User Manual

Version 1.0 – UMLC4501INT

# Evercode™ Low Input Cell and Nuclei Fixation V4 with INTEGRA ASSIST PLUS

For use with

ECLC4501

ECLC4503

ECLC4505

ECLN4501

Support Suite

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# Table of Contents

# Overview

## Workflow

The Evercode Low Input 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.

Two different throughput options provide the flexibility to process up to 96 samples in parallel:

- 96 Reactions workflows uses ECFC4501/ECFN4501

This workflow is designed to efficiently process between 10,000 and 100,000 cells/nuclei, accommodating up to 96 samples simultaneously. The fixation protocol preserves cell/nuclei structure, prevents RNA degradation, and locks RNA inside the cells/nuclei, essential for downstream processing with Evercode's 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.

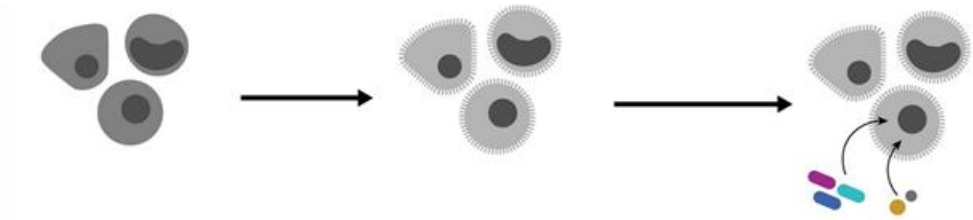


**Figure 1**: Evercode Cell Fixation. Cells in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

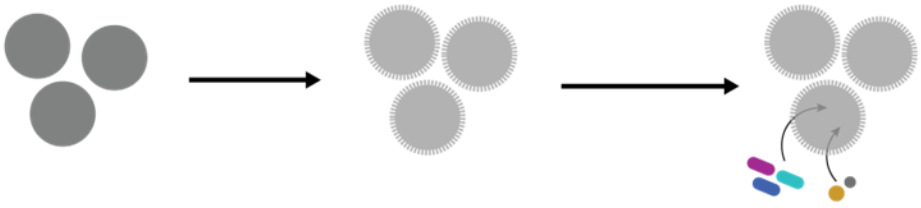


**Figure 2**: Evercode Nuclei Fixation. Nuclei in suspension are fixed and permeabilized before undergoing the split-pool combinatorial barcoding steps.

# 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 10,000 and 100,000 cells/nuclei can be fixed in a single reaction. However, we recommend using the highest number available up to 100,000 total. Exceeding 100,000 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 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 | Minimum Post-Capture Concentration to Fully Load Kit/µL |
| Evercode WT | 372 cells/nuclei |
| Evercode WT Mega | 1,860 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.

## Rigid Plate Strainers

- An example video of using a Rigid Plate Strainer can be found in our Support Suite. We recommend watching this video and practicing using the Rigid Plate Strainer before processing your samples.
- Before using the Rigid Plate Strainer, remember to remove the plastic cover under the strainer.

## 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 v4 kits.
- When first using Evercode Fixation v4 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. We recommend Trypan Blue for all counting up until the bead binding step. After the beads are bound, we recommend using fluorescent staining such as Acridine Orange/Propidium Iodide (AO/PI) or Acridine Orange/DAPI (AO/DAPI) (Figure 3).

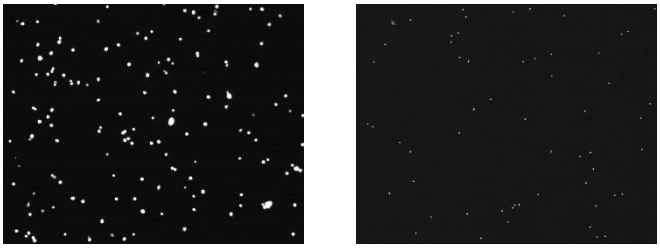


Figure 3: Example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

- 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

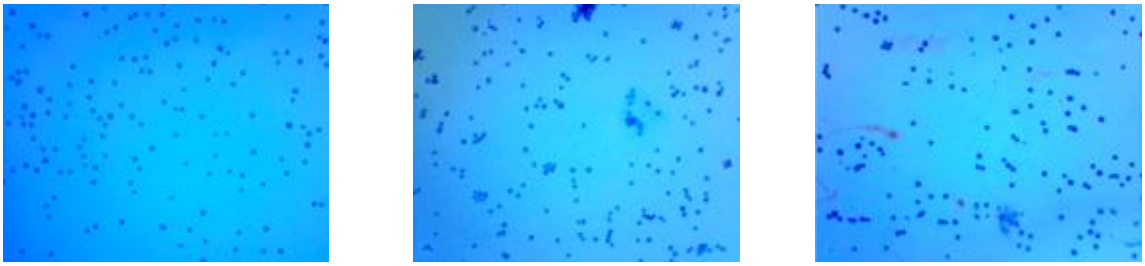


Figure 4: Example of trypan blue stained fixed cells.

## Centrifugation

- A range of centrifugation speeds and durations are given in this protocol rather than a single speed. When using Evercode Fixation v4 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 in 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

- If the kit is going to be used more than once, 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.

- The Prefixation Enhancer and the Cell/Nuclei Binding Beads must be stored at 4°C and should not be frozen. It is critical to never freeze the beads or vortex them for an extended period of time.

- Reagent master mixes should be made fresh and used the same day.

## Storage of Fixed Samples

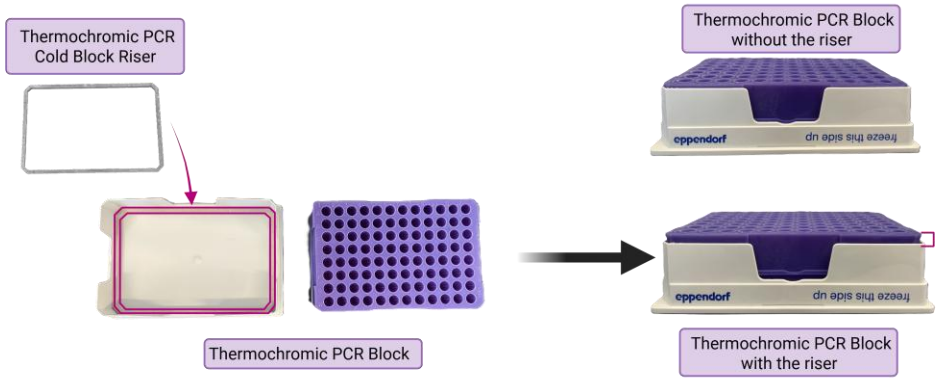
- Fixed samples can be stored at -80°C for up to 6 months. Fixed samples should not be refrozen after thawing.

## 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.

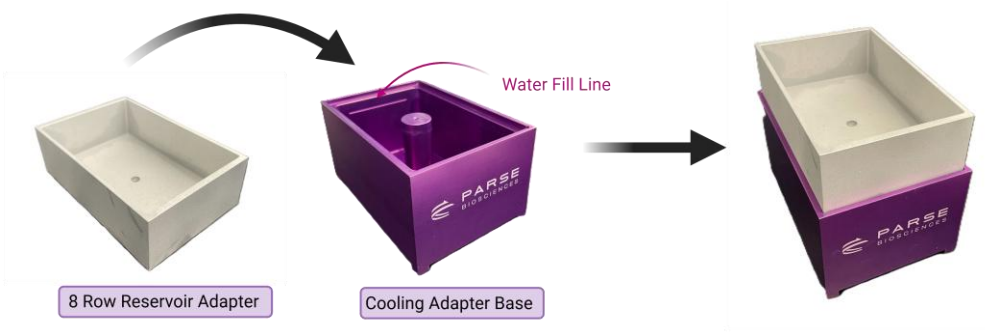
- Tip pinching may occur when using a fully frozen Thermochromic PCR Cold Block. To minimize tip pinching, a fully frozen Thermochromic PCR Cold Block should be warmed

slightly by a few degrees by leaving it at room temperature for 10 minutes before using it on the INTEGRA ASSIST PLUS Deck.

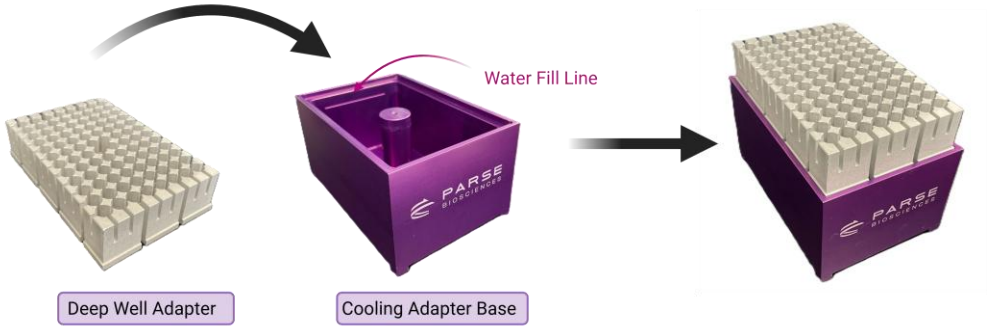
- To ensure a secure positioning within the Thermochromic PCR Cold Block, apply firm pressure on the PCR plates until they are securely seated. Avoid touching the top of the open wells.

## Cooling Adapter Bases

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




8 Row Reservoir Adapter with Cooling Adapter Base.




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 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.

# Part List

## Evercode Low Input Cell Fixation Kit Part List, 96 Reactions

The Evercode Low Input Cell Fixation v4, 96 reactions workflow requires Low Input Cell Fixation Reagents and Low Input Cell Prefixation and Binding Reagents.

**Low Input Cell Fixation Reagents, 96 reactions.** Store at -20°C, PN LCF700

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|  | Prefixation Buffer | CF301 | 15 mL bottle | 1 |
|  | Storage Buffer | CF302 | 8 mL bottle | 2 |
|  | Fixative Solution A | CF303 | 8 mL bottle | 1 |
|  | Fixative Solution B | CF304 | 8 mL bottle | 1 |
|  | Permeabilization Solution | CF319 | 8 mL bottle | 1 |
|  | Fix and Perm Stop Buffer | CF306 | 15 mL bottle | 1 |
|  | RNase Inhibitor | CF311 | 1.5 mL tube | 1 |
|  | DMSO | CF308 | 2 mL tube | 1 |
|  | Cell Binding Bead Wash Buffer | CF313 | 8 mL bottle | 1 |

**Cell Prefixation and Binding Reagents, 96 reactions.** Store at 4°C, PN LCF800

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|  | Prefixation Enhancer | CF401 | 2 mL tube | 1 |
|  | Cell Binding Beads | CF309 | 2 mL tube | 1 |

**30 µM Rigid Plate Strainer*.** Store at Room Temperature

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| N/A | Rigid Plate Strainer, 30 µM | RPS1030 | 6-pack | 1 |

**70 µM Rigid Plate Strainer*.** Store at Room Temperature

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| N/A | Rigid Plate Strainer, 70 µM | RPS1070 | 6-pack | 1 |

**100 µM Rigid Plate Strainer*.** Store at Room Temperature

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| N/A | Rigid Plate Strainer, 100 µM | RPS1100 | 6-pack | 1 |

**Note:** * Only one mesh size of Rigid Plate Strainer is required for the Evercode Low Input Cell Fixation v4 workflow. Select an appropriate mesh size for each sample type.

## Low Input Nuclei Fixation Reagents

The Evercode Nuclei Fixation v4, 96 reactions workflow requires Low Input Nuclei Fixation Reagents and Nuclei Prefixation and Binding Reagents boxes.

**Low Input Nuclei Fixation Reagents, 96 reactions.** Store at -20°C, LNF700

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|   | Prefixation Buffer | NF301 | 15 mL bottle | 1 |
|   | Storage Buffer | NF302 | 8 mL bottle | 2 |
|   | Fixative Solution | NF303 | 8 mL bottle | 1 |
|   | Permeabilization Solution | NF314 | 8 mL bottle | 1 |
|   | Fix and Perm Stop Buffer | NF305 | 15 mL bottle | 1 |
|   | RNase Inhibitor | NF310 | 1.5 mL tube | 1 |
|   | DMSO | NF311 | 2 mL tube | 1 |
|   | Nuclei Binding Bead Wash Buffer | NF312 | 8 mL tube | 1 |

**Nuclei Prefixation and Binding Reagents, 96 reactions.** Store at 4°C, LNF800

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
|  | Prefixation Enhancer | NF401 | 2 mL tube | 1 |
|  | Nuclei Binding Buffer | NF308 | 2 mL tube | 1 |

**30 µm Rigid Plate Strainer.** Store at Room Temperature

| LABEL | ITEM | PN | FORMAT | QTY |
|---|---|---|---|---|
| N/A | Rigid Plate Strainer, 30 µM | RPS1030 | 6-pack | 1 |

## 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 | CHECK LIST |
|---|---|---|---|
| Thermochromic PCR Cold Block | NTAC1102 | 2 | ☐ |
| Thermochromic PCR Cold Block Riser | NTAC1103 | 2 | ☐ |
| Deep Well Adapter | NTAC1104 | 1 | ☐ |
| Cooling Adapter Base | NTAC1106 | 1 | ☐ |

## 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 | CHECK LIST |
|---|---|---|---|---|
| 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, 5-125 µL | Pipette | 4632 | 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 | CHECK LIST |
|---|---|---|---|---|
| 5-125 µL GRIPTIPS (Sterile/Filter/Low Retention) | INTEGRA | 6565 | 2 | ☐ |

## 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 | CHECK LIST |
|---|---|---|---|---|
| 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 | If storing fixed samples in a PCR plate. Note that many clear plastic seals are not designed for storage at -80°C. | ☐ |
| Protein LoBind® 1 mL Deepwell Plate | Eppendorf® | 951033308 | | ☐ |
| Rigid Plate Strainer | Parse Biosciences | RPS1030<br>RPS1070<br>RPS1100 | Choose an appropriate mesh size for your sample type. | ☐ |
| 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. | ☐ |
| Pipette Tips<br>RT LTS 20 µL FL 960A/10<br>RT LTS 200 µL FL 960A/10<br>RT LTS 1000 µL FL 768A/8 | Rainin® | 30389226<br>30389240<br>30389213 | Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips. | ☐ |

### Equipment

| ITEM | SUPPLIER | PN | NOTES | CHECK LIST |
|---|---|---|---|---|
| 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. | ☐ |
| Single-channel pipettes: P20, P200, P1000 | Various Suppliers | Varies | | ☐ |
| Hemocytometer | Sigma-Aldrich® | Z359629 | Or other cell counting devices. Ensure the hemocytometer is compatible with the fluorescent microscope. | ☐ |
| Automated Fluorescence Counter | Various Suppliers | Varies | Any automated counter with fluorescent capabilities. | ☐ |
| Polystyrene foam 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 | CHECK LIST |
|---|---|---|---|---|
| Fluorescence dye | Various Suppliers | Varies | Any dye to assess cell viability, such as AO/PI, DAPI, etc. | ☐ |
| RNaseZap™ RNase Decontamination Solution | Thermo Fisher Scientific | AM9780 | Or equivalent RNase decontamination solution. | ☐ |

# 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.

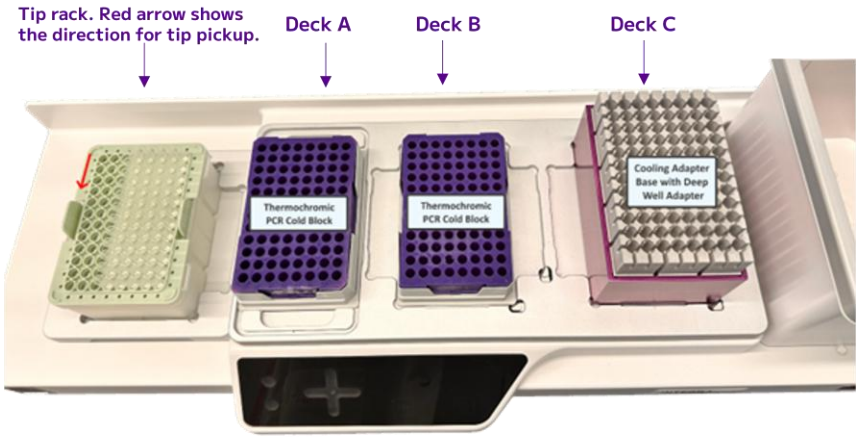
There are three types of figures in this manual.

- 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.

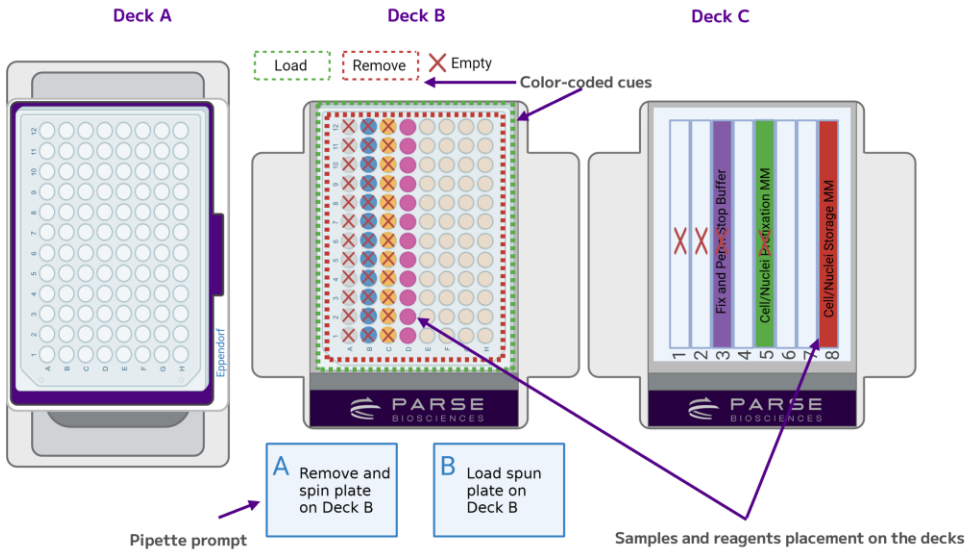



**Blocks and Bases are hardware components that will be placed or removed as needed throughout the workflow.**

## 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.

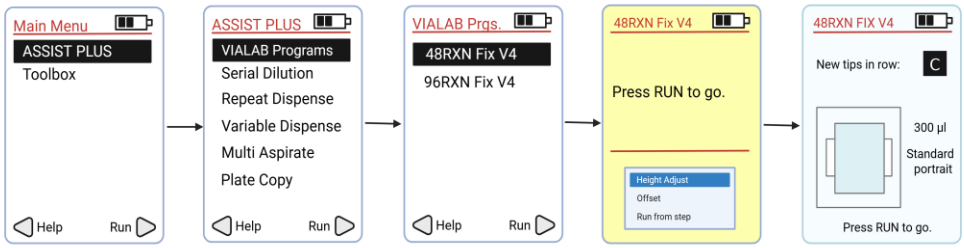

- **Load**: Indicates labware and/or reagents that need be placed or reloaded onto the deck.
- **Remove**: Identifies labware that should be taken off the deck, either for incubation, disposal, or because it is not needed in the upcoming step.
- **Replace**: Highlights labware that has been used and 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. Prepare Master Mixes for Low Input Cell Fixation

Low Input Cell Fixation master mixes should be prepared immediately prior to fixation. The tables in this section show volumes for 96-sample runs.

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| Protein LoBind 1 mL 96 Deepwell Plate | Consumables | 1 | |
| Cooling Adapter Base | Parse-Provided | 1 | If previously frozen, leave at room temperature for 10 minutes prior to use.<br>If not previously frozen, fill with pebble ice prior to use.<br>Replace ice as necessary. |
| 8 Row Reservoir Adapter | Parse-Provided | 1 | |
| Rigid Plate Strainer | Parse | 1 | Choose an appropriate mesh size for your sample type. |
| ○ Prefixation Buffer | Low Input Cell Fixation Reagents (-20°C) | 15 mL bottle | Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex. |
| ○ Storage Buffer | Low Input Cell Fixation Reagents (-20°C) | 8 mL bottle | |
| ○ Fixative Solution A | Low Input Cell Fixation Reagents (-20°C) | 8 mL bottle | |
| ○ Fixative Solution B | Low Input Cell Fixation Reagents (-20°C) | 8 mL bottle | |
| ○ Permeabilization Solution | Low Input Cell Fixation Reagents (-20°C) | 8 mL bottle | |

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ○ Fix and Perm Stop Buffer | Low Input Cell Fixation Reagents (-20°C) | 15 mL bottle | |
| ● DMSO | Low Input Cell Fixation Reagents (-20°C) | 2 mL tube | Thaw and store at room temperature. Mix by inverting the tube. |
| ○ RNase Inhibitor | Low Input Cell Fixation Reagents (-20°C) | 1.5 mL tube | Store on ice immediately before use. Do not vortex. |
| ● Prefixation Enhancer | Cell Prefixation and Binding Reagents (4°C) | 2 mL tube | |

2. Label the new Protein LoBind 1 mL 96 Deepwell Plate as the **Reagent Reservoir**.

3. Prepare the **Cell Prefixation Master Mix** in a new 15 mL tube as follows. Mix thoroughly and pipette **750 µL** into each well of **Row E** of the Reagent 1 mL Deepwell Plate.

| CELL PREFIXATION MASTERMIX | |
|---|---|
| Number of Samples | 96 |
| ○ Prefixation Buffer | 8.7 mL |
| ○ RNase Inhibitor | 109 µL |
| ● Prefixation Enhancer | 590 µL |
| Total Volume | 9.4 mL |

4. Prepare the **Cell Fixative Master Mix** in a new 15 mL tube as follows. Mix thoroughly and pipette **400 µL** into each well of **Row F** of the Reagent 1 mL Deepwell Plate.

| CELL FIXATIVE MASTERMIX | |
|---|---|
| Number of Samples | 96 |
| ○ Fixative Solution A | 2.5 mL |
| ○ Fixative Solution B | 2.5 mL |
| Total Volume | 5.0 mL |

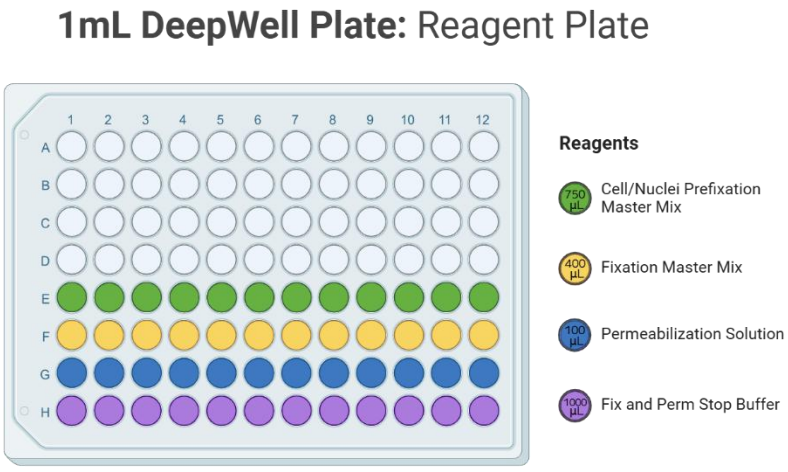
5. Mix thoroughly and pipette **100 µL** the ○ **Permeabilization Solution** into each well of **Row G** of the Reagent 1 mL Deepwell Plate.

| PERMEABILIZATION SOLUTION | |
|---|---|
| Number of Samples | 96 |
| ○ Permeabilization Solution | 100 µL |

6. Prepare the **Cell Fix and Perm Stop Master Mix** in a new 15 mL tube as follows. Mix thoroughly and pipette **1 mL** into each well of **Row H** of the Reagent 1 mL Deepwell Plate.

| FIX AND PERM STOP MASTER MIX | |
|---|---|
| Number of Samples | 96 |
| ○ Fix and Perm Stop Buffer | 11.23 mL |
| ● DMSO | 1.25 mL |
| Total Volume | 12.48 mL |

7. The Reagent Protein LoBind 1mL 96 Deepwell Plate should correspond to the image below. Place the **Reagent Plate** on ice until needed in Section 2.1.



## Section 1.2. Prepare Master Mixes for Low Input Nuclei Fixation

Low Input Nuclei fixation master mixes should be prepared immediately prior to fixation. The tables in this section show volumes for 96-sample runs.

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

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| Protein LoBind 1 mL 96 Deepwell Plate | Consumables | 1 | |
| Cooling Adapter Base | Parse-Provided | 1 | If previously frozen, leave at room temperature for 10 minutes prior to use. If not previously frozen, fill with pebble ice prior to use.<br>Replace ice as necessary. |
| 8 Row Reservoir Adapter | Parse-Provided | 1 | |
| ○ Prefixation Buffer | Low Input Nuclei Fixation Reagents (-20°C) | 15 mL bottle | Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex. |
| ○ Storage Buffer | Low Input Nuclei Fixation Reagents (-20°C) | 8 mL bottle | |
| ○ Fixative Solution | Low Input Nuclei Fixation Reagents (-20°C) | 8 mL bottle | |
| ○ Permeabilization Solution | Low Input Nuclei Fixation Reagents (-20°C) | 2 mL tube | |
| ○ Fix and Perm Stop Buffer | Low Input Nuclei Fixation Reagents (-20°C) | 15 mL bottle | |
| ● DMSO | Low Input Nuclei Fixation Reagents (-20°C) | 1.5 mL tube | Thaw and store at room temperature. Mix by inverting the tube. |

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| ● RNase Inhibitor | Low Input Nuclei Fixation Reagents (-20°C) | 1.5 mL tube | Store on ice immediately before use. Do not vortex. |
| ● Prefixation Enhancer | Nuclei Prefixation and Binding Reagents (4°C) | 2 mL tube | |

2. Label the new Protein LoBind 1 mL 96 Deepwell Plate as the **Reagent Reservoir**.

3. Prepare the ○ **Nuclei Prefixation Master Mix** in a new 15 mL tube as follows. Mix thoroughly and pipette **750 µL** into each well of **Row E** of the Reagent 1 mL Deepwell Plate.

| NUCLEI PREFIXATION MASTERMIX | |
|---|---|
| Number of Samples | 96 |
| ○ Prefixation Buffer | 8.7 mL |
| ● RNase Inhibitor | 109 µL |
| ● Prefixation Enhancer | 590 µL |
| Total Volume | 9.4 mL |

4. Mix thoroughly and pipette **200 µL** of the ○ **Nuclei Fixative Solution** into each well of **Row F** of the Reagent 1 mL Deepwell Plate.

| NUCLEI FIXATIVE SOLUTION | |
|---|---|
| Number of Samples | 96 |
| ○ Fixative Solution | 2.5 mL |

5. Mix thoroughly and pipette **100 µL** the ○ **Permeabilization Solution** into each well of **Row G** of the Reagent 1 mL Deepwell Plate.

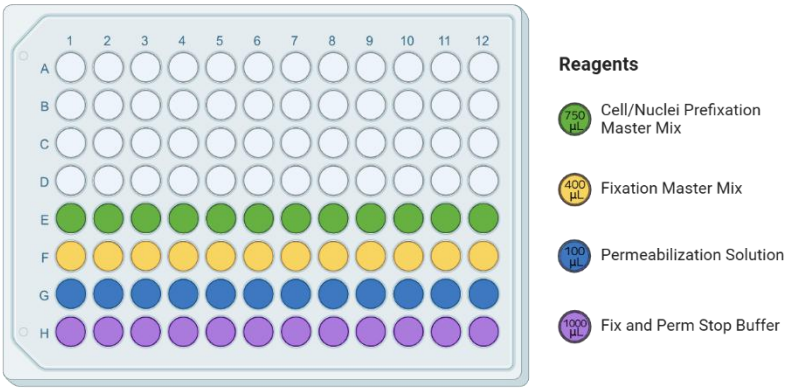
| PERMEABILIZATION SOLUTION | |
|---|---|
| Number of Samples | 96 |
| ○ Permeabilization Solution | 100 µL |

6. Prepare the **Nuclei Fix and Perm Stop Master Mix** in a new 15 mL tube as follows. Mix thoroughly and pipette **1 mL** into each well of **Row H** of the Reagent 1 mL Deepwell Plate.

| FIX AND PERM STOP MASTER MIX | |
|---|---|
| Number of Samples | 96 |
| O Fix and Perm Stop Buffer | 11.23 mL |
| ● DMSO | 1.25 mL |
| Total Volume | 12.48 mL |

7. The Reagent Protein LoBind 1mL 96 Deepwell Plate should correspond to the image below. Place the **Reagent Plate** on ice until needed in Section 2.1.

**1mL DeepWell Plate:** Reagent Plate



# Section 2: Low Input Fixation

## Section 2.1. 96 Reactions Low Input Fixation

This section outlines the protocol for processing 96 samples.

**To fix samples:**

1. Gather the following components:

| ITEM | SOURCE | QTY | HANDLING AND STORAGE |
|---|---|---|---|
| VIAFLO Pipette 12-Ch, 5-125 µL | INTEGRA Component | 1 | |
| Tip Deck for VIAFLO Pipetting Module | INTEGRA Component | 1 | |
| 5-125 µL Tip Rack | INTEGRA-Provided | 2 | |
| 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 | |
| Deep Well Adapter | Parse-Provided | 1 | |
| Rigid Plate Strainer | Parse-Provided | 1 | Choose an appropriate mesh size for your sample type. |
| Cooling Adapter Base | Parse-Provided | 1 | If previously frozen, leave at room temperature for 10 minutes prior to use. If not previously frozen, fill with pebble ice prior to use.<br>Replace ice as necessary. |
| SealPlate® | Consumables | 1 | |
| TempPlate® EXT Sealing Foil | Consumables | 1 | |
| Eppendorf twin.tec® PCR Plate 96 LoBind® | Consumables | 2 | Semi-skirted 96-well plate |

2. Cool a centrifuge with a swinging bucket rotor to 4°C.

3. Fill a bucket with ice.

4. Prepare the cooling hardware:

    a. Assemble and fill one of the Cooling Adapter Bases with pebble ice and place one on 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.

    b. Place the Deepwell Adapter on the Cooling Adapter Base on Deck C.

    c. Remove 2 Thermochromic PCR Cold Block w/ Riser from the freezer. Leave the fully frozen Thermochromic PCR Cold Block with Riser at room temperature for 10 minutes to ensure it is slightly warmed by a few degrees before using it on the INTEGRA ASSIST PLUS Deck.

5. Count the cells/nuclei in the single cell/nuclei 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.

6. Transfer 10,000 to 100,000 cells/nuclei from each sample into the appropriate wells of a new semi-skirted LoBind 96 well PCR plate on ice.

7. Seal the plate with a plastic adhesive plate seal.

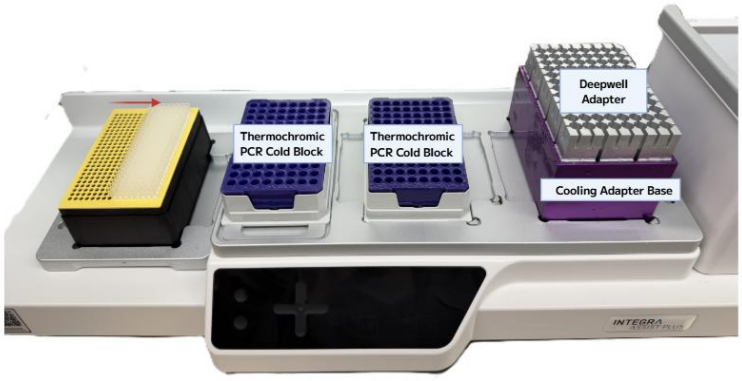
8. Centrifuge the plate in a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C.

9. During centrifugation, set up the deck following the Deck Configuration below.

    a. Deck A: Thermochromic PCR Cold Block w/ Riser.

    b. Deck B: Thermochromic PCR Cold Block w/ Riser.

    c. Deck C: Deepwell Adapter on the Cooling Adapter Base.



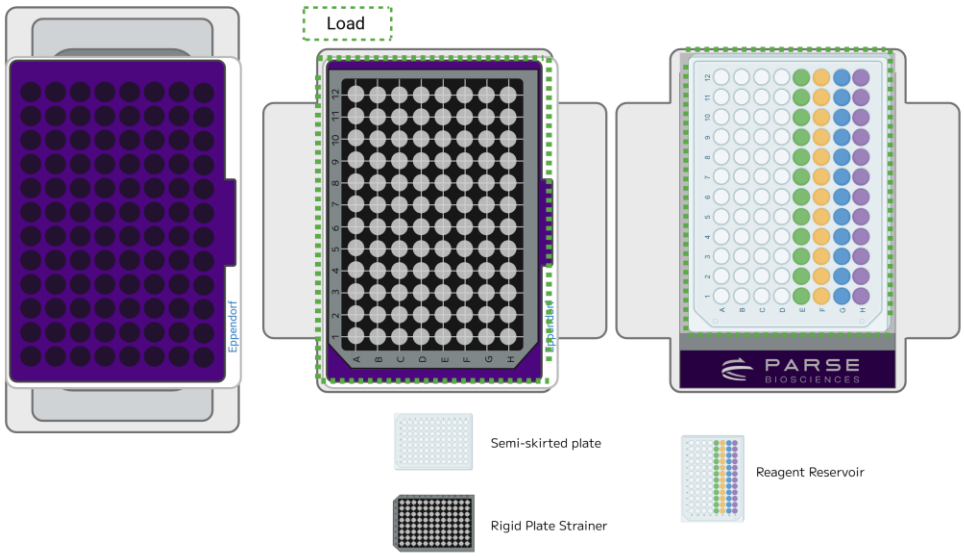
10. Load the following reagents and consumables to their respective positions on Decks A, B and C:

    a. Deck B:

        i. A new semi-skirted PCR plate with A1 corner in the bottom left.

        ii. Remove the film and place a Rigid Plate Strainer on the PCR Plate with A1 on the bottom left. Press down firmly to ensure a snug fit.

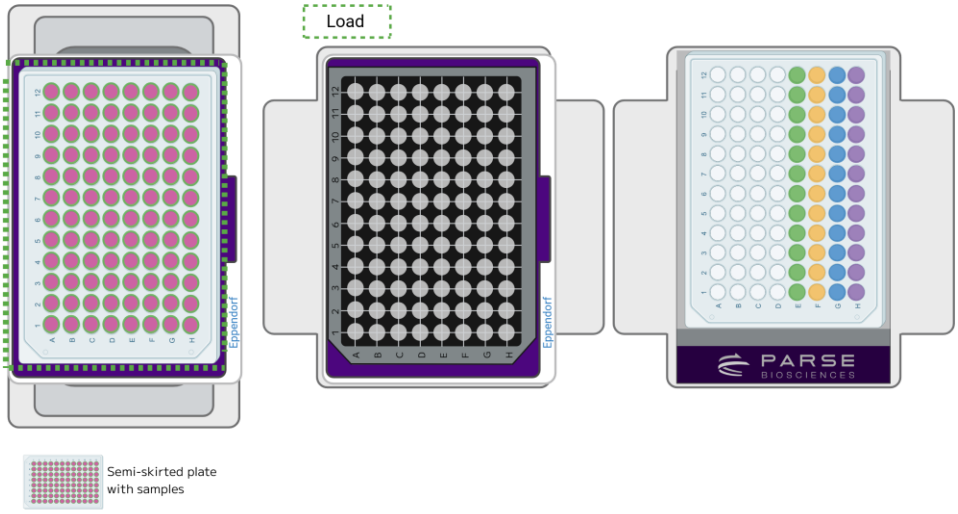
    b. Deck C: **Reagent Reservoir** Plate from Section 1.1 or 1.2. Ensure A1 is on the bottom left.



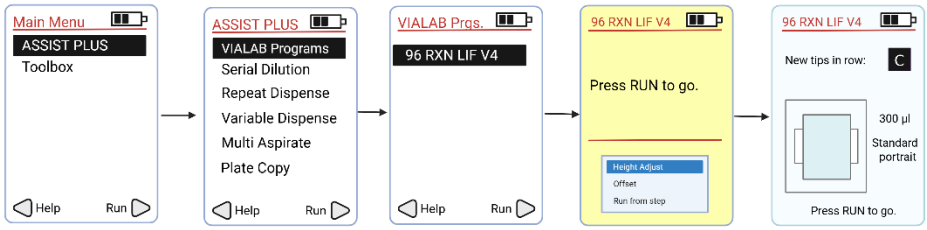

11. Attach VIAFLO 12-Ch 5-125 µL and the corresponding Tip Deck. Ensure the Tip Deck and tip box are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

12. Immediately after the centrifugation is complete, place the Eppendorf semi-skirted plate containing the samples on top of the Thermochromic PCR Cold Block on Deck A, and remove the plate seal.

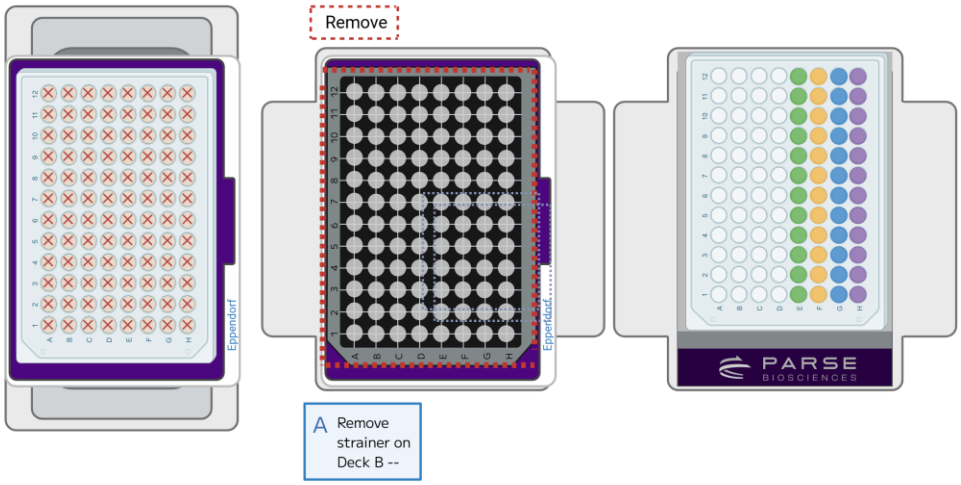
**CRITICAL!** Move quickly and handle the sample gently to avoid dislodging the pellet, which will impact data quality.




13. Immediately run the program **96 RXN LIF V4_0**.




a. If there is excess liquid on the strainer, centrifuge the semi-skirted plate and the strainer on deck B for 100 x g at 4°C for **15 seconds**. Tape the strainer to the semi-skirted plate to reduce the chance of misaligned wells during transportation. When prompted, remove the strainer from the Eppendorf semi-skirted plate on Deck B. Press "Run" to continue.



14. At the conclusion of the run:

    a. The used Eppendorf semi-skirted plate on Deck A can be discarded.

    b. The used **Reagent Reservoir** on Deck C can be discarded.

    c. The Eppendorf semi-skirted 96 well PCR plate on Deck B contains the samples. Label the plate as the **LI Sample Plate**. Seal the semi-skirted plate with a seal that can withstand storage at -80°C. Place the samples in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.

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


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

15. The morning of Barcoding, manually perform Cell/Nuclei Capture according to Section 2.3: 96 Reactions - Cell/Nuclei Capture of the manual Evercode Low Input Cell and Nuclei Fixation v4 User Guide.

# Appendices

## Appendix A: Pipetting Programs

**96 RXN LIF V4_0**

| STEP | ACTION | DURATION |
|---|---|---|
| 1 | Initial Volumes | 0 |
| 2 | Row 1: Remove Soup | 0 min 28 sec |
| 3 | Row 1: Remove Soup | 0 min 43 sec |
| 4 | Row 2: Remove Soup | 0 min 25 sec |
| 5 | Row 2: Remove Soup | 0 min 43 sec |
| 6 | Row 3: Remove Soup | 0 min 25 sec |
| 7 | Row 3: Remove Soup | 0 min 43 sec |
| 8 | Row 4: Remove Soup | 0 min 25 sec |
| 9 | Row 4: Remove Soup | 0 min 43 sec |
| 10 | Row 5: Remove Soup | 0 min 25 sec |
| 11 | Row 5: Remove Soup | 0 min 43 sec |
| 12 | Row 6: Remove Soup | 0 min 25 sec |
| 13 | Row 6: Remove Soup | 0 min 43 sec |
| 14 | Row 7: Remove Soup | 0 min 25 sec |
| 15 | Row 7: Remove Soup | 0 min 43 sec |

| STEP | ACTION | DURATION |
|---|---|---|
| 16 | Row 8: Remove Soup | 0 min 25 sec |
| 17 | Row 8: Remove Soup | 0 min 43 sec |
| 18 | Row 1: Prefixation Mix | 0 min 37 sec |
| 19 | Row 1: Strain | 0 min 46 sec |
| 20 | Row 2: Prefixation Mix | 0 min 37 sec |
| 21 | Row 2: Strain | 0 min 46 sec |
| 22 | Row 3: Prefixation Mix | 0 min 37 sec |
| 23 | Row 3: Strain | 0 min 47 sec |
| 24 | Row 4: Prefixation Mix | 0 min 37 sec |
| 25 | Row 4: Strain | 0 min 47 sec |
| 26 | Row 5: Prefixation Mix | 0 min 37 sec |
| 27 | Row 5: Strain | 0 min 47 sec |
| 28 | Row 6: Prefixation Mix | 0 min 36 sec |
| 29 | Row 6: Strain | 0 min 47 sec |
| 30 | Row 7: Prefixation Mix | 0 min 36 sec |
| 31 | Row 7: Strain | 0 min 47 sec |
| 32 | Row 8: Prefixation Mix | 0 min 36 sec |

| STEP | ACTION | DURATION |
|---|---|---|
| 33 | Row 8: Strain | 0 min 47 sec |
| 34 | Add Fix Soln | 4 min 30 sec |
| 35 | Fixation Incubation | 5 min 30 sec |
| 36 | Add Perm Soln | 4 min 27 sec |
| 37 | Add Stop Mix | 7 min 50 sec |

## Appendix B: Revision History

| Version | Description | Date |
|---|---|---|
| 1.0 | Initial release | July 2026 |

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