation v3 workflow. Select an appropriate mesh size for each sample type.


The Evercode Cell Fixation v3, 96 reactions workflow requires Cell Fixation Reagents and Cell Fixation Enhancer boxes. This kit is used for the 48 reactions script as well.

Cell Fixation Reagents, 96 reactions. Store at -20°C, PN CF300

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Buffer	CF301	15 mL bottle	2
	Storage Buffer	CF302	8 mL bottle	2
	Fixative Solution A	CF303	8 mL bottle	2
	Fixative Solution B	CF304	8 mL bottle	2

LABEL	ITEM	PN	FORMAT	QTY
	Permeabilization Solution	CF305	2 mL tube	2
	Fix and Perm Stop Buffer	CF306	15 mL bottle	2
	RNase Inhibitor	CF307	1.5 mL tube	1
	DMSO	CF308	1.5 mL tube	1

Cell Prefixation Enhancer, 96 reactions. Store at 4°C, PN CF400

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	CF401	2 mL tube	1

30 μM Plate Strainer*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 μM	PS030	Plastic sleeve	2

70 μM Plate Strainer*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 70 μM	PS070	Plastic sleeve	2

100 μM Plate Strainer*. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 100 μM	PS100	Plastic sleeve	2



Note: * Only one mesh size of Plate Strainer is required for the Evercode Cell Fixation v3 workflow. Select an appropriate mesh size for each sample type.

Nuclei Fixation Reagents

The Evercode Nuclei Fixation v3, 12 reactions workflow requires Nuclei Fixation Reagents and Nuclei Fixation Enhancer boxes.

Nuclei Fixation Reagents, 12 reactions. Store at -20°C, NF100





LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Buffer	NF101	8 mL bottle	1
	Storage Buffer	NF102	2 mL tube	1
	Fixative Solution	NF103	1.5 mL tube	1
	Permeabilization Solution	NF104	1.5 mL tube	1
	Fix and Perm Stop Buffer	NF105	8 mL bottle	1
	RNase Inhibitor	NF106	1.5 mL tube	1
	DMSO	NF107	1.5 mL tube	1

Nuclei Fixation Enhancer, 12 reactions. Store at 4°C, NF200

LABEL	ITEM	PN	FORMAT	QTY
	Prefixation Enhancer	NF201	1.5 mL tube	1

The Evercode Nuclei Fixation v3, 96 reactions workflow requires Nuclei Fixation Reagents and Nuclei Fixation Enhancer boxes. This kit is used for the 48 reactions script as well.

Nuclei Fixation Reagents, 96 reactions. Store at -20°C, NF300

LABEL	ITEM	PN	FORMAT	QTY
 Prefix	Prefixation Buffer	NF301	15 mL bottle	2
 Storage	Storage Buffer	NF302	8 mL bottle	2
 Fix	Fixative Solution	NF303	8 mL bottle	2
 Perm	Permeabilization Solution	NF304	2 mL tube	2
 Stop	Fix and Perm Stop Buffer	NF305	15 mL bottle	2
 RNase Inhib	RNase Inhibitor	NF306	1.5 mL tube	1
 DMSO	DMSO	NF307	1.5 mL tube	1

Nuclei Fixation Enhancer, 96 reactions. Store at 4°C, NF400

LABEL	ITEM	PN	FORMAT	QTY
 Prefix Enhancer	Prefixation Enhancer	NF401	2 mL tube	1

30 µm Plate Strainer. Store at Room Temperature

LABEL	ITEM	PN	FORMAT	QTY
N/A	Adhesive Plate Strainer, 30 µM	PS030	Plastic sleeve	2

Parse Equipment

The following is a list of equipment provided by Parse Biosciences, required to successfully perform the Evercode workflows on the INTEGRA ASSIST PLUS.

ITEM	PN	QTY
Thermochromic PCR Cold Block	NTAC1102	2
Thermochromic PCR Cold Block Riser	NTAC1103	2
Fixation Deep Well Adapter	NTAC1104	2
Fixation 8 Row Reservoir Adapter	NTAC1105	1
Fixation Cooling Adapter Base	NTAC1106	3

INTEGRA Components

The following are required INTEGRA ASSIST PLUS components needed to run the Evercode Fixation workflow on the INTEGRA ASSIST PLUS.

ITEM	ITEM TYPE	PN	QTY
Pipette Communication Module for VIAFLO / VOYAGER Pipettes	Accessory	4221	1
ASSIST PLUS Base Unit	Main	4505	1
Communication/Charging Cable for VIAFLO	Accessory	4226	1
VIAFLO Pipette 12-Channel, 10-300 μ L	Pipette	4633	1

INTEGRA Consumables

The following is a list of consumables available from INTEGRA, required to successfully perform Evercode Fixation workflow on the INTEGRA ASSIST PLUS.

ITEM	ITEM TYPE	PN	QTY
8 Row Reagent Reservoirs, Partitioned (32 mL/row) with SUREFLO design	INTEGRA	6373	4
10-300 μ L pipette tips (Sterile/Filter/Low Retention)	INTEGRA	6535	1

Consumables

The following equipment and consumables are required to perform the protocol, but are not provided by INTEGRA or by Parse Biosciences. Note that this list does not include standard laboratory equipment, such as freezers.

Consumables

ITEM	SUPPLIER	PN	NOTES
Semi skirted plates	Thermo Fisher Scientific®	E951020362	Two needed
1 mL Deepwell Plate	Eppendorf	951033308	Two needed
SealPlate®	Excel Scientific®	100-SEAL-PLT	Or equivalent PCR plate seals. Note that many clear plastic seals are not designed for storage at -80°C, so we recommend using foil plate seals if storing fixed samples in PCR plates.
TempPlate® EXT Sealing Foil	USA Scientific®	2998-0100	(Optional) If storing fixed samples in a PCR plate. Note that many clear plastic seals are not designed for storage at -80°C.
Protein LoBind® Plate	Eppendorf®	951033308 (1 mL) 0030504305 (2 mL)	Or equivalent polypropylene, nuclease-free, v-bottom, 1 mL or 2 mL deep well plates. Do not substitute polystyrene plates as it will lead to substantial cell loss. If possible, we recommend using protein low-binding plates.
Reagent basins	Various Suppliers	Varies	(Optional) If blocking deep well plates with BSA. Sterile, nuclease-free reagent basins.
Eppendorf twin.tec® PCR Plate 96 LoBind®	Eppendorf	0030129504	Or equivalent DNA low-binding, nuclease-free PCR plate or 0.2 mL tube strips.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097 (15 mL)	Or equivalent polypropylene centrifuge tubes.

ITEM	SUPPLIER	PN	NOTES
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.

Equipment

ITEM	SUPPLIER	PN	NOTES
Centrifuge with Swinging Bucket Rotor	Various Suppliers	Varies	Compatible with 96 deep well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
1-channel Pipette: P20, P200, P1000	Various Suppliers	Varies	
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting devices. We recommend validating alternatives relative to a hemocytometer.
Styrofoam Cooler	Various Suppliers	Varies	(Optional) If storing fixed samples before processing with an Evercode Whole Transcriptome kit.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.

Reagents

ITEM	SUPPLIER	PN	NOTES
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific		Or equivalent RNase decontamination solution.
Gibco™ Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific		(Optional) If blocking tubes with BSA. Chosen due to its low RNase activity. Contact applications support for alternatives.
TrypLE™ Express OR TrypLE Select	Thermo Fisher Scientific	12605010 OR 12563011	(Optional) If using adherent cells. Trypsin is not recommended due to variable RNase levels.

Reading and Understanding Visuals and Diagrams

Understanding the figures in this user manual is essential for accurately interpreting the information presented.

While the figures are different, they share consistent formats, use standardized visual elements and follow common conventions.

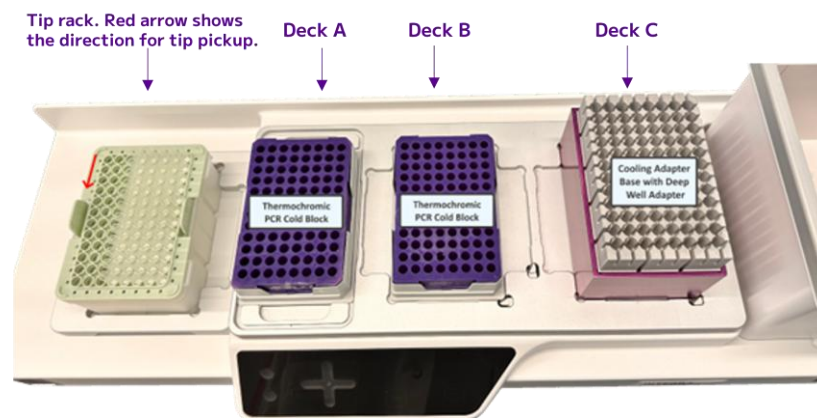
There are three types of figures in this manual.

- Photos of the hardware configurations, including tip rack, and Decks A, B, and C.
- Visual representation of Decks A, B, and C where consumable labware, reagents, and samples are placed.
- Diagrams of the pipette illustrating the steps to select the appropriate program.

We recommend reviewing this section and the illustrations throughout the manual to familiarize yourself with the visual language used.

Understanding the Hardware Configurations

The hardware configurations appear at the beginning of each subsection and show how to set up the INTEGRA platform to complete the associated steps. This photo displays the tip racks, Deck A, Deck B, and Deck C. Red arrows indicate the order in which tips are picked up by the INTEGRA platform, and required hardware is highlighted with text boxes.

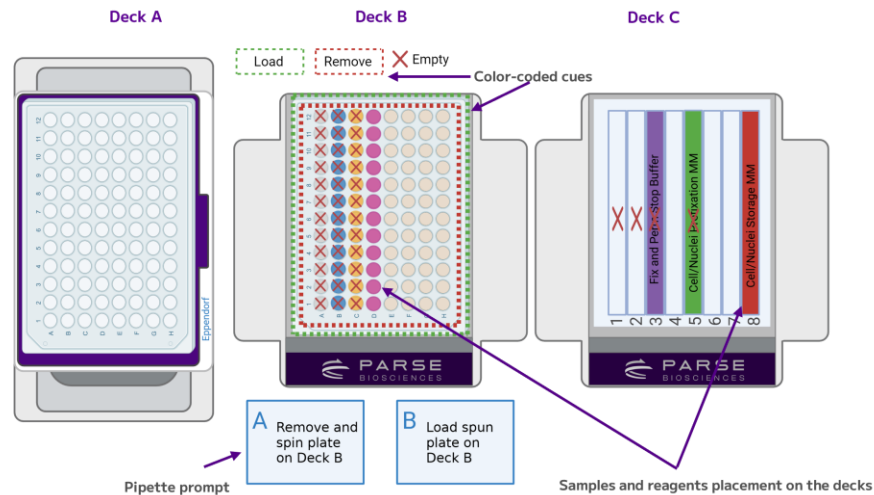


Blocks and Bases are hardware components that will be placed or removed as needed throughout the workflow.

Reading and Understanding the Deck Configurations

Each subsection includes multiple deck configurations that illustrate the steps executed by the program. These configurations display the exact placement of reagents and samples on the decks, list the consumables required at each step, and highlight pipette prompts for manual

intervention. Color-coded cues guide the user through actions such as loading, moving, or removing hardware, reagents, and consumables, while also indicating the specific deck locations where these actions should occur.



Color-Coded Cues

This workflow is semi-automated and still requires user interaction and input. Prompts will appear on the pipette display, instructing the user to perform tasks such as loading, moving, removing, or replacing hardware, reagents, and consumables, as well as mixing and pipetting specific reagents. In this user guide, each prompt is color-coded and shown in the deck configurations illustrating the required actions. Additional explanatory text accompanies each figure to provide further detail on the steps involved.



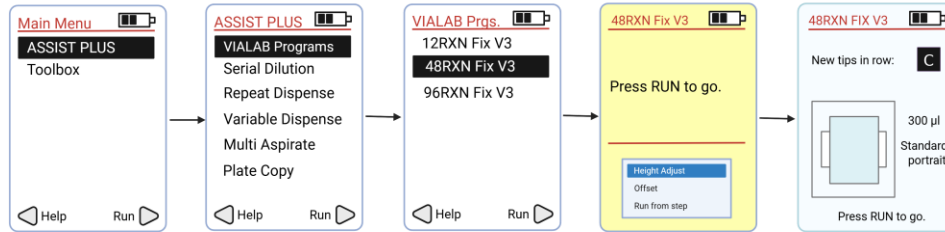
- **Load:** Indicates labware and/or reagents that need be placed or reloaded onto the deck.
- **Remove:** Identifies labware that should be taken off the deck, either for incubation, disposal, or because it is not needed in the upcoming step.
- **Replace:** Highlights labware that has been used and needs to be swapped out for clean or new consumables.
- **Move:** Indicates labware that should be relocated between deck positions.
- **X Emptied:** Reminds that the content has been used and the vessel is now empty.



Note: The position of the color-coded cues on top of the figures does not necessarily correspond to the location where the action should be taken.

Pipette Programs Diagram

At various points in the procedure, the user will need to switch pipettes and tip racks. In each subsection, the pipette is pre-programmed to carry out specific tasks defined by a designated script. The correct program must be loaded onto the pipette before beginning each set of steps. A diagram showing the path to load the appropriate program is provided in every subsection.



Section 1: Set Up

Section 1.1. Block Plates with BSA

Although not required, blocking plates with BSA can increase cell retention. When Protein LoBind plates are not available, we recommend blocking plates, especially for samples with low cell inputs or cells prone to aggregation.

To block plates:

1. Prepare a fresh 1% BSA as follows, depending on the number of samples being processed.

1% BSA			
Number of Samples	12	48	96
Nuclease-free water (not supplied)	21.8 mL	87 mL	175 mL
Gibco Bovine Albumin Fraction V (7.5% solution) (not supplied)	3.4 mL	13 mL	27 mL
Total Volume	25.2 mL	100 mL	202 mL

2. Fill a new basin with 1% BSA, refilling as needed in step 3.
3. For each sample, add **1 mL** of 1% BSA to a well of a polypropylene, nuclease-free, v-bottom, 1 mL or 2 mL Deepwell Plate.
4. Repeat step 3 with a second Deepwell Plate.
5. Add new plate seals and invert once to fully coat the wells.
6. Incubate for **30 minutes** at room temperature.
7. Remove the plate seals. Decant and discard the 1% BSA.
8. Add new plate seals.
9. Centrifuge the plates for **1 minute** at 100 x g at room temperature.
10. Remove the plates from the centrifuge and remove the plate seals.
11. Remove any remaining solution from the bottom of the wells with a multichannel P200.

12. Without sealing the plates, air dry for **30 minutes** in a biosafety cabinet at room temperature.
13. Proceed to Section 1.2 or store sealed BSA-coated plates at 4°C for up to 4 weeks.

Section 1.2. Prepare Master Mixes for Cell Fixation

Cell fixation master mixes should be prepared just prior to fixation.

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY (12 or 48/96)	HANDLING AND STORAGE
8 Row Reservoir	INTEGRA-Provided	1	Individually wrapped consumable
1 mL 96 Deepwell Plate	Eppendorf	1	
Fixation Cooling Adapter Base	Parse-Provided	1	
Fixation 8 Row Reservoir Adapter	Parse-Provided	1	
○ Prefixation Buffer	Cell Fixation Reagents (-20°C)	8 mL or 15 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
● or ○ Storage Buffer	Cell Fixation Reagents (-20°C)	2 mL tube or 8 mL bottle	
● or ○ Fixative Solution A	Cell Fixation Reagents (-20°C)	1.5 mL tube or 8 mL bottle	
● or ○ Fixative Solution B	Cell Fixation Reagents (-20°C)	1.5 mL tube or 8 mL bottle	
● Permeabilization Solution	Cell Fixation Reagents (-20°C)	1.5 mL or 2 mL tube	
○ Fix and Perm Stop Buffer	Cell Fixation Reagents (-20°C)	8 mL or 15 mL bottle	
● DMSO	Cell Fixation Reagents (-20°C)	1.5 mL tube	
● RNase Inhibitor	Cell Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Cell Fixation Reagents (4°C)	1.5 mL or 2 mL tube	

2. Prepare the Cell Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and pipette into **row 5** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

CELL PREFIXATION MASTERMIX			
Number of Samples	12	48	96
○ Prefixation Buffer	2.67 mL	10.7 mL	21.3 mL
● RNase Inhibitor	36 µL	144 µL	288 µL
● Prefixation Enhancer	180 µL	720 µL	1.4 mL
Total Volume	2.9 mL	11.5 mL	23 mL

3. Prepare the Cell Fixative Master Mix in a new tube as follows. Mix thoroughly by pipetting and pipette into **row 6** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice. For the 12 Reactions Fixation only: add **85 µL** of Cell Fixative Master Mix into each well of Row C of the sample 96 Deepwell Plate.

CELL FIXATIVE MASTERMIX			
Number of Samples	12	48	96
● or ○ Fixative Solution A	526 µL	2.4 mL	4.8 mL
● or ○ Fixative Solution B	526 µL	2.4 mL	4.8 mL
Total Volume	1.05 mL	4.8 mL	9.6 mL

4. Prepare the Cell Storage Master Mix in a new tube as follows. Mix thoroughly by pipetting and pipette into **row 8** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

CELL STORAGE MASTERMIX			
Number of Samples	12	48	96
● or ○ Storage Buffer	1.732 mL	6.3 mL	12.6 mL
● RNase Inhibitor	23.1 µL	84 µL	168 µL
● DMSO	92.4 µL	336 µL	672 µL
Total Volume	1.848 mL	6.72 mL	13.44 mL

- Dispense all of the ● Permeabilization Solution into **row 7** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice. For the 12 Reactions Fixation only: add **45 μ L** of ● Permeabilization Solution into each well of Row B of the sample 96 Deepwell Plate.

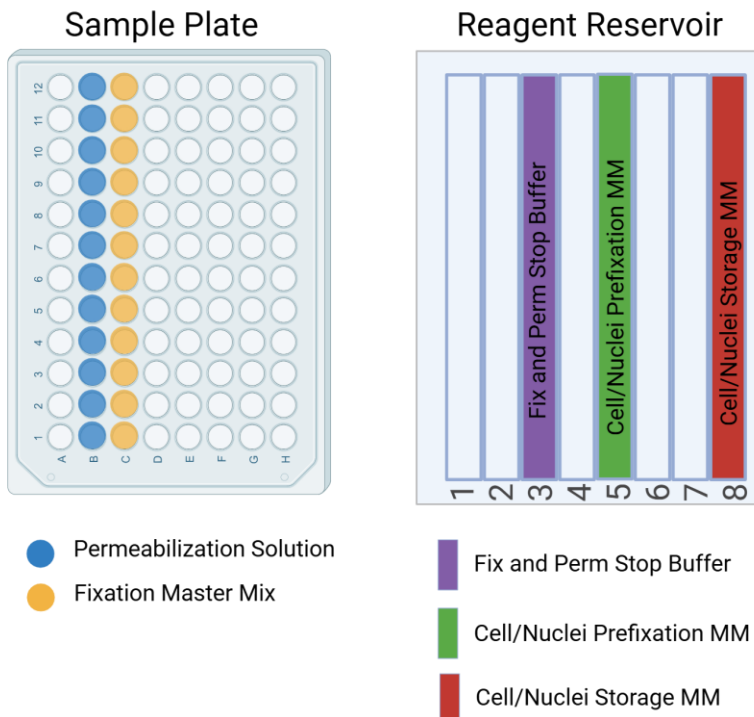
PERMEABILIZATION SOLUTION			
Number of Samples	12	48	96
● Permeabilization Solution	~550 μ L	~2 mL	~4 mL

- Dispense all of the ○ Fix and Perm Stop Buffer into **row 3** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

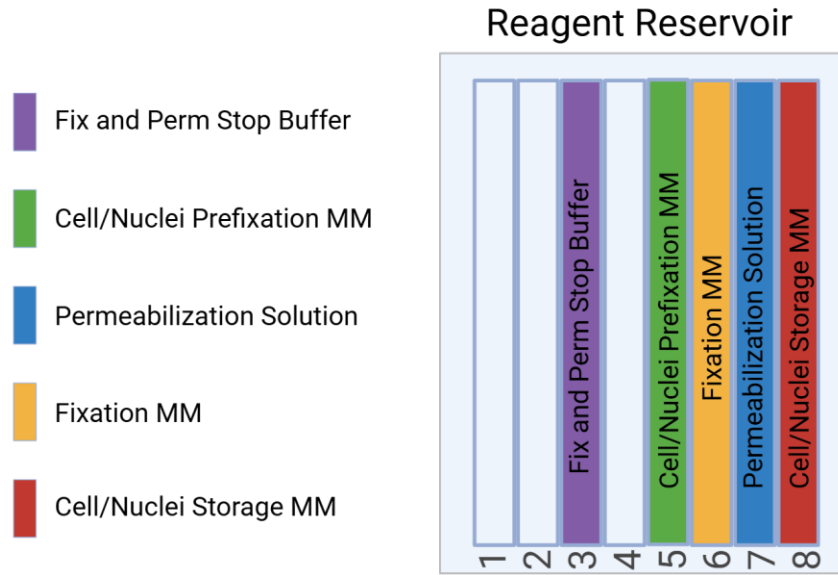
FIX AND PERM STOP BUFFER			
Number of Samples	12	48	96
○ Fix and Perm Stop Buffer	~3.5 mL	~14 mL	~28 mL

- The 8-Row Reservoir and the deep-well plate configured for 12 reactions should correspond to the figures below.

For 12 Cell Fixation Reactions



For 48/96 Cell Fixation Reactions



CRITICAL! Ensure that the liquid is distributed across the bottom of the reservoir.

Section 1.3. Prepare Master Mixes for Nuclei Fixation

Nuclei fixation master mixes should be prepared just prior to fixation.

1. Fill a bucket with ice. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY (12 or 48/96)	HANDLING AND STORAGE
8 Row Reservoir	INTEGRA-Provided	1	Individually wrapped consumable
1 mL 96 Deepwell plate	Eppendorf	1	
Fixation Cooling Adapter Base	Parse	1	
Fixation 8 Row Reservoir Adapter	Parse	1	
○ Prefixation Buffer	Nuclei Fixation Reagents (-20°C)	8 mL or 15 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
● or ○ Storage Buffer	Nuclei Fixation Reagents (-20°C)	2 mL tube or 8 mL bottle	
● or ○ Fixative Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL tube or 8 mL bottle	
● Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1.5 mL or 2 mL tube	
○ Fix and Perm Stop Buffer	Nuclei Fixation Reagents (-20°C)	8 mL or 15 mL bottle	
● DMSO	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Thaw and store at room temperature. Mix by inverting the tube.
● RNase Inhibitor	Nuclei Fixation Reagents (-20°C)	1.5 mL tube	Store on ice immediately before use. Do not vortex.
● Prefixation Enhancer	Nuclei Fixation Reagents (4°C)	1.5 mL or 2 mL tube	

- Prepare the Nuclei Prefixation Master Mix in a new tube as follows. Mix thoroughly by pipetting and pipette into **row 5** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

NUCLEI PREFIXATION MASTER MIX			
Number of Samples	12	48	96
○ Prefixation Buffer	2.53 mL	10.7 mL	21.3 mL
● RNase Inhibitor	34.79 μ L	144 μ L	288 μ L
● Prefixation Buffer	173.94 μ L	720 μ L	1.4 mL
Total Volume	2.7 mL	11.5 mL	23 mL

- Dispense all of the ●/○ Fixative Solution Nuclei Fixative Solution into **row 6** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice. For the 12 Reactions Fixation only: add **80 μ L** of Nuclei Fixative Solution into each well of Row C of the sample 96 Deepwell Plate.

NUCLEI FIXATIVE SOLUTION			
Number of Samples	12	48	96
● or ○ Fixative Solution	~1 mL	~5.2 mL	~10.4 mL

- Prepare the Nuclei Storage Master Mix in a new tube as follows. Mix thoroughly by pipetting and pipette into **row 8** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

NUCLEI STORAGE MASTER MIX			
Number of Samples	12	48	96
● or ○ Storage Buffer	1.732 mL	6.3 mL	12.6 mL
● RNase Inhibitor	23.1 μ L	84 μ L	168 μ L
● DMSO	92.4 μ L	336 μ L	672 μ L
Total Volume	1.848 mL	6.72 mL	13.44 mL

- Dispense all of the ● Permeabilization Solution into **row 7** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice. For the 12 Reactions Fixation only: add **45 μL** of ● Permeabilization Solution into each well of Row B of the sample 96 Deepwell Plate.

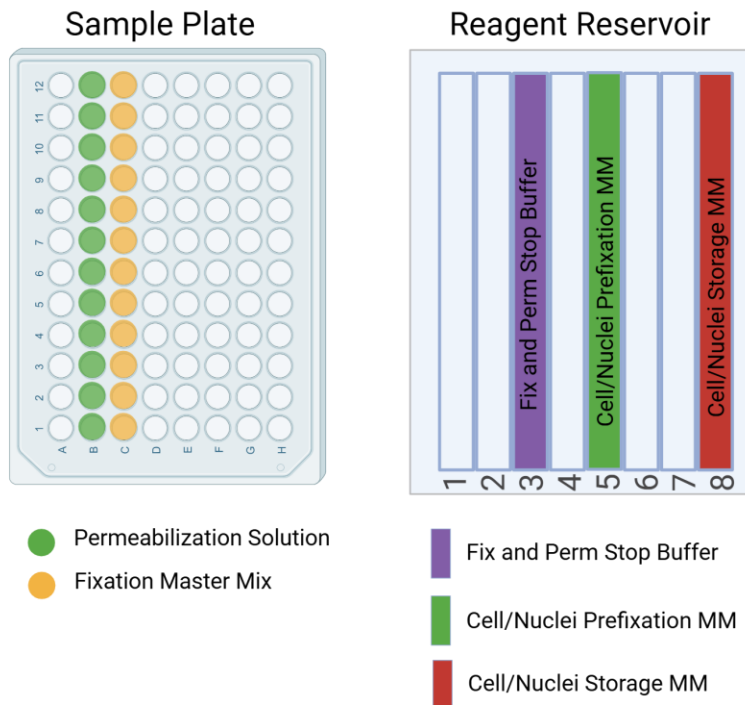
PERMEABILIZATION SOLUTION			
Number of Samples	12	48	96
● Permeabilization Solution	~ 550 μL	~2 mL	~4 mL

- Dispense all of the ○ Fix and Perm Stop Buffer into **row 3** of the 8 Row Reservoir. Store the 8 Row Reservoir on ice.

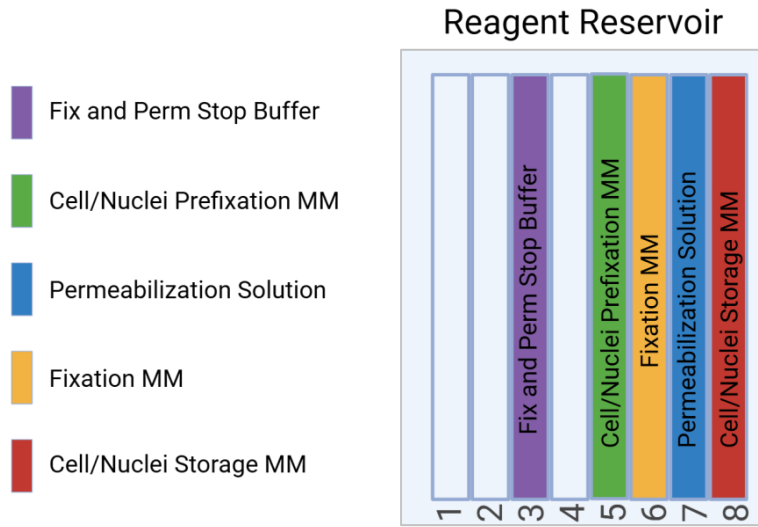
FIX AND PERM STOP BUFFER			
Number of Samples	12	48	96
○ Fix and Perm Stop Buffer	~3.5 mL	~14 mL	~28 mL

- The 8-Row Reservoir and the deep-well plate configured for 12 reactions should correspond to the figures below.

For 12 Reactions Nuclei Fixation



For 48/96 Reactions Nuclei Fixation



CRITICAL! Ensure that the liquid is distributed across the bottom of the reservoir.

Section 2: Fixation

Section 2.1. 12 Reactions Fixation

This section outlines the protocol for processing 12 samples. The procedure takes approximately 20 minutes to complete, with an additional 10 minutes required for centrifugation.

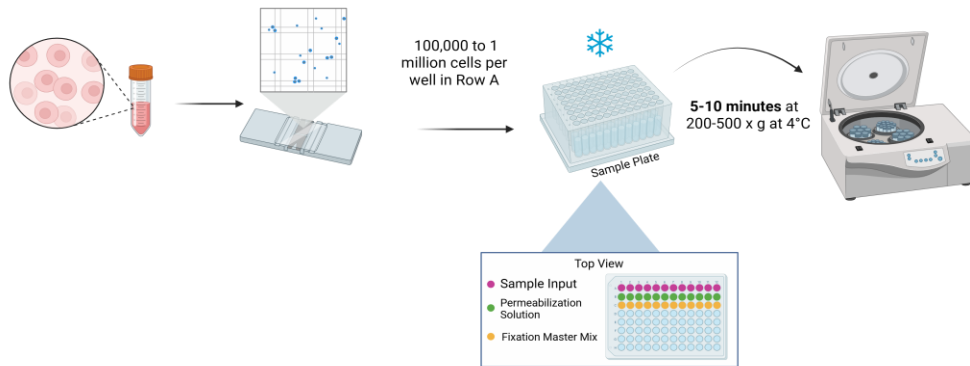
To fix samples:

1. Gather the following components:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 10-300 µL	INTEGRA Component	1	
Tip Deck for VIAFLO Pipetting Module	INTEGRA Component	1	
10-300 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	1	Pull the Freezer Block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	1	
Fixation Deep Well Adapter	Parse	1	
Fixation 8 Row Reservoir Adapter	Parse	1	
Fixation Cooling Adapter Base	Parse	2	

2. Cool the centrifuge with a swinging bucket rotor to 4°C.
3. Fill a bucket with ice.

4. Prepare a cell sample input:



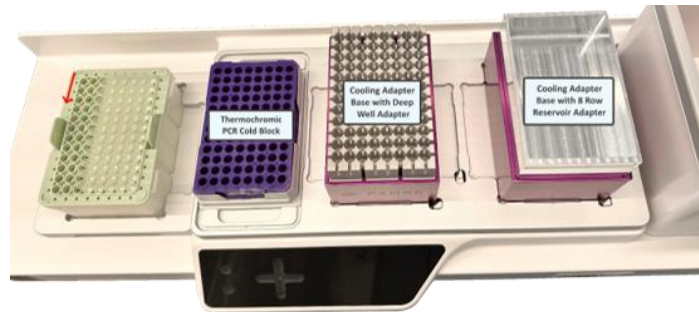
- Prepare a hemocytometer, flow cytometer, or other cell counting device.
- Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Keep cells on ice during counting and work quickly to minimize time on ice prior to fixation.
- With the plate on ice, transfer 100,000 to 1 million cells from each sample into the wells of a 1 mL Deepwell Plate (or BSA-coated deep well plate if prepared in Section 1.1). All samples should be in Row A. Maximum sample volume per well is 1 mL.
- Add a new plate seal on the deep well plate with the cell suspension.
- Centrifuge the plate in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Proceed to INTEGRA deck preparation during centrifugation.

CRITICAL! Using a fixed-angle rotor in this protocol will lead to substantial cell loss.



CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See the Important Guidelines section for details.

5. Prepare the cooling hardware:



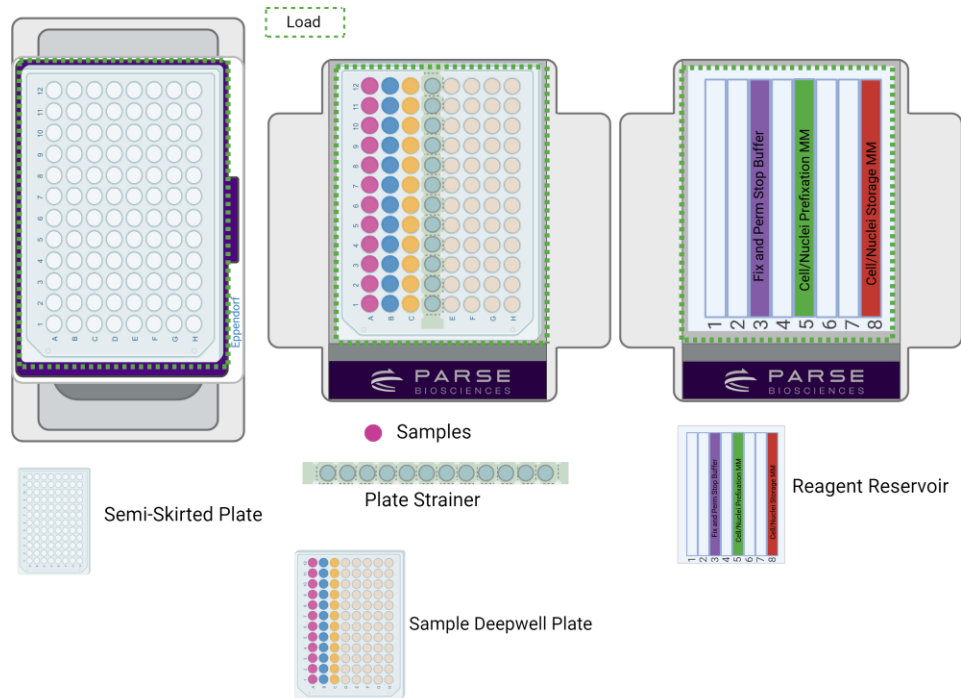
- a. Place a ThermoChromic PCR Cold Block with Riser on Deck A.
- b. Assemble and fill two of the Cooling Adapter Bases with pebble ice and place one on Deck B and Deck C. If the Cooling Adapter Bases were filled with water and frozen the night before, ensure that the Cooling Adapter Base is thawed at room temperature for at least **10 minutes** before use.



CRITICAL! If using the pebble ice filled Cooling Adapter Bases for more than 45 minutes, refill the cooling base with more pebble ice at the 40 minute mark. If the Cooling Adapter Bases were frozen overnight, refill the cooling base with pebble ice at the 90 minute mark.

- b. Place the Fixation Deep Well Adapter on the Fixation Cooling Adapter Base on Deck B.
- c. Place the Fixation 8 Row Reservoir Cooling Adapter on the Cooling Adapter Base on Deck C.

6. Set up the deck following the Deck Configuration below.



- Place a PCR semi-skirted plate on Deck A.
- Place the 8 Row Reservoir with Reagents prepared Section 1 on the Fixation 8 Row Reservoir Adapter on Deck C with **row 1** on the left.
- Once the centrifugation is complete, place the 1mL Deepwell Plate containing the sample on top of the Fixation Deep Well Plate Adapter on Deck B and remove the plate seal.



CRITICAL! Move quickly and handle the sample gently to avoid dislodging the pellet, which will impact data quality.

- d. Cut an individual row of the plate strainer. Adhere one row of the plate strainer on Row D of the Deepwell Plate (Figure 3).



CRITICAL! Ensure the plate strainer is properly aligned to the top of your wells. The pipette's tips are positioned near the top of the well during cell straining to minimize the risk of introducing strain-related errors.

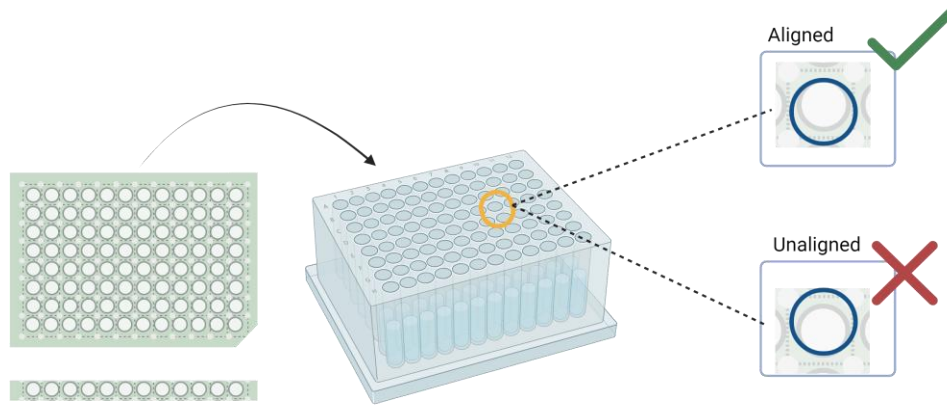
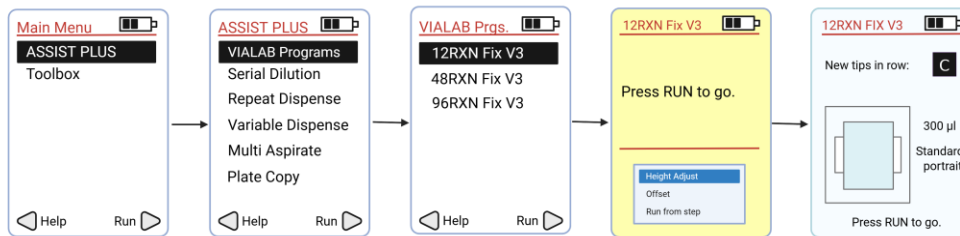


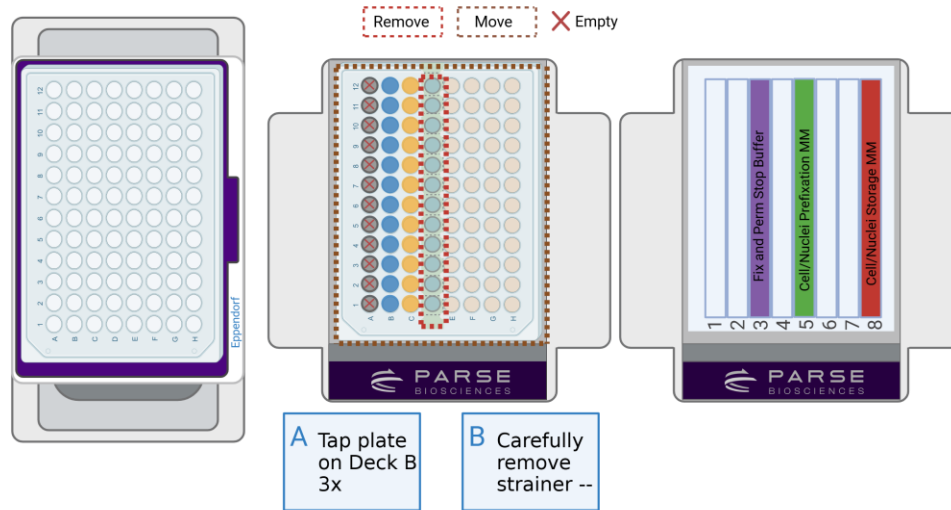
Figure 3: The tops of the strainer and the well openings line up to avoid sample loss.

- 8. Attach VIAFLO Pipetting Module 12-Ch, 10-300 μ L and the corresponding Tip Deck.
- 9. Run the program **12RXN Fix V3_5** following the diagram below.



Note: During the supernatant removal from the 1 mL Deepwell plate containing the samples, the pipette tip touch should not touch the sides of the 8 row reservoir. The tip touch motion is intended to remove large droplets on the outside of the pipette tip.

10. Press "Run" to continue the program. Follow the program prompts for manual intervention:



- a. Tap the plate on Deck B firmly 3x on the deck to move the liquid to the bottom of the wells.
- b. Carefully remove the strainer on row D of the deep well plate on Deck B.

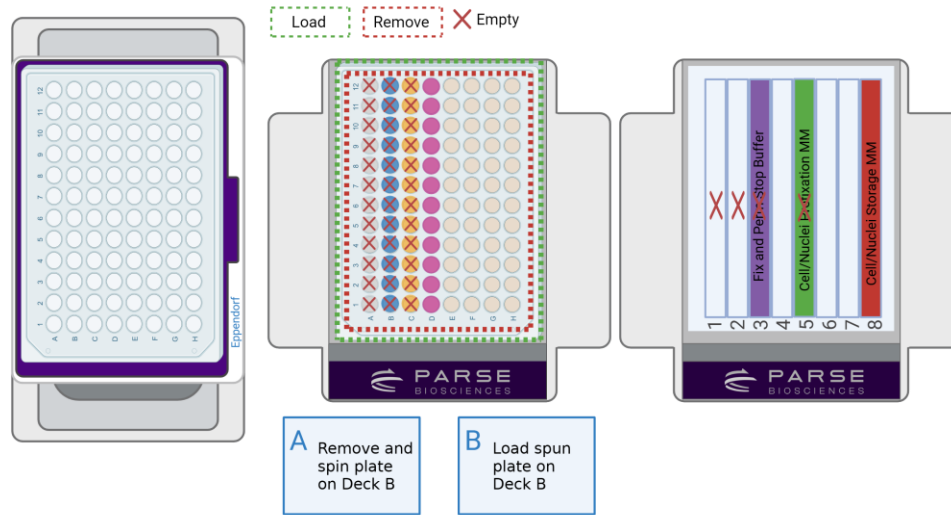


Note: Some volume will be removed from the top of the strainer to reduce the chance of contamination during strainer removal.



CRITICAL! Remove the strainer with one swift motion to reduce the chances of contamination.

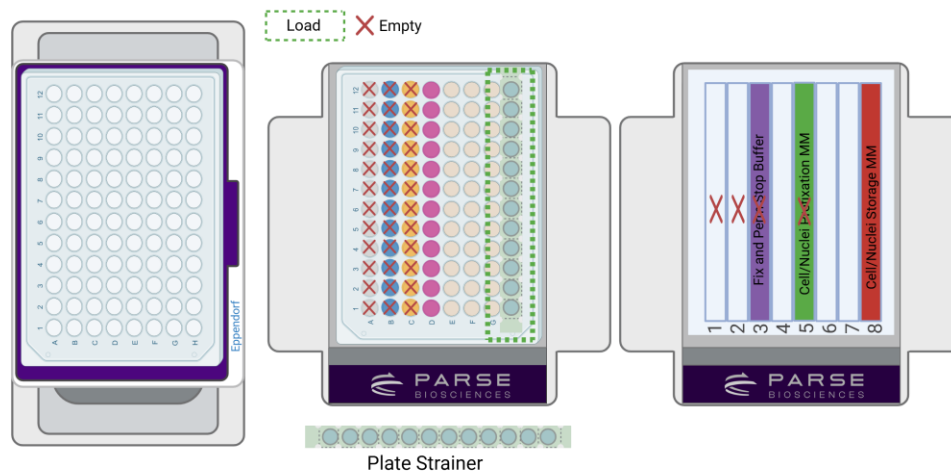
11. Press "Run" to continue the program. Follow the program prompts for manual intervention:



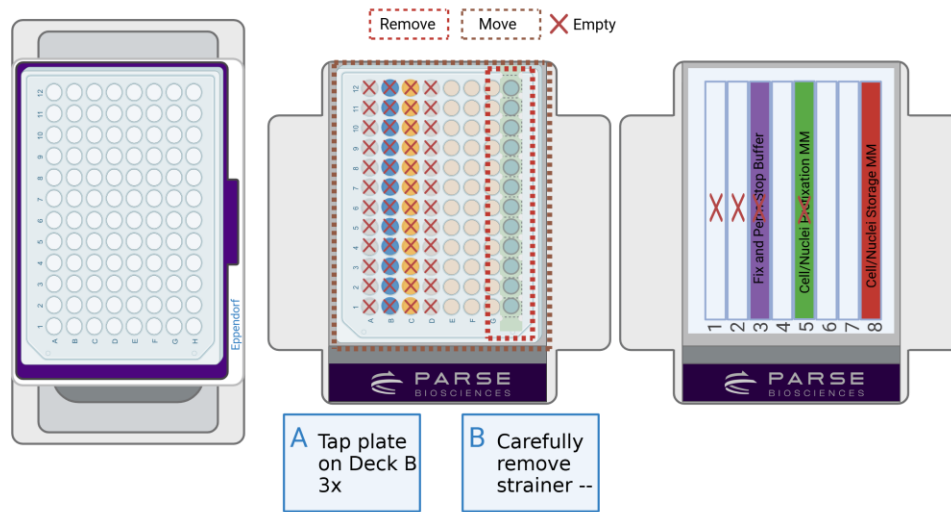
1. Seal and centrifuge the plate on Deck B in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.
 - a. When the centrifugation is complete, place the 1 mL Deepwell Plate on top of the Fixation Deepwell Adapter on Deck B and remove the plate seal, being careful not to disturb the cell pellet.
 - b. Add a plate strainer on Row H as shown in the configuration below.



Note: The strainer can be applied off deck for a more accurate placement.



12. Press "Run" to continue the program. Follow the program prompts for manual intervention:

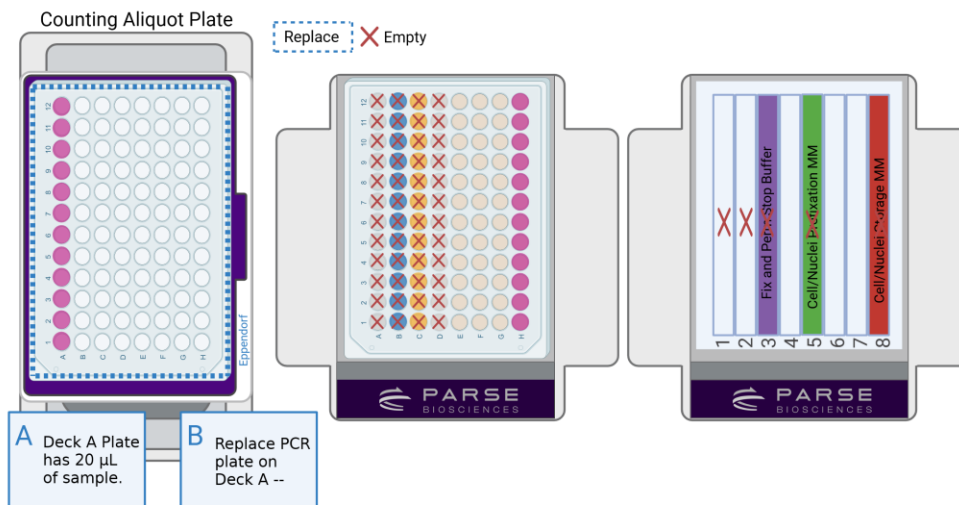


- a. Tap the plate on Deck A firmly 3x on the deck to move liquid to the bottom of the wells.
- b. Carefully peel off the plate strainer and discard it.

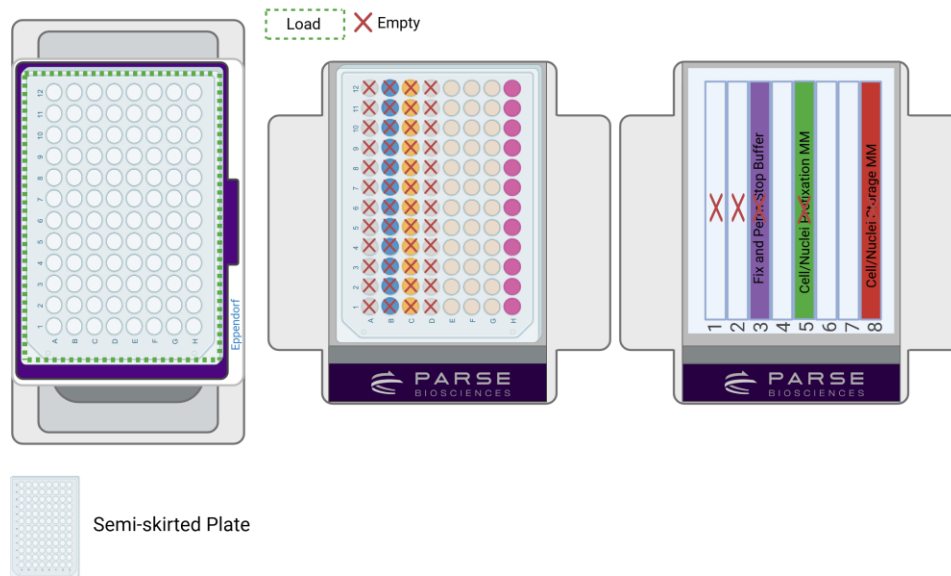


CRITICAL! See guidelines "Storage of Fixed Samples" if you prefer to skip the following steps and abort the program. Proceed directly to step 15.

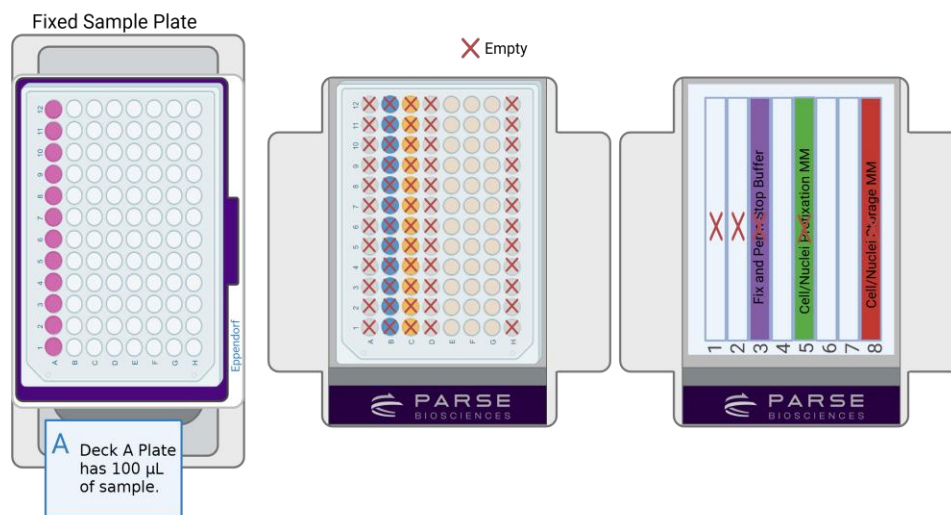
CRITICAL! Remove the strainer with one swift motion to reduce chances of contamination.



13. **When prompted**, replace the Eppendorf semi-skirted plate on deck A with a new Eppendorf semi-skirted plate on the Thermochromic PCR Cold Block on Deck A. The plate that was removed contains a counting aliquot. Store the plate on ice. The deck layout should correspond to the configuration below. Press “Run” to continue the program.



Note: These aliquots can be thawed and counted separately from the remaining sample for downstream processing with Evercode kits.



14. After the program is complete, seal the semi-skirted plates with the aluminum plate seal that can withstand storage at -80°C or transfer the sample to 0.2 mL tube strip(s). The

first semi-skirted plate on Deck A contains a 20 μ L counting aliquot. The second semi-skirted plate on Deck A contains the fixed cell suspension mix.



Note: If proceeding from step 12, and aborted the program before transfer into the semi-skirted plates, seal the 1 mL deep well plate containing the samples or transfer the samples into a container with a seal that can withstand storage at -80°C .



CRITICAL! Many clear plastic seals are not designed for storage at -80°C , so we recommend using 96 Well Aluminum Plate Seal if storing the samples in PCR plates.

15. Place the samples in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.



Safe stopping point: samples are stable for up to 6 months at -80°C .

16. The day before running the downstream Evercode Whole Transcriptome kit, thaw the 20 μ L aliquots in the semi-skirted plate in a water bath set to 37°C . Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Discard any remaining sample from the counting aliquot.

Section 2.2. 48/96 Reactions Fixation

This section outlines the protocol for processing 48 and/or 96 samples. When processing 48 samples the procedure takes approximately 45 minutes to complete, with an additional 10 minutes required for centrifugation. If processing 96 samples the procedure requires about 80 minutes plus 10 minutes for centrifugation.

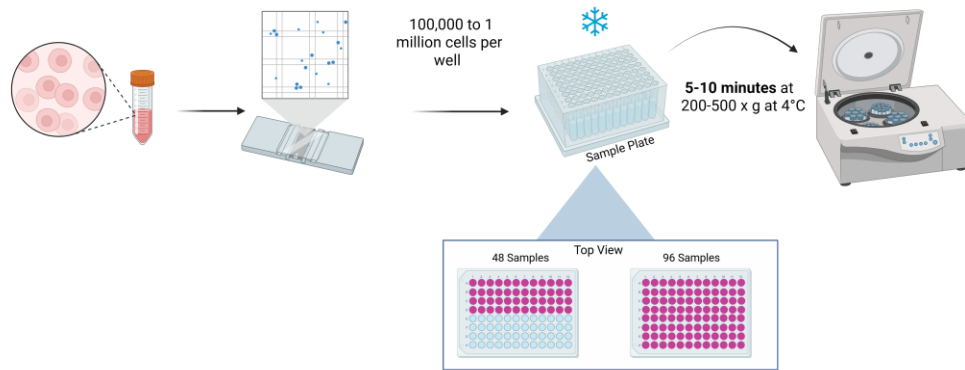
To fix samples:

1. Gather the following components:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 10-300 μ L	INTEGRA Component	1	
Tip Deck for VIAFLO Pipetting Module	INTEGRA Component	1	
10-300 μ L Tip Rack	INTEGRA-Provided	1	
Thermochromic PCR Cold Block	Parse-Provided	2	Pull the Freezer Block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	2	
Fixation Deep Well Adapter	Parse-Provided	2	
Fixation 8 Row Reservoir Adapter	Parse-Provided	1	
Fixation Cooling Adapter Base	Parse-Provided	3	

2. Cool the centrifuge with a swinging bucket rotor to 4°C.
3. Fill a bucket with ice.

4. Prepare sample input:



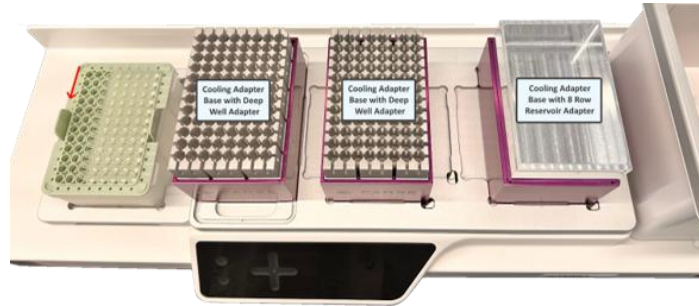
- a. Prepare a hemocytometer, flow cytometer, or other cell/nuclei counting device.
- b. Count the cells/nuclei in the single cell suspension with a hemocytometer or alternative counting device and record the count. Keep cells/nuclei on ice during counting and work quickly to minimize time on ice prior to fixation.
- c. With the plate on ice, transfer 100,000 to 1 million cells/nuclei from each sample into the wells of a 1 mL deep well plate (or BSA-coated deep well plate if prepared in Section 1.1). Maximum sample volume per well is 1 mL.
 - i. Row A - Row D for 48 Samples
 - ii. Row A - Row H for 96 Samples
- d. Place a new plate seal on the deep well plate containing the cell suspension.
- e. Centrifuge the plate in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Proceed to Integra deck preparation during centrifugation.

CRITICAL! Using a fixed-angle rotor in this protocol will lead to substantial cell loss.



CRITICAL! Ideal centrifugation speed and duration should be determined for each sample type to optimize retention and resuspension efficiencies. See the Important Guidelines section for details.

5. Prepare the cooling hardware:



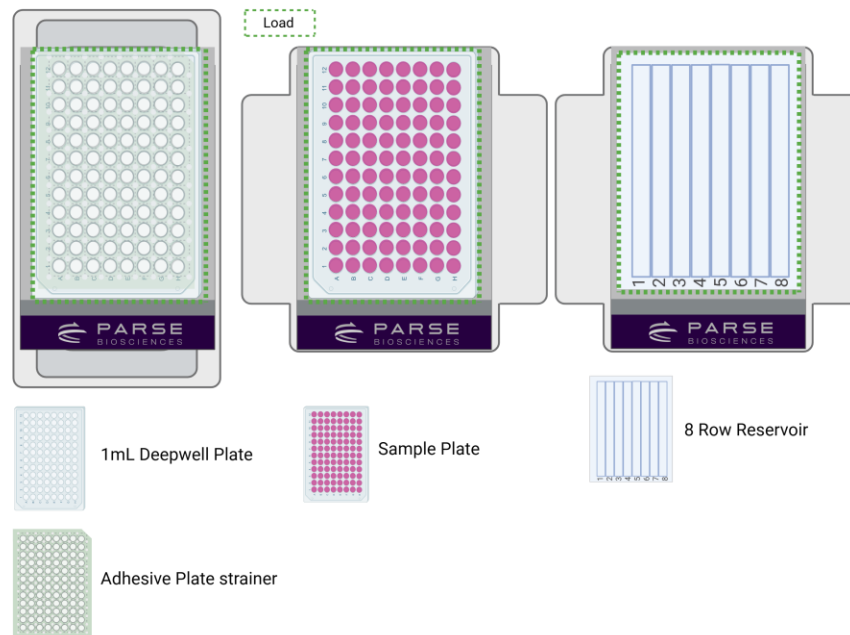
- a. Assemble and fill three of the Cooling Adapter Bases with pebble ice and place one on Deck A, Deck B, and Deck C. If the Cooling Adapter Bases were filled with water and frozen the night before, ensure that the Cooling Adapter Base is thawed at room temperature for at least **10 minutes** before use.



CRITICAL! If using the pebble ice filled Cooling Adapter Bases for more than 45 minutes, refill the cooling base with more pebble ice at the 40 minute mark. If the cooling adapters were frozen overnight, refill them with pebble ice at the 90 minute mark.

- b. Place the Fixation Deep Well Adapter on the Cooling Adapter Bases on Deck A and Deck B.
- c. Place the Fixation 8 Row Reservoir Adapter on the Cooling Adapter Base on Deck C.

6. Set up the deck following the Deck Configuration below.



- a. Place a **new** 8 Row Reservoir on the Fixation 8 Row Reservoir Adapter on Deck C.
- b. Put a plate strainer on a **clean** 1 mL deep well plate and place it on top of the Fixation Deep Well Adapter on Deck A with A1 on the bottom left corner (Figure 4). If processing 48 samples, cut the strainer to fit the 48-reaction workflow.
 - i. Row A - Row D for 48 Samples
 - ii. Row A - Row H for 96 Samples



CRITICAL! Ensure the plate strainer is properly aligned to the top of your wells. The pipette's tips are positioned near the top of the well during cell straining to minimize the risk of introducing strain-related errors.

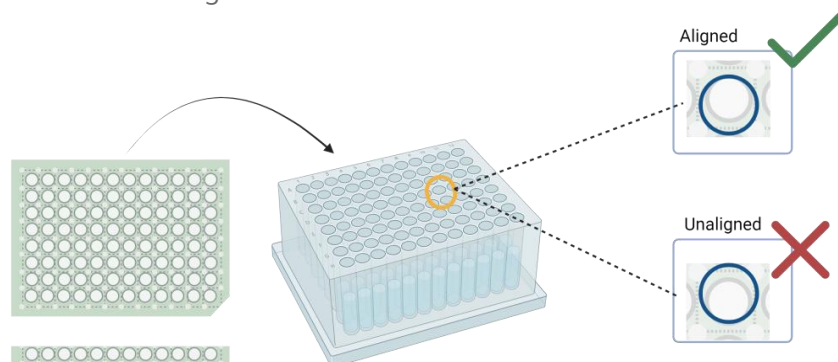


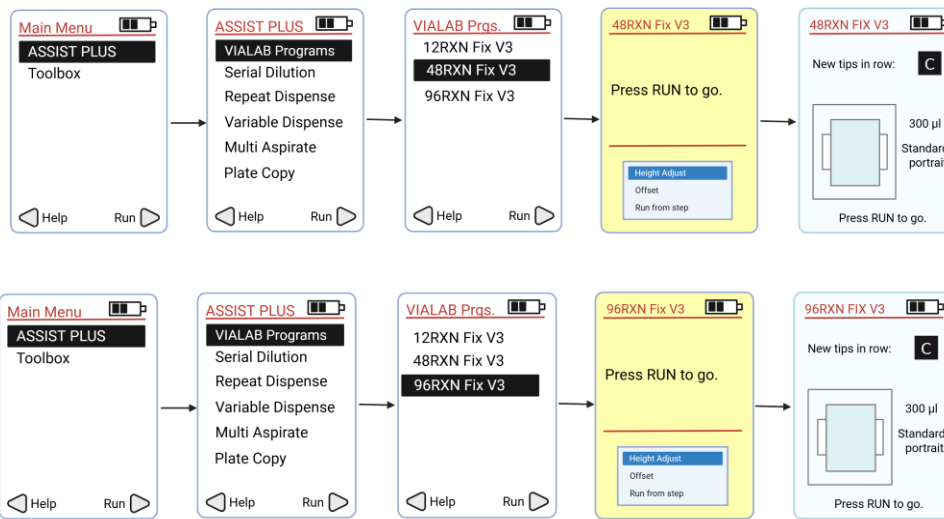
Figure 4: The tops of the strainer and the well openings line up to avoid sample loss.

- b. Once the centrifugation is complete, place the 1 mL deep well plate containing the samples on top of the Fixation Deep Well Adapter on Deck B, and remove the plate seal.



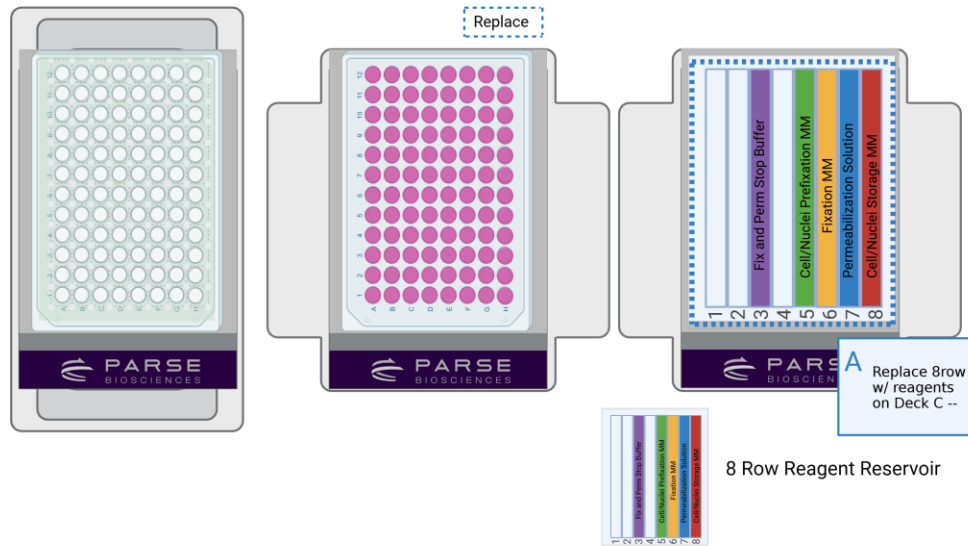
CRITICAL! Move quickly and handle the sample gently to avoid dislodging the pellet, which will impact data quality.

7. Attach VIAFLO Pipetting Module 12-Ch, 10-300 μ L and the corresponding Tip Deck.
8. Run the program **48RXN Fix V3_5** if running up to 48 samples, or program **96RXN Fix V3_5** if running up to 96 samples.

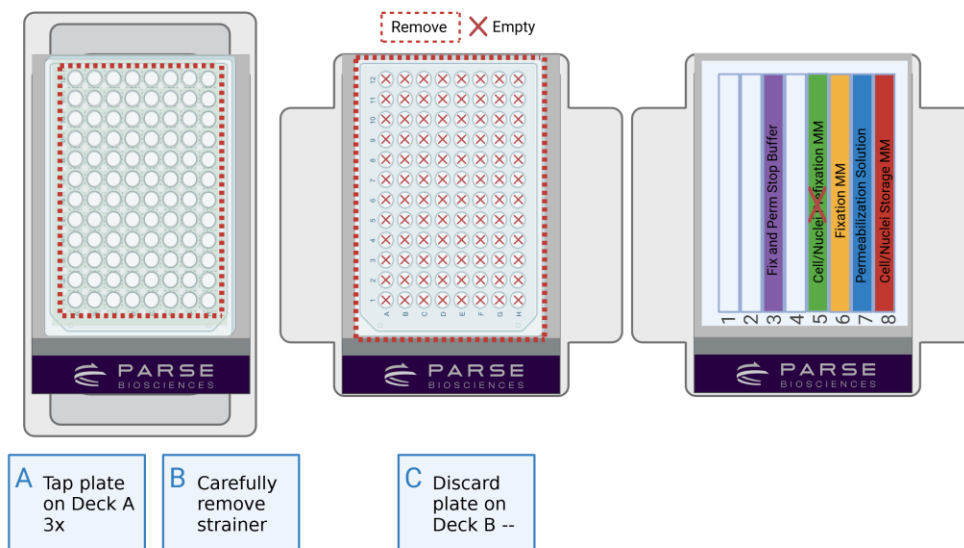


Note: During the supernatant removal from the 1 mL deep well plate containing the samples, the pipette tip touch should not touch the sides of the 8 row reservoir. The tip touch is intended to remove large droplets on the outside of the pipette tip.

9. **When prompted**, replace the cell waste reservoir on Deck C with the Reagent 8 Row Reservoir prepared in Section 1. The deck layout should correspond to the configuration below.



10. Press "Run" to continue. Follow the program prompts for manual intervention:



- a. Tap the plate on Deck A firmly 3x on the deck to move liquid to the bottom of the wells.
- b. Carefully remove the strainer on the 96 deep well plate on Deck A.



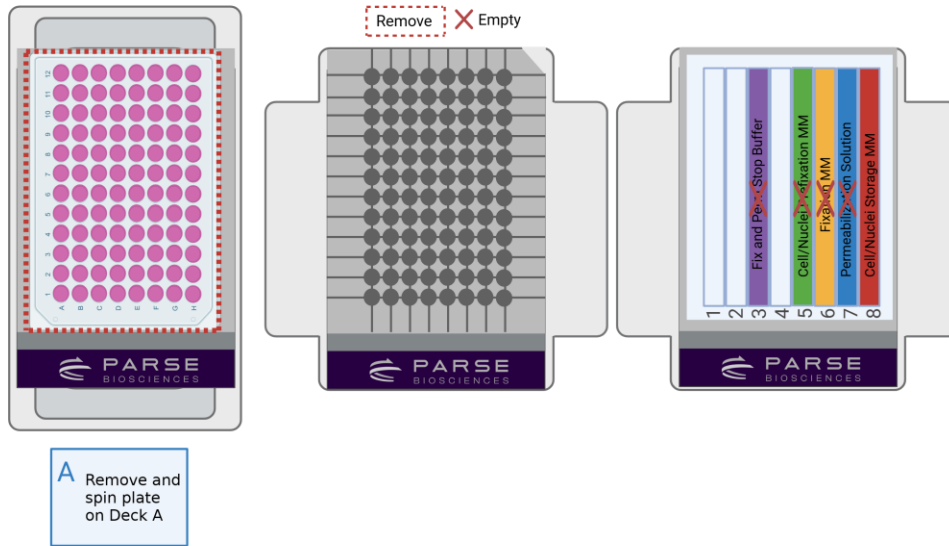
Note: Some volume will be removed from the top of the strainer to reduce the chance of contamination during strainer removal.



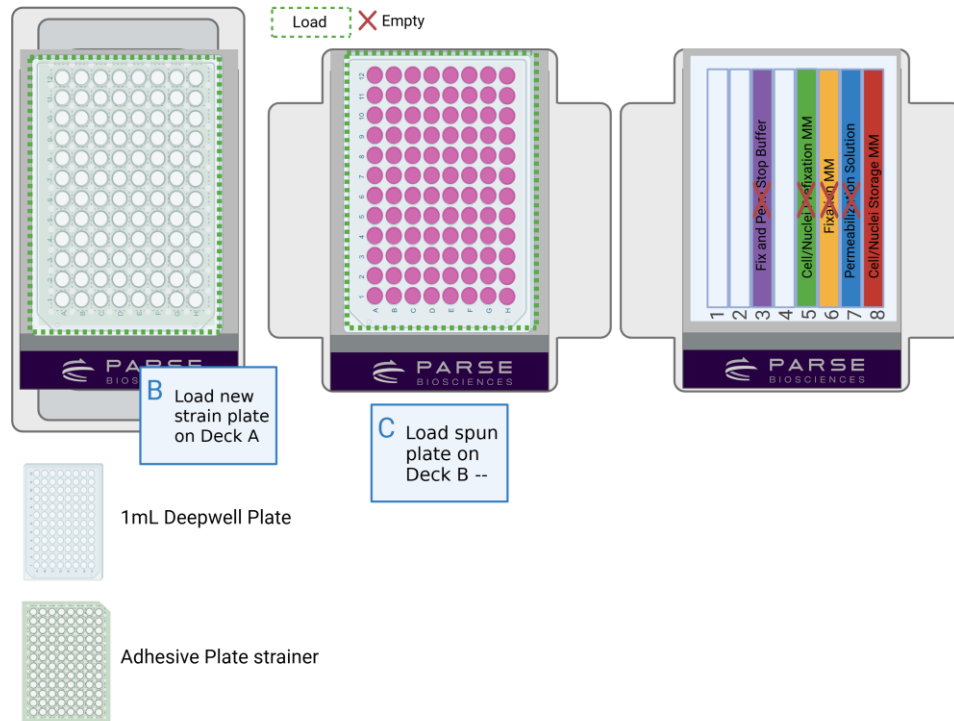
CRITICAL! Remove the strainer with one swift motion to reduce the chances of contamination.

c. Discard the 96 deep well plate on Deck B.

10. Press "Run" to continue the program. Follow the program prompts for manual intervention:



a. Seal and centrifuge the plate on Deck A in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Continue to the next step during centrifugation.



- b. Apply a Plate Strainer to a new 1 mL 96 deep well plate by peeling off the backing, carefully aligning over the wells A1-A12, and placing on the surface of the plate. Place this plate on the Deep Well Plate Cooling Adapter on Deck A with A1 towards the bottom left (Figure 4). If processing 48 samples, cut the strainer to fit the 48-reaction workflow and position it on rows A-D.

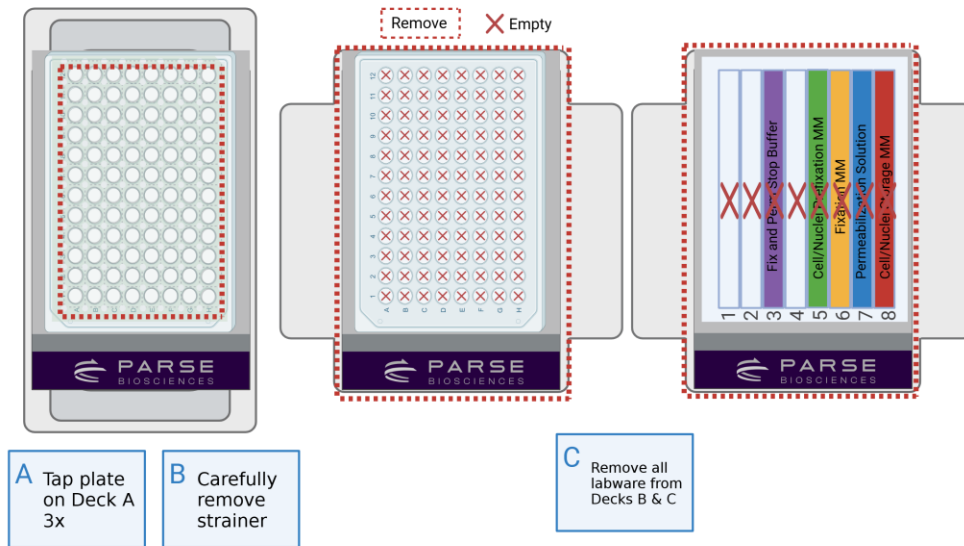


CRITICAL! Ensure your plate strainer is properly aligned to the top of your wells.

CRITICAL! Move quickly and handle the sample gently to avoid dislodging the pellet, which will impact data quality.

- c. Once the centrifugation is complete, place the 1 mL deep well plate containing the sample on top of the Eppendorf 1mL Deep Well Plate Cooling Adapter on Deck B and remove the plate seal.

11. Press “Run” to continue the program. Follow the program prompts for manual intervention:



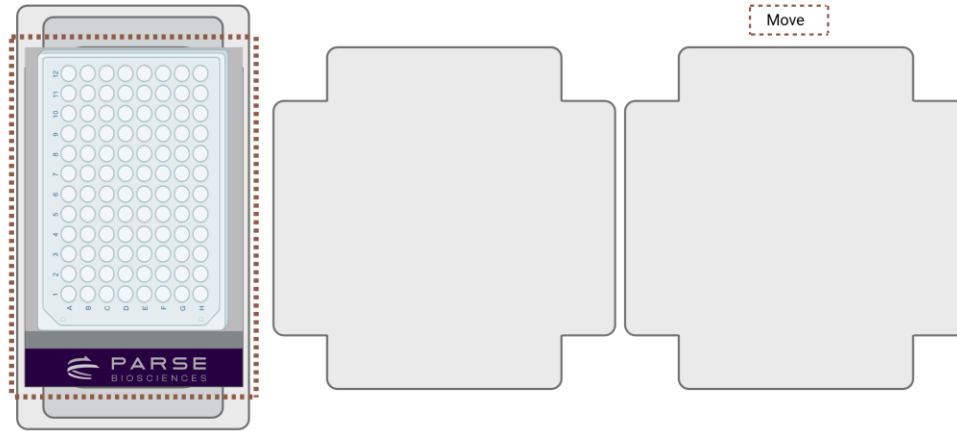
- Tap the plate on Deck A 3x on the deck to move liquid to the bottom of the wells.
- Carefully peel off the plate strainer and discard.



CRITICAL! See guidelines "Storage of Fixed Samples" if you prefer to skip the following steps and abort the program. Proceed directly to step 13.

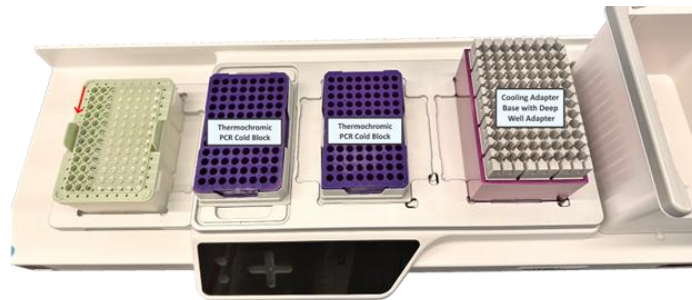
CRITICAL! Remove the strainer with one swift motion to reduce chances of contamination

- Remove all labware on Decks B and C.

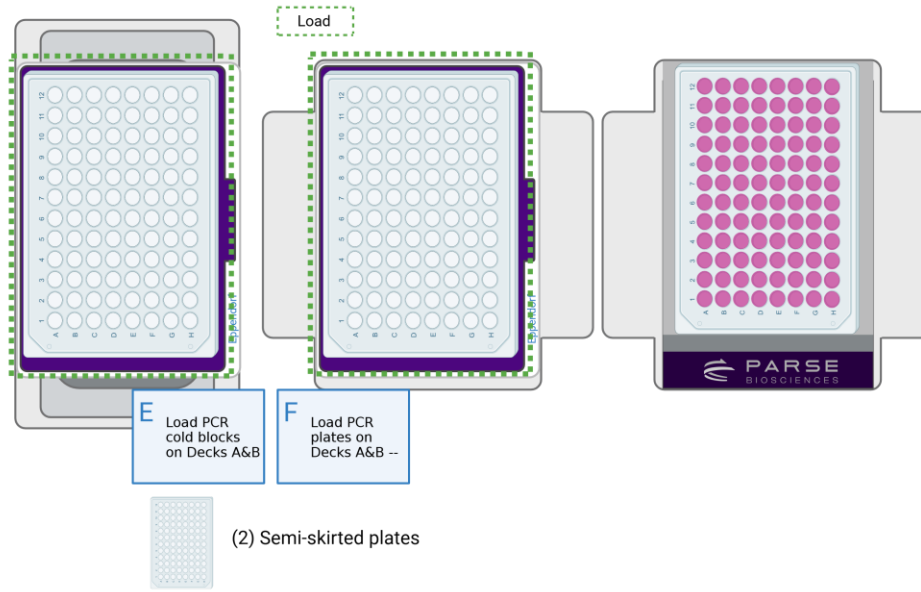


D Move all labware on Deck A to C

- d. Move all labware from Deck A to Deck C. This includes the Cooling adapter base with the Deepwell Plate Cooling Adapter and the sample deep well plate.



- e. Place 2 ThermoChromic PCR Cold Blocks with Risers on both Decks A and B.



- f. Place 2 new Eppendorf semi-skirted plates on the ThermoChromic PCR Cold Blocks on both Decks A and B.
12. At the end of the program, seal the two semi-skirted plates with a seal that can withstand storage at -80°C . The semi-skirted plate on Deck A contains a $20\ \mu\text{L}$ counting aliquot. The semi-skirted plate on Deck B contains the fixed cell suspension mix.



Note: If proceeding from step 11, and aborted the program before sample transfer into the semi-skirted plates, seal the 1 mL Deep Well plate containing the samples or transfer the samples into a container with a seal that can withstand storage at -80°C .

Note: The counting aliquots can be thawed and counted separately from the remaining sample for downstream processing with Evercode kits.



CRITICAL! Many clear plastic seals are not designed for storage at -80°C , so we recommend using foil plate seals if storing fixed samples in PCR plates.

13. Place the samples in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.



Safe stopping point: samples are stable for up to 6 months at -80°C .

14. The day before running the downstream Evercode Whole Transcriptome kit, thaw the $20\ \mu\text{L}$ aliquots in the semi-skirted plate in a water bath set to 37°C . Count the cells in the single cell suspension with a hemocytometer or alternative counting device and record the count. Discard any remaining sample from the counting aliquot.

Appendices

Appendix A: Pipetting Programs

12_RXN_FIX V3_5

STEPS	DESCRIPTION	DURATION
1	Initial Volumes	
2	Row 1: Remove SN 1	0 min 48 sec
3	Row 1: Remove SN 2	0 min 15 sec
4	Row 1: Remove SN 3	0 min 19 sec
5	Volume Modification	0
6	Row 1: Cell Prefixation Buffer	1 min 4 sec
7	Row 1: Strain	0 min 11 sec
8	Row 1: Remove Excess	0 min 17 sec
9	Prompt to tap plate	0
10	Prompt to remove strainer.	0
11	Add Fixation Solution	0 min 40 sec
12	9.5 min Incubation	9 min 30 sec
13	Add Perm Solution	0 min 43 sec
14	2.5 min Incubation	2 min 30 sec
15	Add Stop Mix	0 min 44 sec
16	Prompt to add strainer.	0
17	Prompt to check plate.	0
18	Volume modification	0
19	Row 1: Remove SN1	0 min 22 sec
20	Row 1: Remove SN2	0 min 15 sec

STEPS	DESCRIPTION	DURATION
21	Row 1: Remove SN3	0 min 18 sec
22	Row 1: Cell Storage Master Mix Addition	0 min 36 sec
23	Row 1: Resusp 1	0 min 18 sec
24	Row 1: Resusp 2	0 min 18 sec
25	Row 1: Strain	0 min 40 sec
26	Row 1: Remove Excess	0 min 17 sec
27	Prompt to tap plate	0
28	Prompt to remove cell strainer.	0
29	Aliquot counting aliquots.	0 min 34 sec
30	Sample Plate Notification	0
31	Replace PCR plate	0
32	Aliquot counting aliquots.	0 min 37 sec
33	Sample Plate Notification	0

48_RXN_FIX V3_5

STEPS	DESCRIPTION	DURATION
1	Initial Volumes	0
2	Row 1: Remove SN 1	0 min 48 sec
3	Row 1: Remove SN 2	0 min 15 sec
4	Row 1: Remove SN 3	0 min 19 sec
5	Row 2: Remove SN 1	0 min 45 sec
6	Row 2: Remove SN 2	0 min 15 sec
7	Row 2: Remove SN 3	0 min 19 sec
8	Row 3: Remove SN 1	0 min 45 sec
9	Row 3: Remove SN 2	0 min 15 sec

STEPS	DESCRIPTION	DURATION
10	Row 3: Remove SN 3	0 min 19 sec
11	Row 4: Remove SN 1	0 min 45 sec
12	Row 4: Remove SN 2	0 min 15 sec
13	Row 4: Remove SN 3	0 min 19 sec
14	Volume Modification	0
15	Row 1: Cell Prefixation Master Mix	1 min 4 sec
16	Row 1: Strain Cells 1	0 min 12 sec
17	Row 1: Remove Excess	0 min 17 sec
18	Row 2: Cell Prefixation Master Mix	1 min 4 sec
19	Row 2: Strain Cells 1	0 min 12 sec
20	Row 2: Remove Excess	0 min 17 sec
21	Row 3: Cell Prefixation Master Mix	1 min 4 sec
22	Row 3: Strain Cells 1	0 min 12 sec
23	Row 3: Remove Excess	0 min 17 sec
24	Row 4: Cell Prefixation Master Mix	1 min 4 sec
25	Row 4: Strain Cells 1	0 min 12 sec
26	Row 4: Remove Excess	0 min 17 sec
27	Prompt to tap plate	0
28	Prompt to remove strainer	0
29	Prompt to discard plate on Deck B	0
30	Add Fixation Solution	2 min 49 sec
31	Fixation Soln Incubation	7 min 0 sec
32	Add Perm Solution	2 min 48 sec
33	Perm Incubation	0 min 30 sec

STEPS	DESCRIPTION	DURATION
34	Add Fix and Perm Stop Buffer	3 min 3 sec
35	Row 1: Remove SN 1	0 min 22 sec
36	Row 1: Remove SN 2	0 min 15 sec
37	Row 1: Remove SN 3	0 min 19 sec
38	Row 2: Remove SN 1	0 min 22 sec
39	Row 2: Remove SN 2	0 min 15 sec
40	Row 2: Remove SN 3	0 min 19 sec
41	Row 3: Remove SN 1	0 min 23 sec
42	Row 3: Remove SN 2	0 min 15 sec
43	Row 3: Remove SN 3	0 min 18 sec
44	Row 4: Remove SN 1	0 min 22 sec
45	Row 4: Remove SN 2	0 min 15 sec
46	Row 4: Remove SN 3	0 min 18 sec
47	Row 1: Cell Storage Master Mix	0 min 33 sec
48	Row 1: Resusp 1	0 min 18 sec
49	Row 1: Resusp 2	0 min 18 sec
50	Row 1: Strain Cells 1	0 min 41 sec
51	Row 1: Remove Excess	0 min 17 sec
52	Row 2: Cell Storage Master Mix	0 min 33 sec
53	Row 2: Resusp 1	0 min 18 sec
54	Row 2: Resusp 2	0 min 18 sec
55	Row 2: Strain Cells 1	0 min 41 sec
56	Row 2: Remove Excess	0 min 17 sec
57	Row 3: Cell Storage Master Mix	0 min 33 sec

STEPS	DESCRIPTION	DURATION
58	Row 3: Resusp 1	0 min 18 sec
59	Row 3: Resusp 2	0 min 18 sec
60	Row 3: Strain Cells 1	0 min 41 sec
61	Row 3: Remove Excess	0 min 17 sec
62	Row 4: Cell Storage Master Mix	0 min 33 sec
63	Row 4: Resusp 1	0 min 18 sec
64	Row 4: Resusp 2	0 min 18 sec
65	Row 4: Strain Cells 1	0 min 41 sec
66	Row 4: Remove Excess	0 min 17 sec
67	Prompt to tap plate.	0
68	Prompt to remove the cell strainer.	0
69	Prompt to remove labware.	0
70	Prompt to move labware	0
71	Create Counting Aliquot Plate	2 min 24 sec
72	Create Sample Storage Plate	2 min 23 sec

96_RXN_FIX V3_5

STEPS	DESCRIPTION	DURATION
1	Initial Volumes	0
2	Row 1: Remove SN 1	0 min 48 sec
3	Row 1: Remove SN 2	0 min 15 sec
4	Row 1: Remove SN 3	0 min 19 sec
5	Row 2: Remove SN 1	0 min 45 sec
6	Row 2: Remove SN 2	0 min 15 sec

STEPS	DESCRIPTION	DURATION
7	Row 2: Remove SN 3	0 min 19 sec
8	Row 3: Remove SN 1	0 min 45 sec
9	Row 3: Remove SN 2	0 min 15 sec
10	Row 3: Remove SN 3	0 min 19 sec
11	Row 4: Remove SN 1	0 min 45 sec
12	Row 4: Remove SN 2	0 min 15 sec
13	Row 4: Remove SN 3	0 min 19 sec
14	Row 5: Remove SN 1	0 min 45 sec
15	Row 5: Remove SN 2	0 min 15 sec
16	Row 5: Remove SN 3	0 min 19 sec
17	Row 6: Remove SN 1	0 min 45 sec
18	Row 6: Remove SN 2	0 min 15 sec
19	Row 6: Remove SN 3	0 min 19 sec
20	Row 7: Remove SN 1	0 min 45 sec
21	Row 7: Remove SN 2	0 min 15 sec
22	Row 7: Remove SN 3	0 min 19 sec
23	Row 8: Remove SN 1	0 min 45 sec
24	Row 8: Remove SN 2	0 min 15 sec
25	Row 8: Remove SN 3	0 min 19 sec
26	Volume Modification	0
27	Row 1: Cell Prefixation Master Mix	1 min 3 sec
28	Row 1: Strain Cells 1	0 min 12 sec
29	Row 1: Remove Excess	0 min 17 sec
30	Row 2: Cell Prefixation Master Mix	1 min 4 sec

STEPS	DESCRIPTION	DURATION
31	Row 2: Strain Cells 1	0 min 12 sec
32	Row 2: Remove Excess	0 min 17 sec
33	Row 3: Cell Prefixation Master Mix	1 min 4 sec
34	Row 3: Strain Cells 1	0 min 12 sec
35	Row 3: Remove Excess	0 min 17 sec
36	Row 4: Cell Prefixation Master Mix	1 min 4 sec
37	Row 4: Strain Cells 1	0 min 12 sec
38	Row 4: Remove Excess	0 min 17 sec
39	Row 5: Cell Prefixation Master Mix	1 min 3 sec
40	Row 5: Strain Cells 1	0 min 12 sec
41	Row 5: Remove Excess	0 min 17 sec
42	Row 6: Cell Prefixation Master Mix	1 min 3 sec
43	Row 6: Strain Cells 1	0 min 12 sec
44	Row 6: Remove Excess	0 min 17 sec
45	Row 7: Cell Prefixation Master Mix	1 min 3 sec
46	Row 7: Strain Cells 1	0 min 12 sec
47	Row 7: Remove Excess	0 min 17 sec
48	Row 8: Cell Prefixation Master Mix	1 min 3 sec
49	Row 8: Strain Cells 1	0 min 12 sec
50	Row 8: Remove Excess	0 min 17 sec
51	Prompt to tap plate	0
52	Prompt to remove strainer	0
53	Prompt to discard plate on Deck B	0
54	Add Fixation Solution	5 min 37 sec

STEPS	DESCRIPTION	DURATION
55	Fixation Soln Incubation	5 min 0 sec
56	Add Perm Solution	5 min 34 sec
57	Add Fix and Perm Stop Buffer	6 min 3 sec
58	Row 1: Remove SN 1	0 min 22 sec
59	Row 1: Remove SN 2	0 min 15 sec
60	Row 1: Remove SN 3	0 min 19 sec
61	Row 2: Remove SN 1	0 min 22 sec
62	Row 2: Remove SN 2	0 min 15 sec
63	Row 2: Remove SN 3	0 min 19 sec
64	Row 3: Remove SN 1	0 min 23 sec
65	Row 3: Remove SN 2	0 min 15 sec
66	Row 3: Remove SN 3	0 min 18 sec
67	Row 4: Remove SN 1	0 min 22 sec
68	Row 4: Remove SN 2	0 min 15 sec
69	Row 4: Remove SN 3	0 min 18 sec
70	Row 5: Remove SN 1	0 min 22 sec
71	Row 5: Remove SN 2	0 min 14 sec
72	Row 5: Remove SN 3	0 min 18 sec
73	Row 6: Remove SN 1	0 min 23 sec
74	Row 6: Remove SN 2	0 min 15 sec
75	Row 6: Remove SN 3	0 min 18 sec
76	Row 7: Remove SN 1	0 min 22 sec
77	Row 7: Remove SN 2	0 min 15 sec
78	Row 7: Remove SN 3	0 min 18 sec

STEPS	DESCRIPTION	DURATION
79	Row 8: Remove SN 1	0 min 22 sec
80	Row 8: Remove SN 2	0 min 15 sec
81	Row 8: Remove SN 3	0 min 18 sec
82	Row 1: Cell Storage Master Mix	0 min 33 sec
83	Row 1: Resusp 1	0 min 18 sec
84	Row 1: Resusp 2	0 min 18 sec
85	Row 1: Strain Cells 1	0 min 41 sec
86	Row 1: Remove Excess	0 min 17 sec
87	Row 2: Cell Storage Master Mix	0 min 33 sec
88	Row 2: Resusp 1	0 min 18 sec
89	Row 2: Resusp 2	0 min 18 sec
90	Row 2: Strain Cells 1	0 min 41 sec
91	Row 2: Remove Excess	0 min 17 sec
92	Row 3: Cell Storage Master Mix	0 min 33 sec
93	Row 3: Resusp 1	0 min 18 sec
94	Row 3: Resusp 2	0 min 18 sec
95	Row 3: Strain Cells 1	0 min 41 sec
96	Row 3: Remove Excess	0 min 17 sec
97	Row 4: Cell Storage Master Mix	0 min 33 sec
98	Row 4: Resusp 1	0 min 18 sec
99	Row 4: Resusp 2	0 min 18 sec
100	Row 4: Strain Cells 1	0 min 41 sec
101	Row 4: Remove Excess	0 min 17 sec
102	Row 5: Cell Storage Master Mix	0 min 33 sec

STEPS	DESCRIPTION	DURATION
103	Row 5: Resusp 1	0 min 18 sec
104	Row 5: Resusp 2	0 min 18 sec
105	Row 5: Strain Cells 1	0 min 41 sec
106	Row 5: Remove Excess	0 min 17 sec
107	Row 6: Cell Storage Master Mix	0 min 33 sec
108	Row 6: Resusp 1	0 min 18 sec
109	Row 6: Resusp 2	0 min 18 sec
110	Row 6: Strain Cells 1	0 min 41 sec
111	Row 6: Remove Excess	0 min 17 sec
112	Row 7: Cell Storage Master Mix	0 min 33 sec
113	Row 7: Resusp 1	0 min 18 sec
114	Row 7: Resusp 2	0 min 18 sec
115	Row 7: Strain Cells 1	0 min 41 sec
116	Row 7: Remove Excess	0 min 17 sec
117	Row 8: Cell Storage Master Mix	0 min 33 sec
118	Row 8: Resusp 1	0 min 18 sec
119	Row 8: Resusp 2	0 min 18 sec
120	Row 8: Strain Cells 1	0 min 41 sec
121	Row 8: Remove Excess	0 min 17 sec
122	Prompt to tap plate.	0
123	Prompt to remove the cell strainer.	0
124	Prompt to remove labware.	0
125	Prompt to move labware	0
126	Create Counting Aliquot Plate	4 min 48 sec

STEPS	DESCRIPTION	DURATION
127	Create Sample Storage Plate	4 min 45 sec

Appendix B: Revision History

Version	Description	Date
1.0	Initial release	October 2024
1.1	Updated pipette references in deck configurations	October 2024
1.2	Updated 12 Reactions Fixation deck configurations	January 2025
1.3	Updated scripts	June 2025

Appendix C: Acknowledgements

All graphics were created with BioRender.com.



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