

User Manual



Version 1.0 – UMMW4300INT

Evercode™ MAX WT v4

Cell Barcoding M on

INTEGRA ASSIST PLUS

For use with
ECMW4300



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Overview

Workflow

The Evercode MAW WT v4 workflow is compatible with the INTEGRA ASSIST PLUS to enable large-scale single cell RNA-Seq experiments through a robust, semi-automated process.

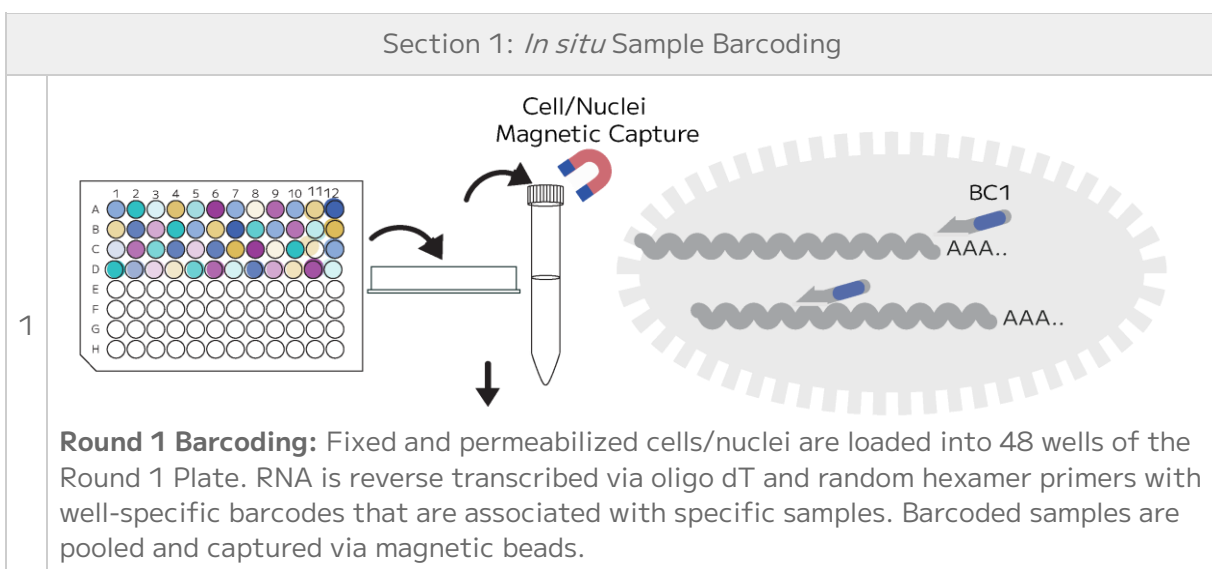
Evercode MAX WT v4 Cell Barcoding kits add further flexibility by separating cell barcoding from library construction, so each dimension can be scaled independently based on experimental needs.

- Four sizes of Evercode MAX WT v4 Cell Barcoding kits determine the number of samples that can be indexed in a given run.
- Two sizes of Evercode MAX WT v4 Library Prep kits determine the number of sublibraries that can be prepared from the indexed samples.

Barcoding kits can be paired with any sublibrary kit. This lets users choose cell number and sublibrary count based on experimental design.

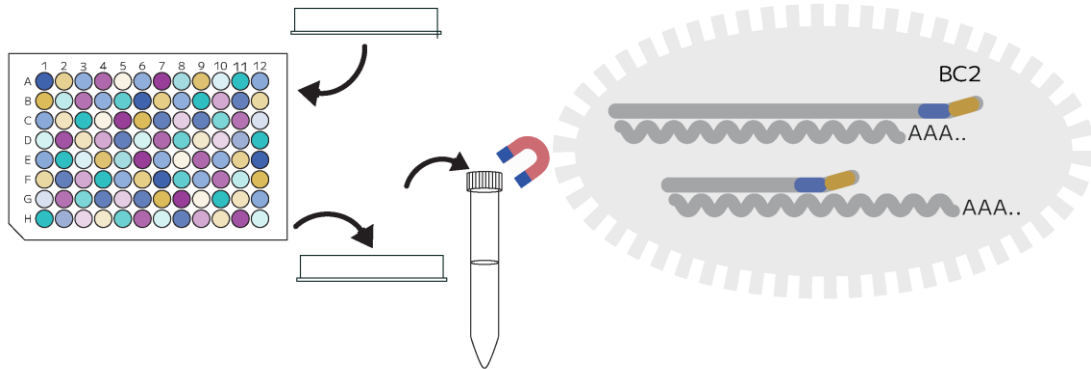
The Evercode MAX WT v4 Cell Barcoding M Barcoding kit can index up to 512,000 cells/nuclei across up to 48 different samples. The Evercode MAX WT v4 Library Prep INTEGRA workflow facilitates 8, 16, or 32 sublibraries following barcoding. Different quantities of sublibraries may need to be run manually.

This manual describes the first 3 rounds of barcoding and the generation of sublibraries as lysates. Users can either store the lysates or proceed directly to library preparation.



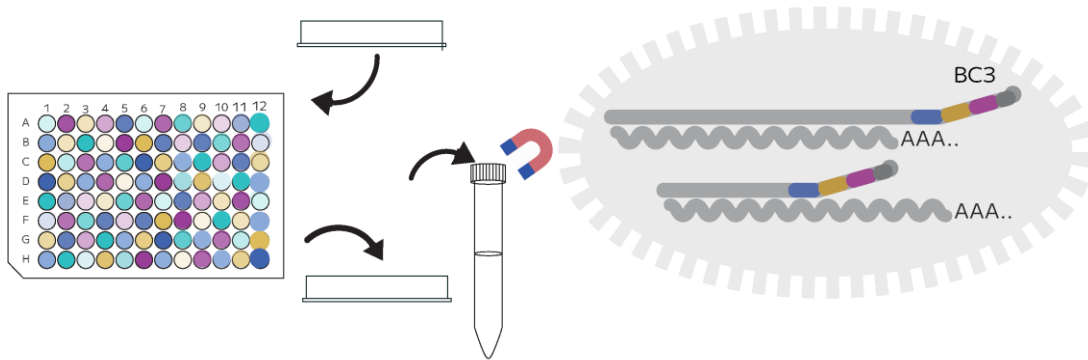
Section 1: *In situ* Sample Barcoding

2



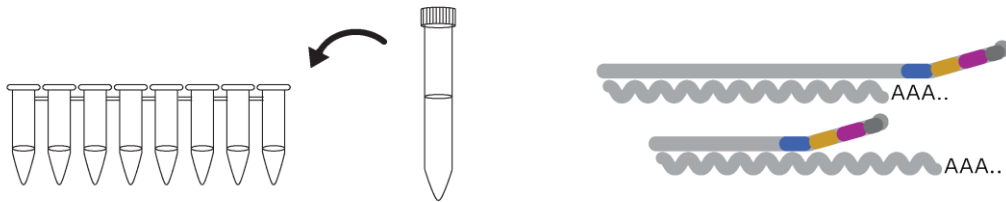
Round 2 Barcoding: The pooled cells/nuclei are loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. Cells/nuclei are pooled and then captured via magnet.

3



Round 3 Barcoding: The cells/nuclei are pooled and loaded into the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina TruSeq Read 2 sequence, and biotin. Cells/nuclei are pooled and then captured via magnet.

4



Lysis and Sublibrary Generation: Cells/nuclei are split across the desired number of sublibraries and lysed.

Important Guidelines

The following guidelines provide additional information to obtain optimal performance with the Evercode MAX WT v4 M with INTEGRA ASSIST PLUS barcoding workflow.

For further information on the experimental or automation workflow, please contact support@parsebiosciences.com. Please contact support-us@integra-biosciences.com for any questions regarding the INTEGRA ASSIST PLUS instrument.

Sample Input

- This protocol begins with cells or nuclei previously fixed with an Evercode Cell Fixation v4 or Evercode Nuclei Fixation v4 kit.
- Samples fixed with the Standard Fixation and the Low Input Fixation kits are fully compatible with the INTEGRA automated barcoding workflow.
- Even if samples were counted before freezing, we strongly recommend counting cells/nuclei again after thawing to account for any changes in cell/nuclei concentration during storage and freeze/thaw. Typically, a 5-15% decrease in concentration after thawing should be expected. These counts are used to determine how cells/nuclei are loaded in the Round 1 Plate, and counting accuracy at this stage is critical to recover the desired number of cells/nuclei following barcoding.
- Standard Fixation samples only: when planning on processing many fixed samples, we recommend setting aside a small "counting aliquot" of each sample at the end of fixation. These counting aliquots can be counted the day prior to starting Section 1 of any Evercode MAX WT v4 Cell Barcoding kit. The Evercode Fixation User Guides outline recommendations for generating aliquots. Because aliquots have undergone a similar storage time and a freeze/thaw, cell/nuclei counts from these aliquots will be more representative than using counts from immediately after fixation.
- Aliquots should be thawed in a water bath set to 37°C in sets of 2-4 and counted with a hemocytometer or alternative counting device. Cell/nuclei counts should be recorded in the Sample Loading Table, and any remaining cell/nuclei material in the thawed counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.
- On the Assist Plus samples will be diluted into a dilution plate prior to Round 1 Barcoding. The Sample Loading Table will recommend the appropriate volume of dilution buffer needed to achieve the correct cell concentration in the required volume. If the required "Number of Sample Dilution Tubes" is greater than 1, three additional dilution buffer tubes for highly concentrated samples can be purchased as a Sample Dilution

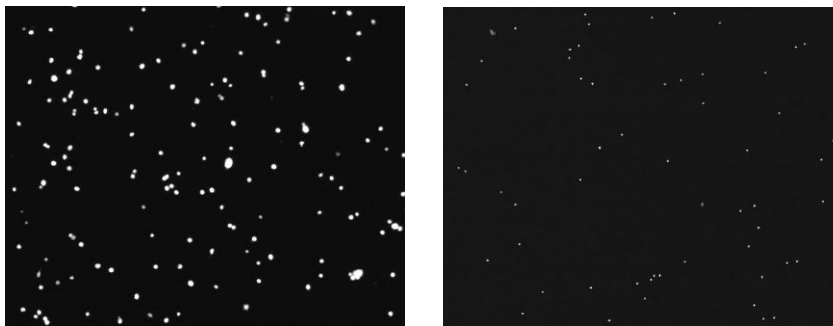
Accessory Kit (PN ECAC3901). This kit is included in the initial INTEGRA bundle, but can be separately purchased for subsequent orders.

Low Input Fixation Sample Input

- Samples fixed with the Low Input Fixation Workflow can be uploaded after cell/nuclei capture. Low Input samples are prepared manually up to step 1.1.12 of the manual Evercode MAX WT v4 M user guide. The diluted low input fixed samples can then enter the Integra automated barcoding workflow at step 1.3.5b in place of the Diluted Sample Plate in this user guide.

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 against a hemocytometer when using Evercode kits for the first time.
- We suggest saving images of cells/nuclei at each counting step. 20x and 40x magnification images provide the most useful information.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI). However, after barcoding a fluorescent dye should be used to count the cells/nuclei as the Sample Capture Beads make counting with trypan blue difficult.
- Examples of AO/DAPI stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells/nuclei.



Example of AO/DAPI stained HEK cells (left) and PBMCs (right).

Avoiding RNase Contamination

- Take standard precautions 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. These can be sprayed on benchtops and pipettes. The Assist Plus Base Unit and hardware can also be wiped down with RNase Zap to inactivate RNases.
- Low retention, filtered pipette tips should be used to reduce RNase contamination from pipettes.

Sample Loading Table

- Ensure that the Evercode™ MAX WT v4 Cell Barcoding M INTEGRA Sample Loading Table being used is the most up-to-date version. The Sample Loading Table can be downloaded from the Parse Biosciences [Customer Support Suite](#). Customer log-in is required to access the Sample Loading Table. The [Sample Loading Table module](#) of Trailmaker™ supports the generation and export of Sample Loading Tables for Evercode MAX WT v4 Cell Barcoding.
- The Evercode™ MAX WT v4 Cell Barcoding M INTEGRA Sample Loading Table should be completed before starting the experiment.
- If the table isn't working as expected, ensure that Macros are enabled in the Sample Loading Table. Be sure to only edit the colored cells in the table to avoid disturbing any calculations.
- In the unlikely event that samples are not concentrated enough according to the Sample Loading Table (Excel spreadsheet), you can either call your local FAS at support@parsebiosciences.com or:
 - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error. This will maintain the desired final library proportion between samples but barcode fewer total cells/nuclei.
 - Proceed as-is. The INTEGRA pipette will dispense the highest possible number of cells based on the existing concentration. This may lead to reduced library yields for samples with particularly low concentrations.

Plate Sealing

- Use clear plastic sticky plate seals. When sealing or unsealing 96 well plates, do not splash liquid onto the PCR plate seal or between wells. Securing plates in PCR tube racks will minimize this occurrence.
- PCR plate seals may be difficult to remove. Carefully peel off the PCR plate seal while applying downward pressure on the edges of the plate to keep it in the PCR plate rack. Avoid touching the top of the open wells.

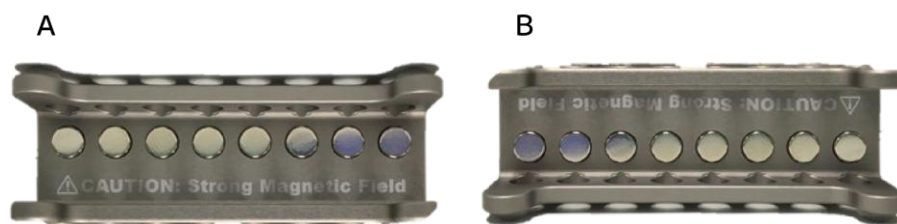
Sample Concentrations

- For an Evercode MAX WT v4 Cell Barcoding M kit, samples can be pre-diluted with Sample Dilution Buffer to a more appropriate working range of 520-3,500 cells/nuclei per μL prior to Sample Dilution on the ASSIST PLUS.
- On the Assist Plus, samples will be diluted with Sample Dilution Buffer to the desired concentration based on the Sample Loading Table.
- For each Standard Fixation sample we recommend preparing a 20 μL post-fixation counting aliquot and two 30-50 μL post-fixation sample aliquots. Prior to aliquoting, the samples should be thoroughly resuspended. The counting aliquot may be thawed in advance of the barcoding day to evaluate post-thaw cell recovery. The cell/nuclei loss observed in this aliquot is expected to closely reflect that of the sample aliquots. Concentrations measured from the counting aliquot can then be used to update the Sample Loading Table accordingly.
- If the sample stock concentrations are too high the Sample Loading Table may indicate that more than 1 tube of Sample Dilution Buffer is needed. Additional Sample Dilution Buffer can be purchased in the Dilution Accessory Box (Parse PN ECAC3901) before starting the barcoding workflow.
- The input format for samples into Sample Dilution is a 96-well plate format. If samples have been frozen in 1.5 mL Protein LoBind Tubes, they will need to be transferred to a LoBind 96-well plate on the day of Barcoding. Label this plate as the Sample Plate.

Magnetic Racks and Bead Cleanups

- Parse Biosciences Magnetic rack for 0.2 mL tubes is required to capture lysed cells/nuclei. The Parse Biosciences Magnetic Rack uses powerful rare earth magnets for rapid and efficient magnetic bead purifications for 0.2 mL tubes. The rack has high and low magnet positions important for optimal yield. We do not recommend substituting alternative racks.

- To alternate between the positions, the rack can be flipped upside down so the magnet is closer to the top (high) or bottom (low) of the 0.2 mL tubes. See the figure below for clarification



Parse Biosciences Magnetic Rack at (A) high and (B) low magnet position.

- To ensure material is not lost during purifications, ensure supernatants are completely clear before moving to the next step. The incubation times at each step are recommendations, but visual confirmation of clearing should be used to make the final determination. See the figure below for an example of completely cleared supernatants.



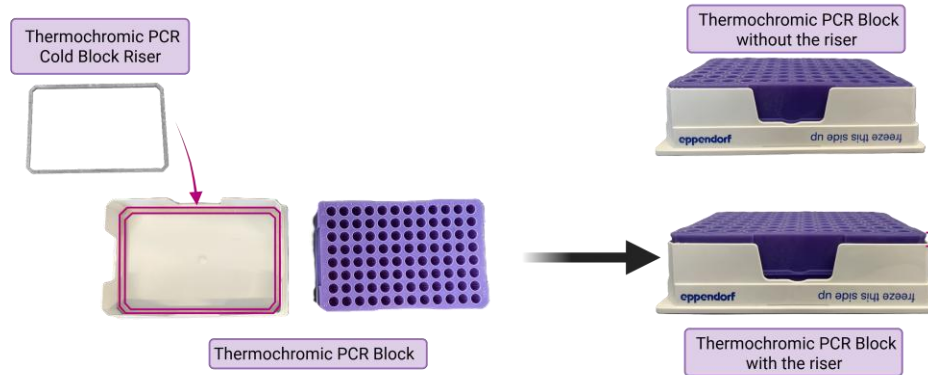
Clear supernatant with compact bead pellets.

- The Evercode MAX WT v4 workflow supports preparation of up to 32 sublibraries using two Evercode MAX WT v4 Library Prep 16-Reaction kits. If planning to barcode samples for more than 16 sublibraries, we recommend using a PCR plate magnet for capturing lysed cells. Dispense lysates into low-bind 96-well plates if using a 96-well plate magnetic rack.

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. Ensure the Block has been left at room temperature for **10 minutes** to avoid freezing and breaking the cells/nuclei.
- 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.

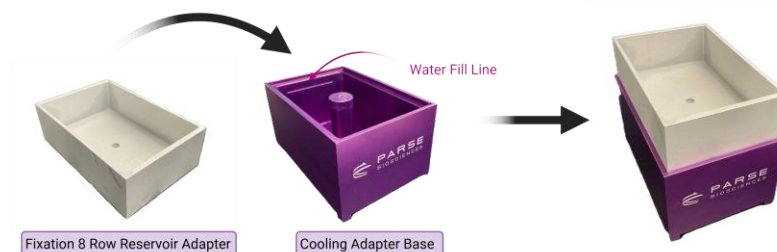


Note: Uneven freezing of the liquid inside the Thermochromic PCR Block can cause slanting of the cooling position. The Thermochromic PCR Cold Block Riser reduces the variability caused by the freezing process. If the PCR Cold Block riser is not used, there will be extra liquid left in the wells of the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

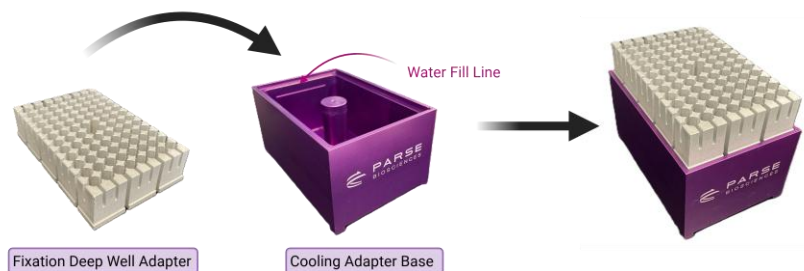
- Tip pinching may occur when using a fully frozen Thermochromic PCR Cold Block for a plate pooling step. To minimize tip pinching, a fully frozen block should be warmed slightly by a few degrees by leaving it at room temperature for 10 minutes before using it on the INTEGRA ASSIST PLUS Deck.
- To ensure 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.

- 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.



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.

INTEGRA ASSIST PLUS Pipetting Programs

- Ensure that the VIALAB program is installed on the same Windows computer that will be used to communicate with the INTEGRA Pipetting Module.
- Ensure that the Evercode workflow precheck script has been confirmed prior to running the INTEGRA + Parse workflow. This can be done by running the [Evercode WT v4 with INTEGRA ASSIST PLUS Precheck](#) Scripts available on the Customer Support Suite.
- Ensure that the .csv worklists generated via the Evercode MAX WT v4 Cell Barcoding M INTEGRA Sample Loading Table ("**CombinedMWorksheet_YYMMDD_HHMMSS.csv** ") are accessible for upload to the VIALAB program.
- 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 and the pipette will keep moving. If the prompt does not contain a double dash, "--", it is followed by another prompt.

Deck Loading

- Always ensure the tip box placed on the deck clicks when put into place. Remove the tip box lid prior to starting the program.
- 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.

Parse Reagents

The Evercode MAX WT V4 Cell Barcoding M kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request

-20°C Reagents Store at -20°C, PN XMM400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	WT101	Green semi-skirted 96 well plate	1
	Round 2 Plate v4	WT142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	WT103	Yellow semi-skirted 96 well plate	1
	Bead Prep Buffer	WT136	1.5 mL tube	1
	Barcoding Buffer	WT137	15 mL bottle	1
	Round 1 Stop Mix	WT138	2 mL tube	1
	Round 2 Stop Mix	WT139	2 mL tube	1
	Ligation Enzyme	WT140	1.5 mL tube	1
	Sample Dilution Buffer	WT105	2 mL tube	1
	Ligation Buffer	WT134	15 mL bottle	1
	Final Stop Mix	WT141	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	WT112	2 mL tube	1
	Pre-Lysis Wash Buffer	WT110	5 mL tube	1
	Lysis Solution	PT146	2 mL tube	1
	Lysis Enzyme	PT109	1.5 mL tube	1

4°C Reagents. Store at 4°C, PN XMM500

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	MG135	0.5 mL tube	1

Parse-Provided Equipment

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

The equipment listed should be stored at -20°C when not in use and taken out when instructed throughout the protocol.

ITEM	PN	QTY	CHECK LIST
Thermochromic PCR Cold Block	NTAC1102	3	☐
Thermochromic PCR Cold Block Riser	NTAC1103	3	☐
Parse Metal Cold Block	NTAC1107	1	☐
Cooling Adapter Base	NTAC1106	1	☐
Self-Standing 8 Row Reservoir Adapter	NTAC1110	1	☐
HEATMAG 8 Row Reservoir Adapter	NTAC1109	1	☐
HEATMAG Cooling Accessory	NTAC1108	1	☐

INTEGRA Components

The following are required INTEGRA components needed to run the Evercode assay on the INTEGRA and should not be substituted.

ITEM	ITEM TYPE	PN	QTY	CHECK LIST
Pipette Communication Module for VIAFLO / VOYAGER Pipettes	Accessory	4222	3	☐
Tip Deck for D-ONE Pipetting Module	Base	4535	1	☐
ASSIST PLUS Labware Pedestal (+24 mm) for D-ONE, portrait, for 10 mL, 25 mL, 100 mL, reservoirs and plates/reservoirs in SLAS/ANSI format (24 mm Labware Pedestal)	Adapter	4551	1	☐
D-ONE Pipetting Module 1-Ch, 5-1250 μ L	Pipette	4532	1	☐
VIAFLO Pipette 12-Ch, 10-300 μ L	Pipette	4633	1	☐
VOYAGER Pipette 8-Channel, 5-125 μ L	Pipette	4722	1	☐
ASSIST PLUS Base Unit	Main	4505	1	☐
Communication/Charging Cable for VIAFLO	Accessory	4226	1	☐
HEATMAG Module	Module	4901	1	☐
96 Well Adapter for HEATMAG module	Adapter	4906	1	☐

Consumables and Reagents

The following is a list of consumables and reagents required to successfully perform Parse Evercode workflows on the INTEGRA ASSIST PLUS.

INTEGRA Consumables

ITEM	PN	QTY	CHECK LIST
8 Row Reservoirs, Polypropylene, Pyramid Bottom (a.k.a. Self-Standing 8 Row Reservoir)	6372	2	☐
1250 µL Pipette Tips	6545	1	☐
300 µL Pipette Tips	6535	4	
125 µL Pipette Tips	6565	2	

Other Consumables

ITEM	SUPPLIER	PN	QTY	CHECK LIST
Rigid Plate Strainer (30 µL, 70 µL, 100 µL)	Parse Biosciences	RPS1030 RPS1070 RPS1100	6-pack	☐
10 mL serological pipette	Various Suppliers	Varies	as needed	☐
1.5 mL Tubes	Genesee Scientific	21-257	2	☐
8-count PCR strip tube	USA Scientific®	1402-4700	as needed	☐
twin.tec® Semi-Skirted PCR Plate96 LoBind®Semi-skirted 96 well plates	Eppendorf®	E951020362	as needed	☐
SealPlate®	Excel Scientific	100-SEAL-PLT	as needed	☐
TempPlate Sealing® Foil	USA Scientific	2923-0100	as needed	☐
Pipette Tips RT LTS 20 µL FL 960A/10 RT LTS 200 µL FL 960A/10 RT LTS 1000 µL FL 768A/8	Rainin®	30389226 30389240 30389213	as needed	☐

Reagents

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific®	AM9780	Or equivalent RNase decontamination solution.	☐
Propidium Iodide (PI)	Various Suppliers	Varies	Or alternative fluorescent dyes to assess viability, such as AO/PI, Hoechst, DAPI, Calcein AM, etc.	☐

Equipment

The following equipment is 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.

Equipment

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Parse Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.	☐
Permagen Bar Magnet	Permagen®	MSPU650	<u>Optional</u> : if preparing >16 sublibraries. We do not recommend alternative magnets, as they may result in lower transcript and gene detection.	☐
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 96 well plates and capable of reaching 4°C.	☐
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes and PCR 8-strip tubes.	☐
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.	☐
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting devices compatible with fluorescence-based cell counting. We recommend validating alternatives relative to a hemocytometer.	☐
Serological pipet controller	Various Suppliers	Varies		☐
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.	☐
T100 Thermal Cycler	Bio-Rad Laboratories®	1861096	Or an equivalent thermocycler compatible with semi-skirted 96 well plates, 0.2mL PCR tubes with up to 100 µL of sample volume, sample heating and cooling from 4-98°C, and a heated lid capable of 30-105°C.	☐

ITEM	SUPPLIER	PN	NOTES	CHECK LIST
Water bath	Various Suppliers	Varies	Or equivalent thermomixer or heat block capable of holding temperature at 37°C.	☐
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM for 30 minutes.	☐
6-inch Platform	Scientific Industries	146-6005-00		
Microplate Foam Insert	Scientific Industries	504-0235-00		

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.

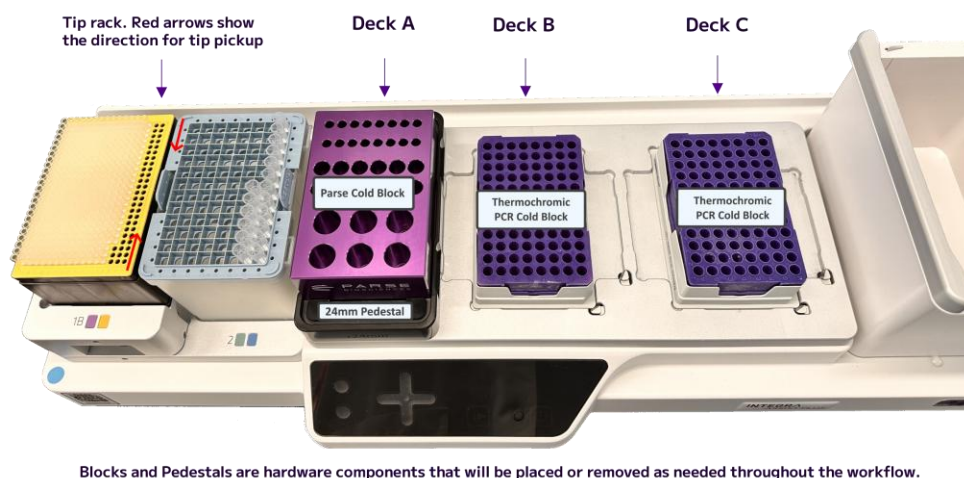
There are three types of figures in this manual.

- 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.



Reading and Understanding the Deck Configurations

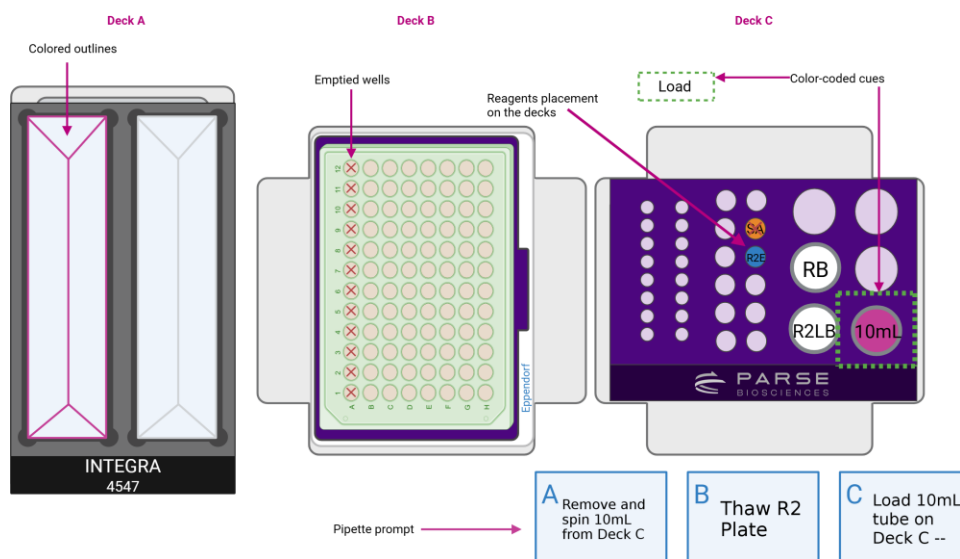
Each subsection includes multiple deck configurations that illustrate the steps executed by the program. These configurations display the exact placement of reagents and samples on the

decks, list the consumables required at each step, and highlight pipette prompts for manual intervention.

Graphical representation of basins and/or tubes with colored outlines show where specific reagents will be added. Each outline color matches the color of the reagent's label.

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.

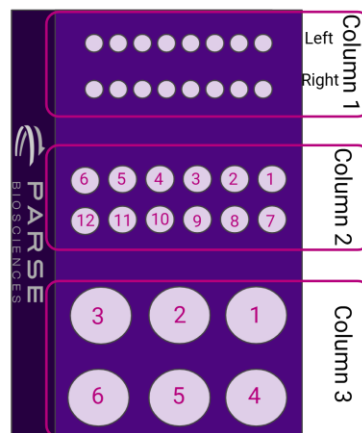
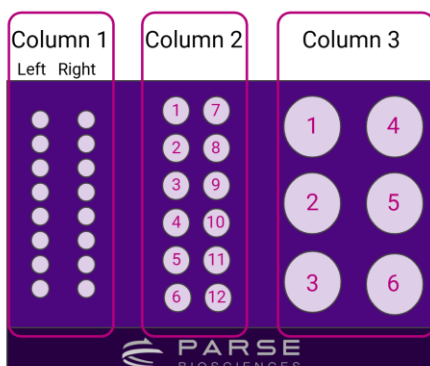
Letter-coded pipette prompts indicate the specific action the robot is about to perform when initiated. A double dash ("--") at the end of a prompt signifies that all prompts have been entered, and the robot will begin executing the programmed movements immediately after.



The Parse Metal Cold Block

It is critical to place reagents, samples, and consumables in the correct positions on the Parse Metal Cold Block.

The Parse Metal Cold Block is organized into three main columns, 1, 2 and 3, each containing two sub-columns (left and right) with slots of varying sizes to accommodate different tube types.



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.



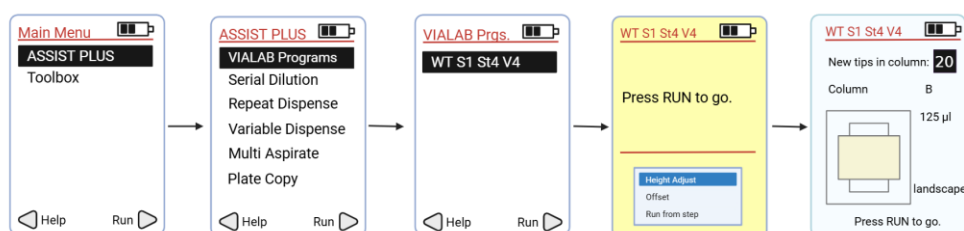
- **Load:** Indicates labware and/or reagents that need to be placed or reloaded onto the deck.
- **Remove:** Identifies labware that should be taken off the deck, either for incubation, disposal, or because it is not needed in the upcoming step.
- **Replace:** Highlights labware that has been used and needs to be swapped out for clean or new consumables.
- **Pipette:** Prompts the user to manually dispense reagents into designated basins.
- **Mix:** Instructs the user to mix specific reagents.
- **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: Automation Setup & In Situ Barcoding

1.1. Sample Normalization

This section is for Standard Fixed samples only. If using **Low Input Fixed samples**, manually capture and dilute the cells. Skip this section and proceed to Section 1.2.

Prior to setting up the Automation workflow, count the cells/nuclei to assess quality and concentration of the fixed sample(s). Samples should be diluted with Sample Dilution Buffer to the target concentrations listed in the Sample Loading Table.

After adjusting the sample(s) to the recommended dilution range, update the Sample Loading Table, and download the Sample Loading Table macro (Section 1.1.6). This .csv file will be used as reference for allocating the fixed cells/nuclei into the 96-well PCR Dilution Plate, to prepare samples to be loaded into the Barcoding Round 1 Plate. The recommended dilution range for M is 520-3,500 cells/nuclei per μL .

The program uses the Sample Dilution Buffer on Deck A to normalize fixed samples from plate format on Deck C into an intermediate dilution plate on Deck B.

To normalize samples:

1. If working with standard fixation samples, remove the ● Sample Binding Beads from the 4°C Reagents box. Gently pulse-vortex (1-2 seconds per pulse) or pipette mix the beads until completely resuspended. Observe the beads to confirm they stay in solution for **at least 2-3 minutes** before beginning to settle. Store the ● Sample Binding Beads at room temperature until use.



CRITICAL! If you observe that the Sample Binding Beads are frozen or dried, and settled at the bottom the tube within ~10 seconds after pulse-vortexing, **do not proceed**. Contact your FAS or support@parsebiosciences.com for assistance

2. Cool a centrifuge with swinging bucket rotors to 4°C.
3. Set a water bath to 37°C.
4. Fill an ice bucket with ice.

5. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
24 mm Labware Pedestal	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	2	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate	Consumables	2	
● Sample Binding Beads	4°C Reagents	1	Keep at room temperature. Ensure beads are not settled before use.
● Sample Dilution Buffer	-20°C Reagents or ECAC3901	1	Thaw at room temperature then store on ice. Mix by inverting 3x.

- Download the Evercode MAX WT v4 Cell Barcoding M INTEGRA Sample Loading Table. The most current version of the Evercode M v4 INTEGRA Sample Loading Table can be found on the Parse Biosciences [Customer Support Suite](#)_0. Customer log-in is required to access the Sample Loading Table. Or generate and export from the [Sample Loading Table module](#) on Trailmaker.
- Thaw the previously fixed cells/nuclei samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.
- If not done beforehand, count the number of cells/nuclei with an automated cell counter or alternative cell counting device. Record the cell/nuclei count. This will be used to fill out the Sample Loading Table in section 1.1.9b.
- Fill the Sample Loading Table tab of the worksheet.

- a. Per the instructions in the worksheet, input number of samples (Figure 1).



Note: For more information on using the Sample Loading Table, see the "Important Guidelines" section of the User Guide.

This sheet should be filled out prior to starting Section 1.

Step	Instructions
1	Ensure Macros are enabled.
2	Input the number of samples.
3	Input the target number of barcoded cells. Note: The default is 100,000 cells for Evercode WT.
4	Input your sample names.
5	Input the target percentage representation of each sample in the final library. CRITICAL: No percentage can be lower than 2.09%.
If not already done, count the samples as described in Section 1.1 of the Evercode WT User Manual.	
6	Input stock cell concentration for each sample.
7	Prepare the dilutions as described. CRITICAL: Ensure that Sample Dilution Buffer is completely thawed before use.
8	Open the "Plate Configuration" sheet. With the plate on ice, add 14 μ L of each diluted sample to the appropriate well(s) of the Round 1 Plate as shown in the plate map. CRITICAL: Follow the instructions in the User Guide with respect to sample mixing and changing tips.

CRITICAL: We do not recommend editing cells highlighted in grey.

Number of Samples (Step 2):

Target Number Barcoded Cells (Step 3):

Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/ μ L) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/ μ L)
1		100.00%		48	100000	520
TOTALS:		100.00%		48	100,000	

Figure 1: Evercode WT Sample Loading Table.

- b. Enter Target Number of Barcoded Cells, Sample Name, Percent of Library and Stock Concentration for corresponding samples. Errors and possible solutions can be found in Appendix D (Figure 2).



Note: If any parameter is out of range, the cells will turn pink in color and/or an error message will appear (Figure 2). Be sure to address and adjust worksheet input values appropriately before continuing.

This sheet should be filled out prior to starting Section 1.

Step	Instructions
1	Ensure Macros are enabled.
2	Input the number of samples.
3	Input the target number of barcoded cells. Note: The default is 100,000 cells for Evercode WT.
4	Input your sample names.
5	Input the target percentage representation of each sample in the final library. CRITICAL: No percentage can be lower than 2.09%.
If not already done, count the samples as described in Section 1.1 of the Evercode WT User Manual.	
6	Input stock cell concentration for each sample.
7	Prepare the dilutions as described. CRITICAL: Ensure that Sample Dilution Buffer is completely thawed before use.
8	Open the "Plate Configuration" sheet. With the plate on ice, add 14 μ L of each diluted sample to the appropriate well(s) of the Round 1 Plate as shown in the plate map. CRITICAL: Follow the instructions in the User Guide with respect to sample mixing and changing tips.

CRITICAL: We do not recommend editing cells highlighted in grey.

Number of Samples (Step 2):

Target Number Barcoded Cells (Step 3):

Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/ μ L) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/ μ L)
1	SA-0001	32.00%	500	14	32000	571
2	SA-0002	48.00%	3,300	24	48000	499
3	SA-0003	20.00%	5,250	10	20000	499
TOTALS:		100.00%		48	100,000	

CRITICAL: This cell stock concentration is too low.

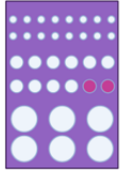
Figure 2: Example error message, noting that the sample stock concentration is too low.

- c. Navigate to the "INTEGRA Loading Table" tab. If the "Required Number of Sample Dilution Tubes" is greater than 1, three additional dilution buffer tubes for highly

concentrated samples can be purchased as a Sample Dilution Accessory Kit (see "Sample Concentrations" in Important Guidelines) (Figure 3).



Note: Example: Sample 1 is loaded into Sample Location A1 and Sample Location A2 with a minimum volume of 131 μ L. More sample volume (up to a maximum of 200 μ L total sample volume) can be loaded to reduce bubbles during mixing.

Sample Name	Sample Location	Min Sample Stock Needed for Dilution (μ L)	*Note: due to semi-skirted plate volumes, multiple wells might be needed for the same samples.	Min Diluent Needed (μ L)	Required Number of Sample Dilution Tubes	Sample Dilution Tube Locations
Sample 1	A1	131.0		1955.0	2	
Sample 1	A2	131.0				
Sample 2	A3	84.0				
Sample 3	A4	84.0				
Sample 4	A5	84.0				
	A6					
	A7					
	A8					
	A9					
	A10					
	A11					
	A12					
	B1					
	B2					

EXTRA SAMPLE DILUTION TUBES REQUIRED TO COMPLETE INTEGRA SAMPLE NORMALIZATION

Figure 3: INTEGRA Loading Table tab noting Minimum Diluent Needed and Sample Locations.

- Referring to the "INTEGRA Loading Table" tab, manually load fixed samples into the directed "Sample Location" well into a new Eppendorf semi-skirted plate. Note that any individual sample may require loading into more than one "Sample Location" well. This is the sample stock plate. Store the sample stock plate on ice for later use.
- Navigate to the "Diluent Volumes" tab and click on the purple box labeled "Generate a Worklist for Import into VIALAB". Save the generated CSV file (called "CombinedMWorksheet_YYYYMMDD_HHMMSS.csv") for later use (Figure 4).

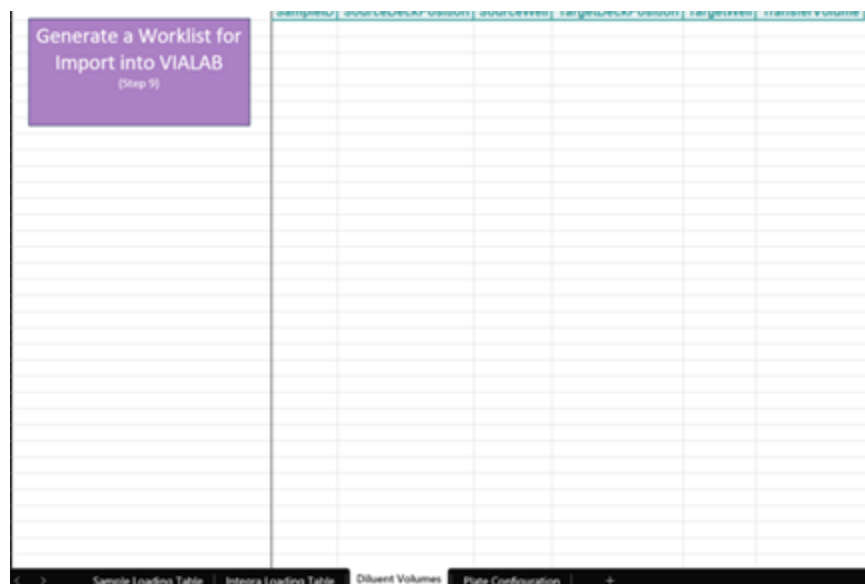


Figure 4: Diluent Buffer Volumes tab for generating a VIALAB worklist.

- f. Navigate to the “Plate Configuration” tab to visualize the final sample location within the 96 well plate format (Figure 5).

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Seattle, WA 98109



Evercode WT Round 1 Barcoding Plate Configuration

- For more details on using this Sample Loading Table, see the Important Guidelines section of the User Manual

Use the following plate layout to load samples into the Round 1 Plate in Section 1.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	2	2	2	2	2	2	2	2	2	2
C	2	2	2	2	2	2	2	2	2	2	2	2
D	2	2	3	3	3	3	3	3	3	3	3	3
E	Do not add samples to these wells.											
F												
G												
H												

Sample Number	Sample Name	Percent Contributing
1	SA-0001	32.00%
2	SA-0002	48.00%
3	SA-0003	20.00%

Figure 5: Plate Configuration tab visualizes the sample locations.

10. Import the generated CSV files into VIALAB to be uploaded on the pipettes.

- a. Open the VIALAB program **M S1 St1 DONE V4_0** and navigate to the “Method” section.



CRITICAL! Delete any previous S1 St1 scripts from the D-ONE pipette to avoid running incorrect scripts.

- b. In the “O2 Worklist”, under the “Worklist and Volumes” tab, upload the “CombinedMWorksheet_YYYYMMDD_HHMMSS.csv” worklist file generated in Step 9e using the “Import” button (Figure 6).

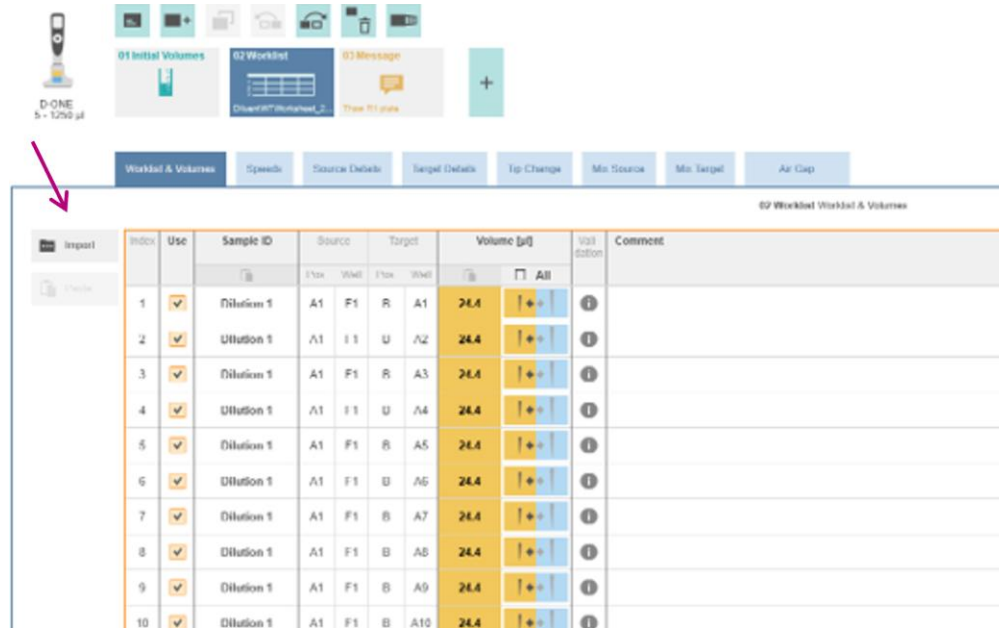
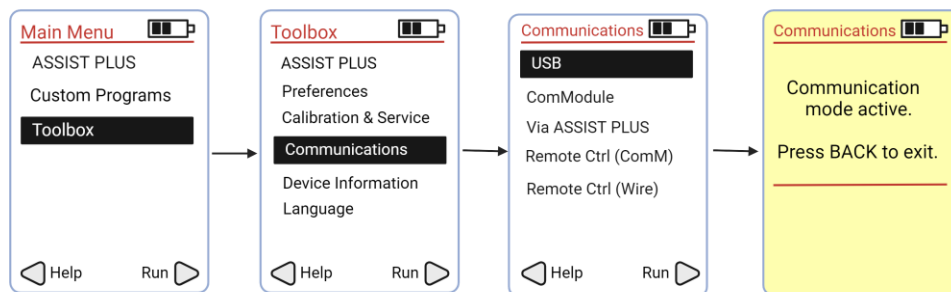


Figure 6: VIALAB worklist generation for diluent volumes using O2 Worklist.

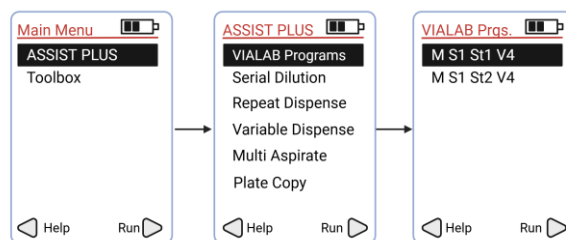
11. Save and transfer your program to your D-ONE Pipetting Module (1-Ch, 5-1250 µL) as follows, ensuring that any program previously uploaded on the pipette is deleted:

- Connect your computer with D-ONE Pipette using Communication/Charging Cable for VIAFLO.
- Follow the instructions on the pipette menu, as shown in the diagram below:



- Select the "Transfer" tab in the opened **M S1 St1 DONE V4_0** VIALAB program.
- Confirm that the pipette has been recognized by the software. Click "Send to pipette". An audible signal will indicate the successful upload of the M S1 St1 V4_0 program to the D-ONE Pipette.
- Disconnect the cable and proceed with the protocol on the INTEGRA ASSIST PLUS platform.

- f. If done correctly, a program named **M S1 St1 V4_0** will be found on your pipette as shown in the diagram below.

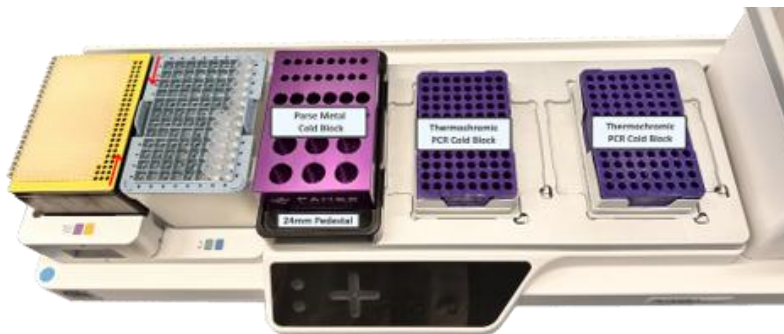


12. Load the hardware on deck.

- a. Load the Parse Cold Block on the 24mm Pedestal on Deck A.
- b. If not done earlier, leave two Thermochromic PCR Cold Blocks with Risers on Deck with A1 at the bottom left corner at room temperature for 10 minutes prior to use. Load one on Deck B and the other on Deck C. The deck should correspond to the Deck Configuration below.



Note: 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.



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

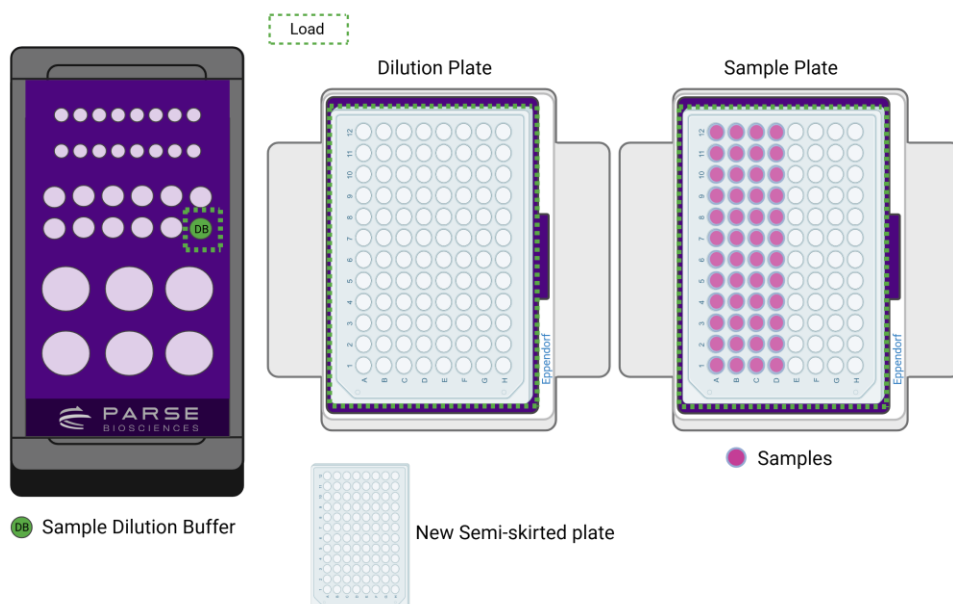
- a. Deck A, column 2
 - i. Pos 7: ● Sample Dilution Buffer.



Note: If the Sample Loading Table states that more than 1 Sample Dilution Buffer tube is required, follow the INTEGRA Loading Table tab Sample Dilution Table Locations image for tube loading (Figure 3).

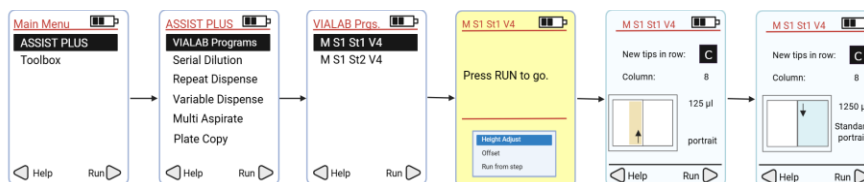
- b. Deck B: a clean semi-skirted plate on Deck B with A1 corner in the bottom left.

- c. Deck C: Sample plate with A1 corner in the bottom left.
- Label the 96-well plate that contains your Parse-fixed samples as the **Sample Plate**.
 - Refer to the Integra Loading Table tab of the Sample Loading Table for correct sample layout in the **Sample Plate**.
 - If samples have been frozen in individual 1.5 mL Protein LoBind Tubes or 8-strip PCR tubes, they will need to be transferred to a LoBind 96-well plate on the day of Barcoding.



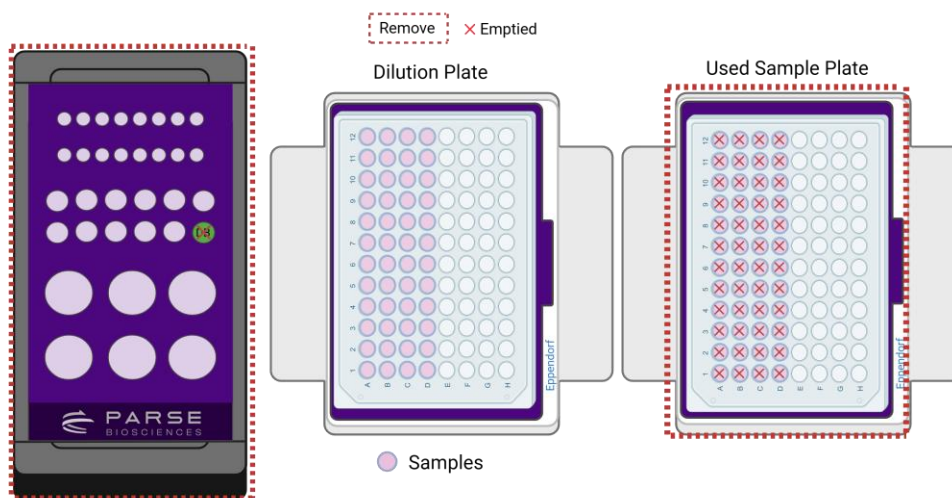
14. Attach D-ONE Pipetting Module 1-Ch, 5-1250 μ L and the corresponding Tip Deck. Ensure the Tip Deck and tip box are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

- a. Remove the reagent caps, then select and run the program **M S1 St1 V4_0**.



15. At the conclusion of the run:

- Store any remaining ● Sample Dilution Buffer from Deck A on ice. Keep the hardware on Deck A. This will be used in the next step.
- Label the plate on Deck B as the **Dilution Plate** and store on ice. This will be used in WT S1 St3. Remove the Thermochromic PCR Cold Block from Deck.
- Remove all labware on Deck C. Discard the used **Sample Plate** on Deck C.



1.2. Reagents Preparation

This program takes reagents from Deck A and moves them into 8 Row Reservoir positions in Decks B and C.

To prepare the reagents:

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
24 mm Labware Pedestal	INTEGRA	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
Cooling Adapter Base	Parse	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. Replace ice as necessary.
Self-Standing 8 Row Reservoir Adapter	Parse	1	
HEATMAG Module	INTEGRA Component	1	
Heatmag 8 Row Reservoir Adapter	Parse	1	
Heatmag Cooling Accessory	Parse	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. Replace ice as necessary.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
6-Tube Magnetic Separation Rack	Equipment	1	
Self-Standing 8 Row Reservoir	Consumables	2	
1.5 mL tube	Consumables	1	
● Round 2 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing. Briefly centrifuge before use.
○ Final Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing. Briefly centrifuge before use.
○ Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
● Round 1 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Ligation Buffer	-20°C Reagents	1	
● Bead Prep Buffer	-20°C Reagents	1	
○ Barcoding Buffer	-20°C Reagents	1	Thaw in 37°C water bath, then store on ice.
● Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

2. Fill a Cooling Adapter Base with pebble ice and place it on Deck B. If the Cooling Adapter Base was 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.
3. Place the Self-Standing 8 Row Reservoir Cooling Adapter on the Cooling Adapter Base on Deck B.
4. Place the HEATMAG with the INTEGRA logo oriented to the left on Deck C. Ensure the wires are not interfering with the HEATMAG position. Ensure HEATMAG power is on for the duration of Section 1.
5. Fill the HEATMAG Cooling Accessory with pebble ice. If the HEATMAG Cooling Accessory was filled with water and frozen the night before, ensure that the HEATMAG Cooling Accessory is thawed at room temperature for at least **10 minutes** before use.

6. If not done already, remove the PCR plate adapter from the HEATMAG. Place the HEATMAG 8 Row Reservoir Adapter and HEATMAG Cooling Accessory on the HEATMAG following the figure below.

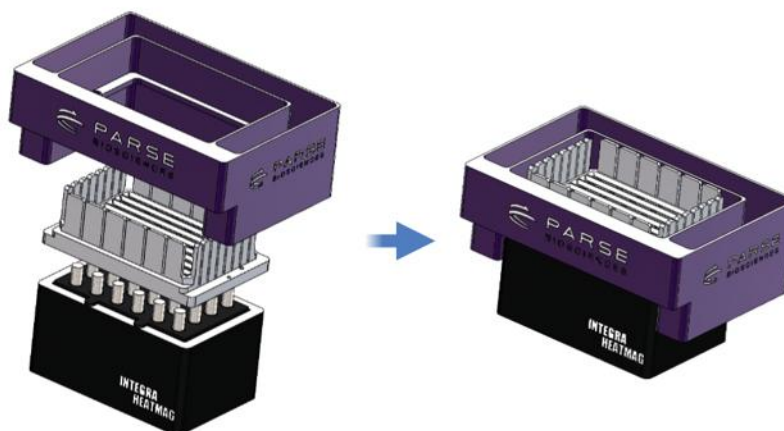
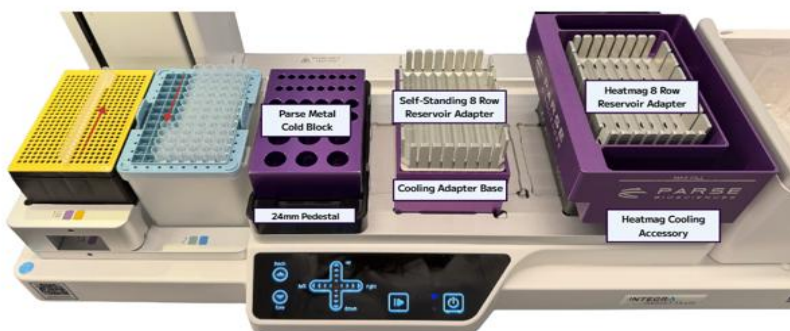


Figure 7: Graphic depicting how the HEATMAG 8 Row Reservoir Adapter and HEATMAG Cooling Accessory fit on the INTEGRA HEATMAG. To check proper alignment, the magnet can be set to high.

7. The deck should match the configuration below.

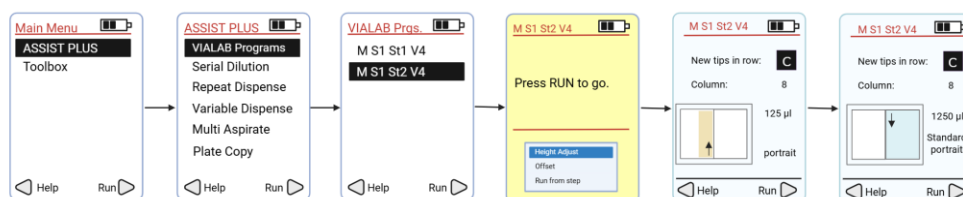


8. Load the consumables, reagents, and samples on Deck:
 - a. Load a clean Self-Standing 8 Row Reservoir on Deck B on the Self-Standing 8 Row Reservoir Cooling Adapter with Row A oriented to the left. Label this reservoir as **Reservoir 1**.
 - b. Load a clean Self-Standing 8 Row Reservoir on Deck C on the HEATMAG 8 Row Reservoir Adapter with Row A oriented to the left. Label this reservoir as **Reservoir 2**.

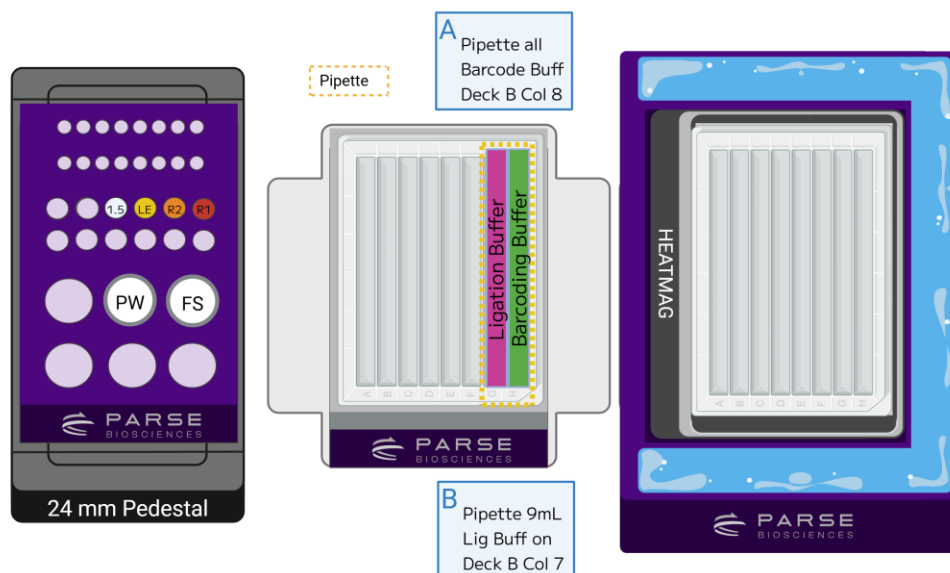
- c. Load the reagents and consumables on the Parse Metal Cold Block on Deck A. If necessary, briefly spin down the tubes to ensure the liquid is at the bottom of the tubes.
- i. Column 2:
 - i. Pos 1: ● Round 1 Stop Mix
 - ii. Pos 2: ● Round 2 Stop Mix
 - iii. Pos 3: ● Ligation Enzyme
 - iv. Pos 4: Clean 1.5 mL tube
- ii. Column 3:
 - i. Pos 1: ○ Final Stop Mix
 - ii. Pos 2: ○ Pre-Lysis Wash Buffer
- c. Load a clean Self Standing 8 Row Reservoir on Deck B on the Self-Standing 8 Row Reservoir Cooling Adapter with Row A oriented to the left. Label this reservoir as **Reservoir 1**.
- d. Load a clean Self Standing 8 Row Reservoir on Deck C on the HEATMAG 8 Row Reservoir Adapter with Row A oriented to the left. Label this reservoir as **Reservoir 2**.



9. Remove the reagent caps, select and run the program **M S1 St2 V4_0** following the diagram below.



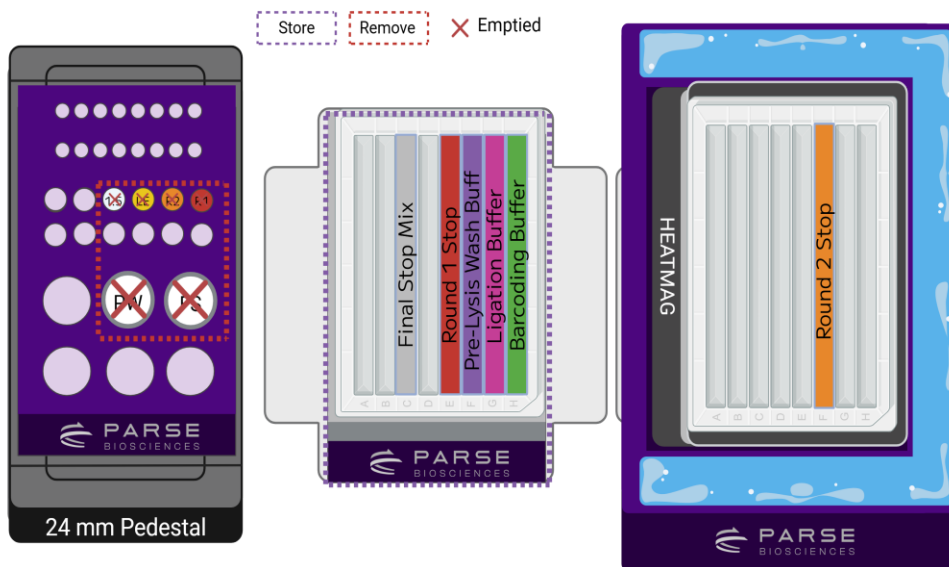
10. Press "Run" to continue the program. Follow the program prompts for manual intervention:



- a. Pipette entire volume of Barcoding Buffer (~13 mL) into Row H of **Reservoir 1** using a clean 10 mL serological pipette.
 - b. Pipette **9 mL** of ○ Ligation Buffer into Row G of **Reservoir 1** using a clean 10 mL serological pipette.
11. While the program is running, manually wash and prep the ● Sample Binding Beads. **If using low input fixation samples, cells/nuclei have been already captured. Skip this step and do not generate the Cell Capture Master Mix.**
- a. Gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **200 µL** of ● Sample Binding Beads to a 1.5 mL tube.
 - b. Place the tube on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).
 - c. While still on the magnet, remove and discard the supernatant.
 - d. Remove the tube from the magnetic rack and fully resuspend the bead pellet in **200 µL** of ● Bead Prep Buffer.
 - e. Place the tube on the magnetic rack until the solution clears (~2 minutes).
 - f. While still on the magnet, remove and discard the supernatant.
 - g. Repeat steps 11d - 11f two more times for a total of 3 washes.

- h. Remove the tube from the magnetic rack. Fully resuspend the pellet in **200 µL** of **○** Barcoding Buffer and store on ice. Barcoding Buffer is in Row H of **Reservoir 1**. Label this tube **CCMM** (Cell Capture Master Mix) and store it on ice for later use.

12. At the conclusion of the run:



- a. Discard all used 1.5 mL, 2 mL, and 5 mL tubes from Deck A. Remove all of the labware on Deck A.
- b. Remove all labware from Deck B. Seal **Reservoir 1** with a PCR plate seal. Store the Cooling Adapter Base, Self-Standing 8 Row Reservoir Cooling Adapter and **Reservoir 1** on ice.

1.3. Round 1, Round 2, Round 3 Plate Loading and Pooling

This program loads samples into the Round 1 Plate on Deck A from the Dilution Plate on Deck B. A deck change occurs where Reservoir 1 is loaded on Deck B. The Round 1 Plate is pooled into Reservoir 1 and the HEATMAG module is used to prepare the sample for Round 2 loading. The Round 2 Plate is then loaded using the prepared sample. The process repeats for Round 2 Pooling and Round 3. At the end of the program, samples will be ready for counting and lysate creation.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch 5-300 µL	INTEGRA Components	1	
Tip Deck for VIAFLO Pipetting Module	INTEGRA Components	1	
300 µL Tip Rack	INTEGRA	3	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	2	
Cooling Adapter Base	Parse	1	Replace ice as necessary.
Self-Standing 8 Row Reservoir Adapter	Parse	1	
HEATMAG Module	INTEGRA Components	1	
Heatmag 8 Row Reservoir Adapter	Parse	1	
Heatmag Cooling Accessory	Parse	1	Replace ice as necessary.
twin.tec® Semi-Skirted PCR 96 wells plate	Consumable	1	
Plate Seals	Consumables	as needed	
 Round 1 Plate	-20°C Reagents	1	Place directly on ice.
 Round 2 Plate V4	-20°C Reagents	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 3 Plate	-20°C Reagents	1	

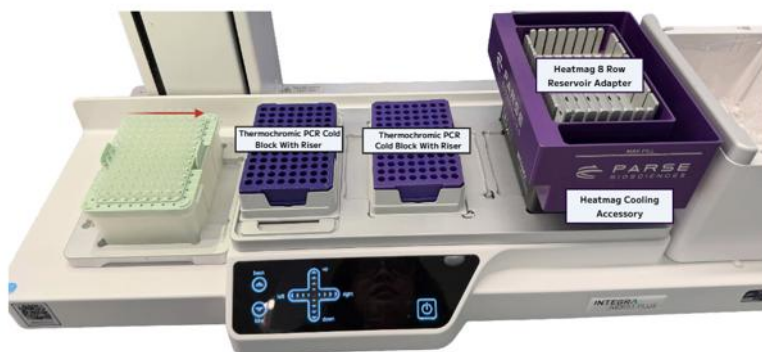
- Thaw the Round 1 Plate using the following thermocycling program. Remove two Thermochromic PCR Cold Blocks to thaw at room temperature for 10 minutes during the following thermocycling program.

THAW ROUND 1 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	26 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

- At the completion of the thermocycler program, centrifuge the Round 1 Plate for **1 minute** at 100 x g at 4°C.
- Load two Thermochromic PCR Cold Blocks with Risers on Deck with A1 on the bottom left. Place one on Deck A and the other on Deck B. The deck should correspond to the Deck Configuration below.

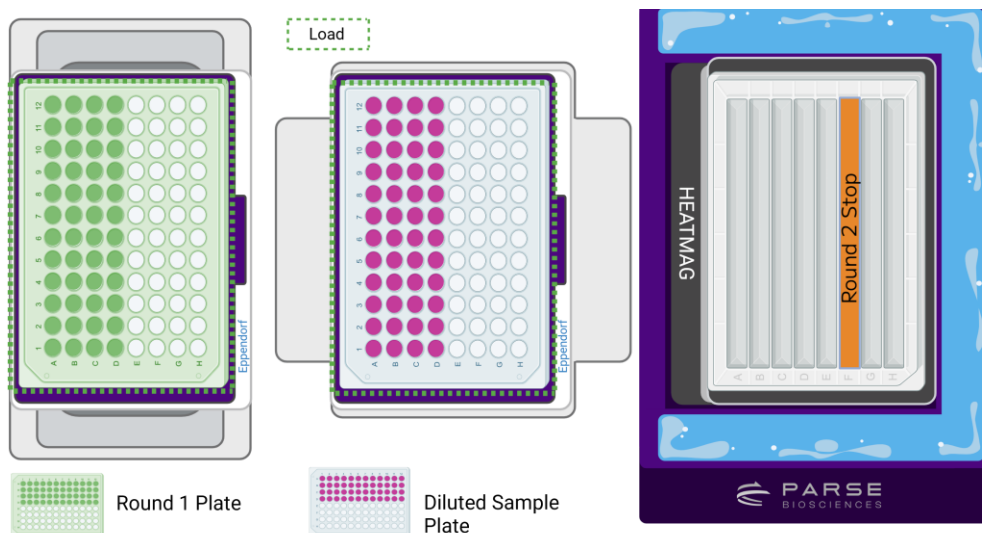


Note: Leave the fully frozen Thermochromic PCR Cold Block with Riser at room temperature for **10 minutes** to ensure it is slightly warmed by a few degrees before using it on the INTEGRA ASSIST PLUS Deck.

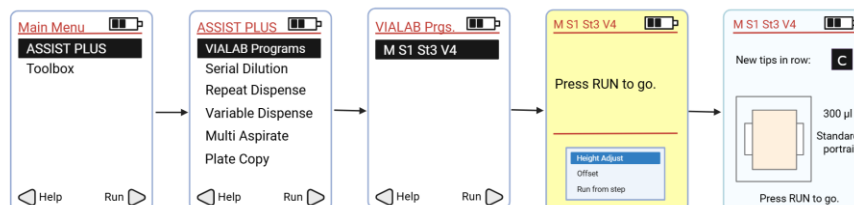


- Load the reagents and samples on deck:

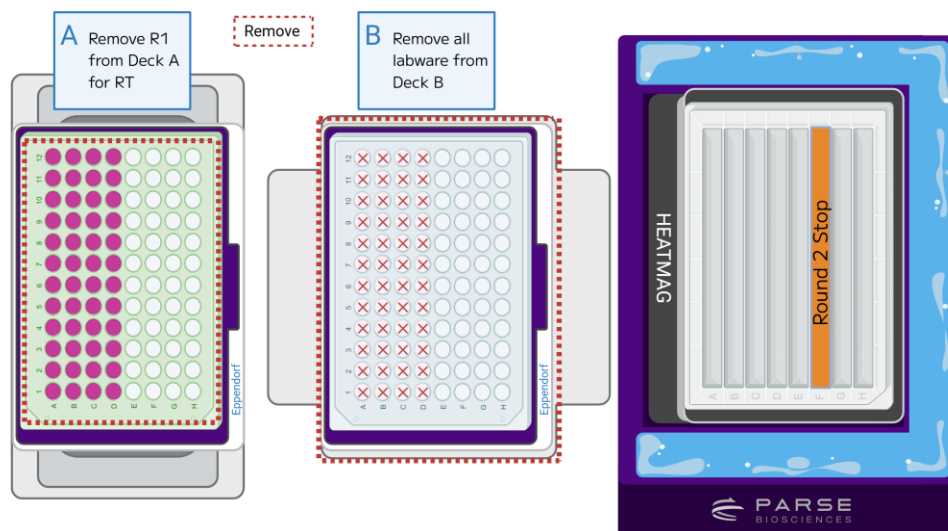
- a. Deck A: While secured in a PCR plate rack on a flat surface, remove the plate seal from the Round 1 Plate and place it on Deck A with A1 at the bottom left.
- b. Deck B: **Dilution Plate** stored from step 1.1.15b. with A1 corner in the bottom left. **If using Low Input Fixed Samples, the captured and diluted samples can be loaded** instead of the **Dilution Plate** from step 1.1.15b. Make sure the layout of the samples in the **Dilution Plate** match the Plate Configuration tab of your Sample Loading Table.



6. Change the pipette that is connected to the INTEGRA ASSIST PLUS:
 - a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 μ L and corresponding Tip Decks. Ensure it is disconnected by pressing the back button until the main menu is displayed.
 - b. Replace it with VIAFLO 12-Ch 10-300 μ L Pipette and 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.
7. Run the program **M S1 St3 V4_0** following the diagram below.



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



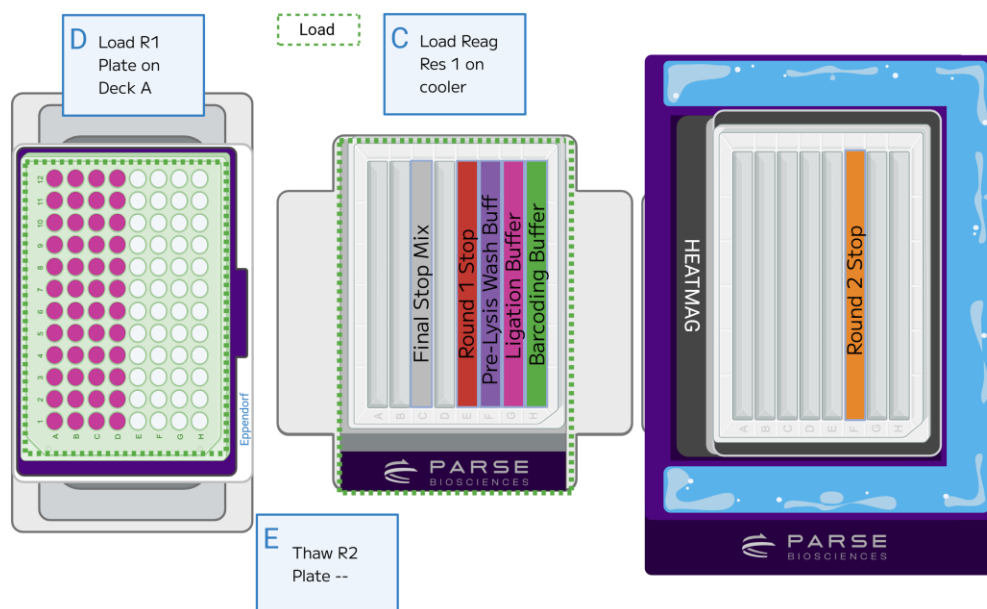
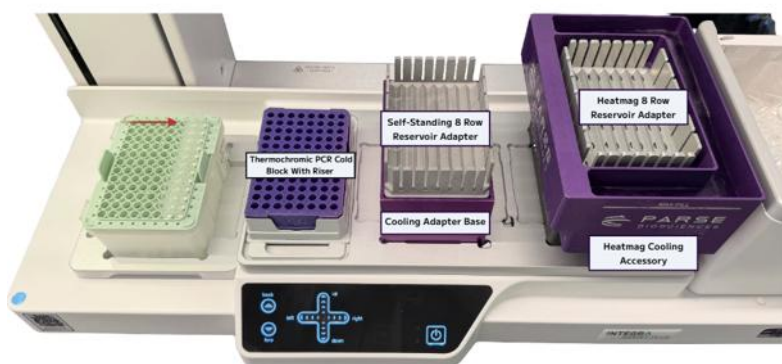
- a. When prompted, remove and seal the Round 1 Plate from Deck A using the Plate Seal Applicator. Place the Round 1 Plate into a thermocycler and run the following program. With the thermal cycler running, press "Run" on the pipette to proceed. Follow the program prompts for manual intervention.

BARCODING ROUND 1			
Total Run Time			40 minutes
Lid Temperature			70°C
Sample Volume			40 µL
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 sec	8°C	3
3	45 sec	15°C	
4	45 sec	20°C	
5	30 sec	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1



CRITICAL! In section 1.4, you will need a thawed Thermochromic PCR Cold Block with a Thermochromic PCR Cold Block Riser. We recommend either leaving one block at room temperature for the remainder of the section 1.3 process, or running it under warm water to thaw quickly prior to section 1.4.

- While the thermocycling program is running, remove all labware from Deck B. The used **Dilution Plate** can be discarded. Remove the Thermochromic PCR Cold Block located on Deck B and store it in the freezer upside down for future use.



10. Load **Reservoir 1** with the Self-Standing 8 Row Reservoir Cooling Adapter on the Cooling Adapter Base stored from step 1.2.12c on Deck B. Ensure row A is facing the left side.
11. When the Barcoding Round 1 Thermocycling program is complete, load the Round 1 Plate on the Thermochromic PCR Cold Block on Deck A with A1 on the bottom left.
12. Gather the following:

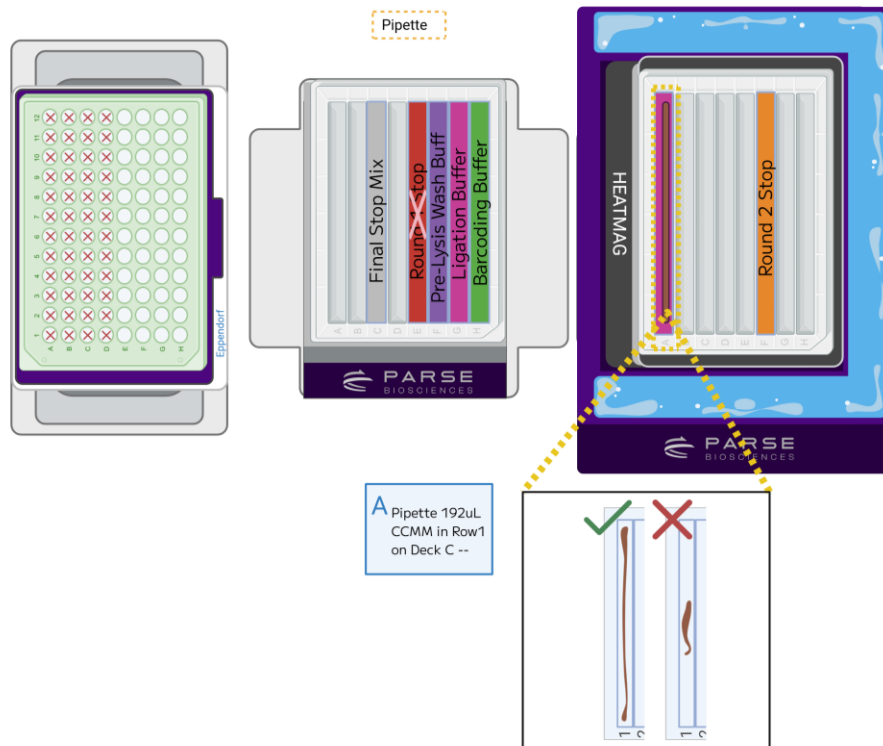
ITEM	SOURCE	QTY	HANDLING AND STORAGE
Thermochromic PCR Cold Block	Parse	1	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block riser	Parse	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Round 2 Plate v4	-20°C Reagents	1	Place directly on ice.

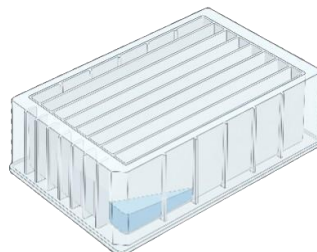
13. Thaw the Round 2 Plate using the program below for later use. While the program is still running, remove a frozen Thermochromic PCR Cold Block with Riser from -20°C freezer and thaw at room temperature for later use.

THAW ROUND 2 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

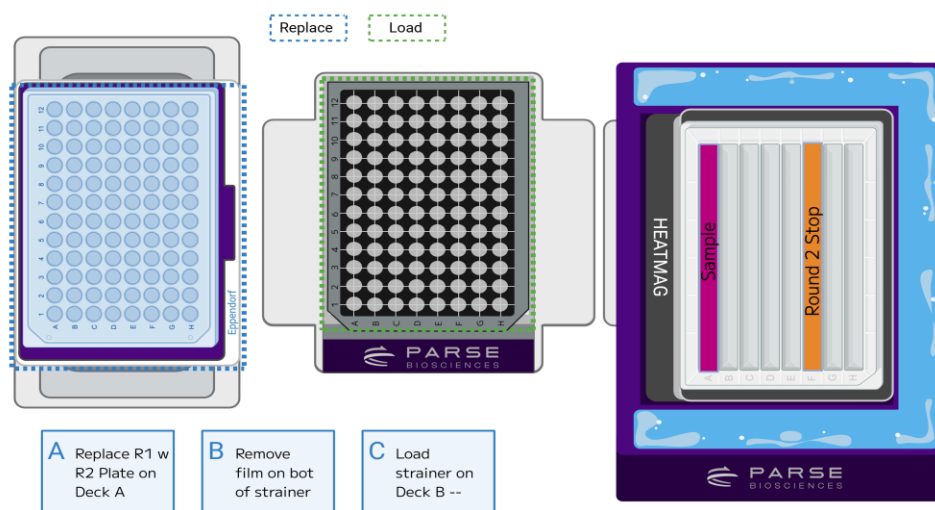
14. At the completion of the thermocycling program, centrifuge the Round 2 Plate for **1 minute** at 100 x g at 4°C. Place the Round 2 Plate on ice for later use
15. While the thermocycling program is running, remove the plate seals from **Reservoir 1** and the Round 1 Plate. Press "Run" to continue the program. Follow the program prompts for manual intervention.



- If using low input fixation samples, cells/nuclei have been already captured. **Skip this step and do not add Cell Capture Master Mix to the reservoir.** If using standard fixation samples, resuspend any settled beads in the Cell Capture Master Mix (CCMM) from step 1.2.11h by pipette mixing. Pipette **192 μ L** of the CCMM across Row A of **Reservoir 2** on Deck C.
- Pipette across the whole row to ensure even cell capture. Tilt the reservoir and mix the CCMM with the sample 20 times with a P1000 pipette set to 1000 μ L.
- Return reservoir to flat position and spread out beads evenly across Row A to ensure even capture.



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



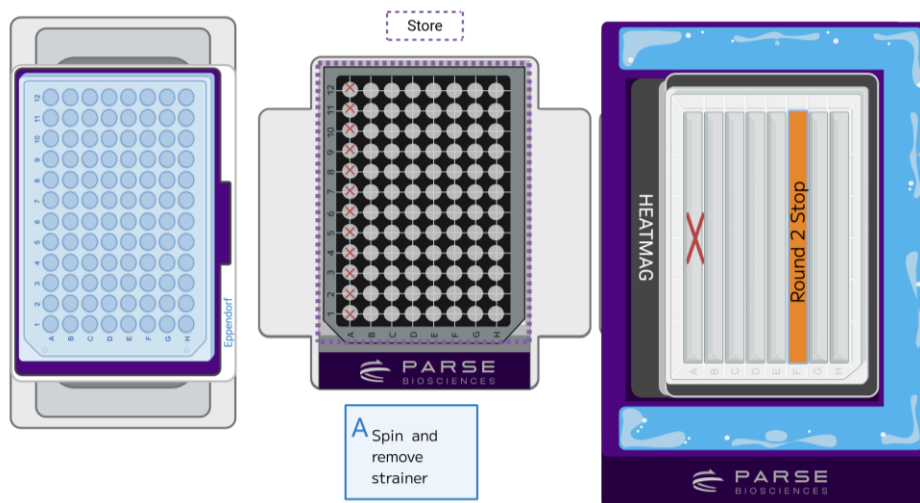
- a. Replace the Round 1 Plate and Thermochromic PCR Cold Block with Riser on Deck A with the Round 2 Plate and Thermochromic PCR Cold Block with Riser thawed in step 1.3.13.
- b. Open a new Rigid Plate Strainer. Remove the film on the bottom of the strainer.



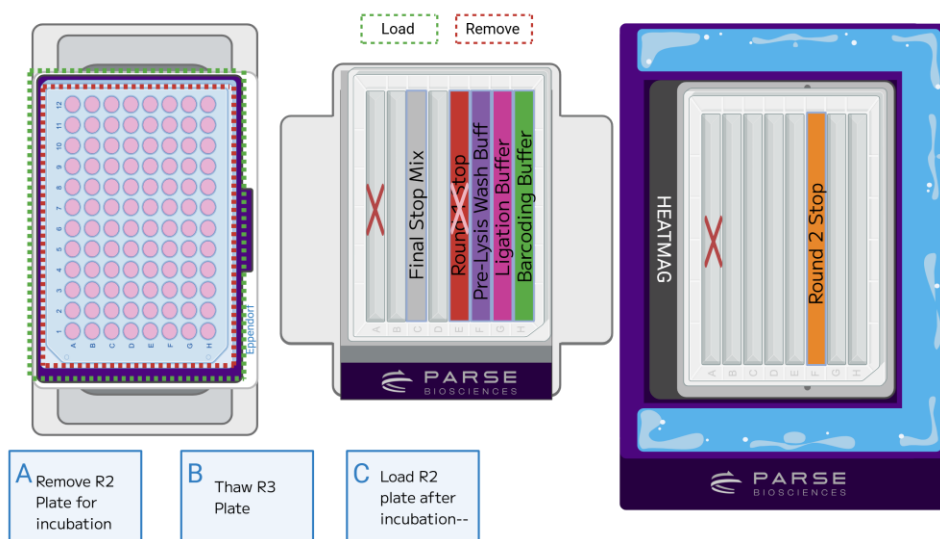
CRITICAL! Choose an appropriate mesh size for your sample type. The wrong size will lead to cell loss.

- c. Load the Rigid Plate Strainer and press firmly on top of **Reservoir 1** on Deck B. Ensure A1 is on the bottom left.

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



- a. Ensuring the strainer is not jostled, carefully remove **Reservoir 1** with the strainer on top from deck B. Centrifuge the strainer on **Reservoir 1** at 100g x **10 seconds**. Remove and store the strainer on a clean semi-skirted plate for later use. Reload the **Reservoir 1** on Deck B.
18. Press "Run" to continue the program. Follow the program prompts for manual intervention.



19. Remove and seal the Round 2 Plate from Deck A using the Plate Seal Applicator. Place the Round 2 Plate into a thermocycler and run the following program

BARCODING ROUND 2		
Run Time	15 minutes	
Lid Temperature	50°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

20. Gather the following:

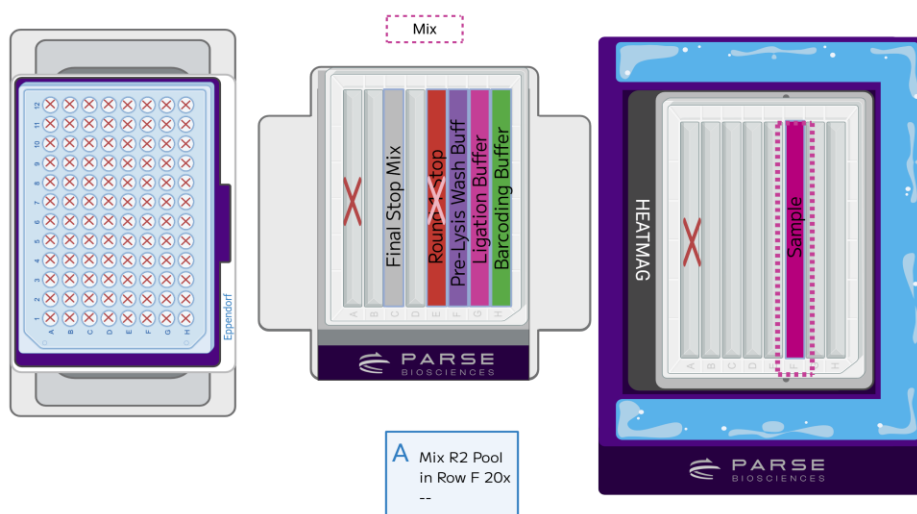
ITEM	SOURCE	QTY	HANDLING AND STORAGE
Hemocytometer	Equipment	1	
Propidium Iodide (PI)	Reagents	1	
Thermochromic PCR Cold Block	Parse	1	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block riser	Parse	1	
Round 3 Plate	-20°C Reagents	1	Place directly on ice.
6-Tube Magnetic Separation Rack	Equipment	1	
1.5 mL tube	Consumables	1	
8-count PCR tube	Consumables	as needed	<u>Optional</u> : if preparing 24 or 32 sublibraries, you can use a low-bind semi-skirted plate.
● Pre-Lysis Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.

21. At the completion of the Barcoding Round 2 thermocycling program, thaw the Round 3 Plate using the program below for later use. While the Thaw Round 3 Plate program is still running, remove a frozen Thermochromic PCR Cold Block with Riser from -20°C

freezer and thaw at room temperature for later use. Press “Run” to continue the pipette program.

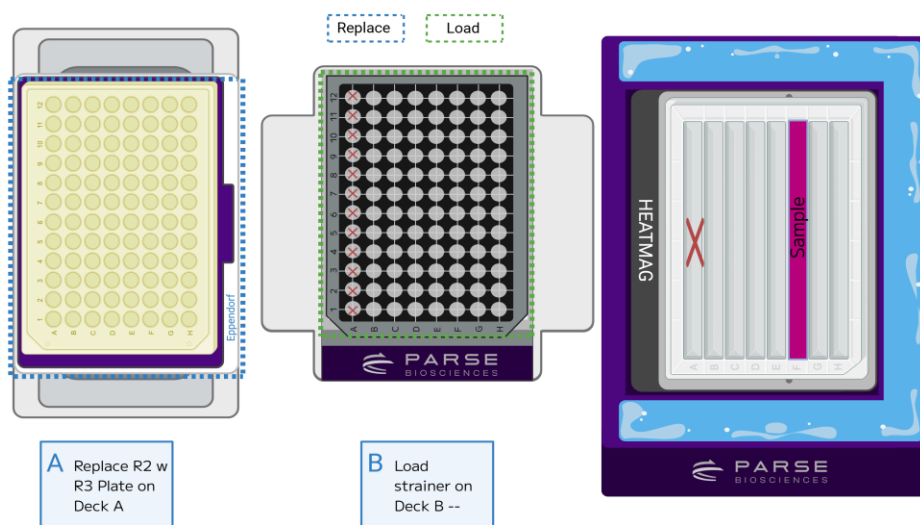
THAW ROUND 3 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

22. At the completion of the thermocycling program, centrifuge the Round 3 Plate for **1 minute** at 100 x g at 4°C. Place the Round 3 Plate on ice for later use.
23. Load the Round 2 Plate on the Thermochromic PCR Cold Block on Deck A and remove the seal. Ensure A1 is on the bottom left. Press “Run” to continue the program.
24. Follow the program prompts for manual intervention.

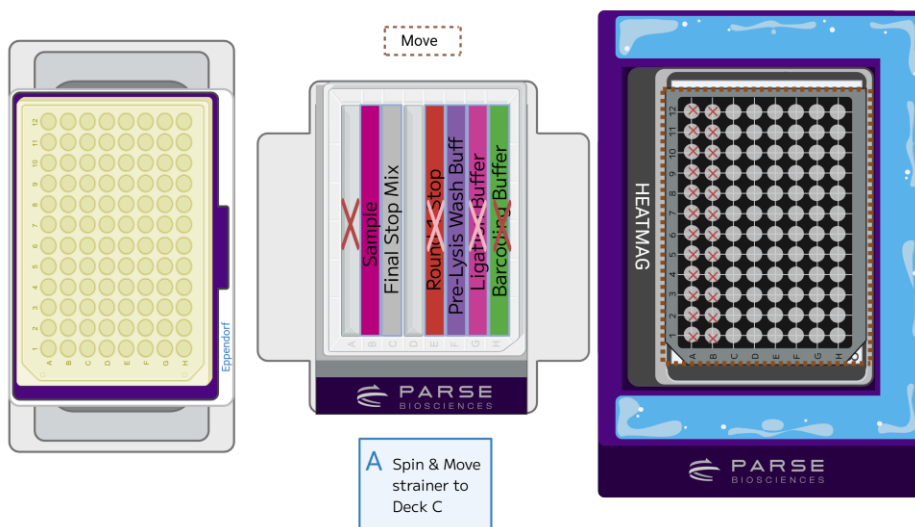


- a. Tilt **Reservoir 2** on Deck C and mix the sample pool in Row F 20 times with a P1000 pipette set to 1000 µL. Place back on Deck C and ensure the sample is evenly spread across the row.

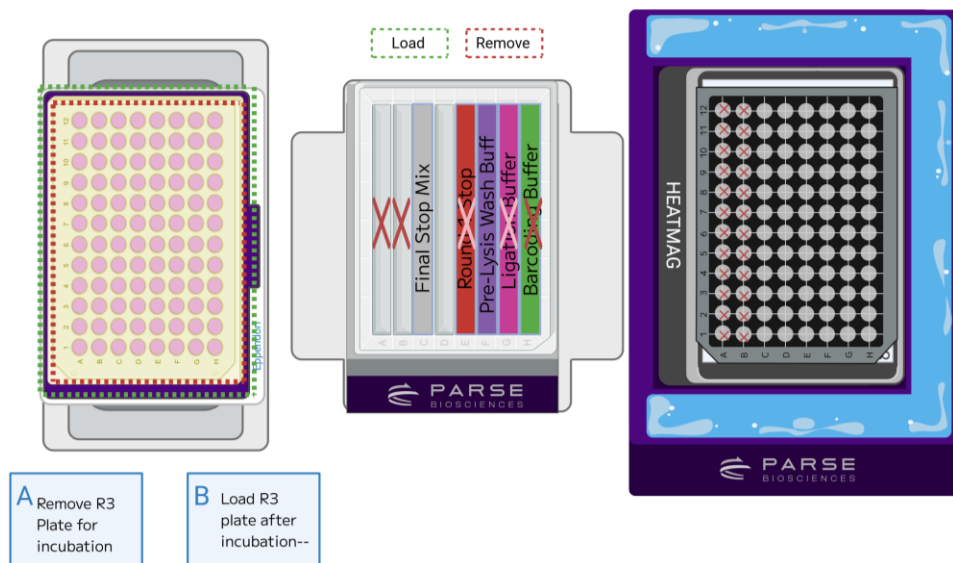
25. Press “Run” to continue the program. Follow the program prompts for manual intervention.



- Replace the Round 2 Plate and Thermochromic PCR Cold Block with Riser on Deck A with the Round 3 Plate and Thermochromic PCR Cold Block with Riser thawed in step 1.3.21.
 - Load the Rigid Plate Strainer stored from step 1.3.17a on top of **Reservoir 1** on Deck B. Ensure A1 is on the bottom left. Press firmly to ensure proper loading.
26. Press “Run” to continue the program. Follow the program prompts for manual intervention.



- a. Ensuring the strainer is not jostled, carefully remove **Reservoir 1** with the strainer on top from deck B. Centrifuge the strainer on **Reservoir 1** at 100g x **10 seconds**. Move the strainer to **Reservoir 2** on Deck C. Reload the **Reservoir 1** on Deck B.
27. Press "Run" to continue the program. Follow the program prompts for manual intervention.

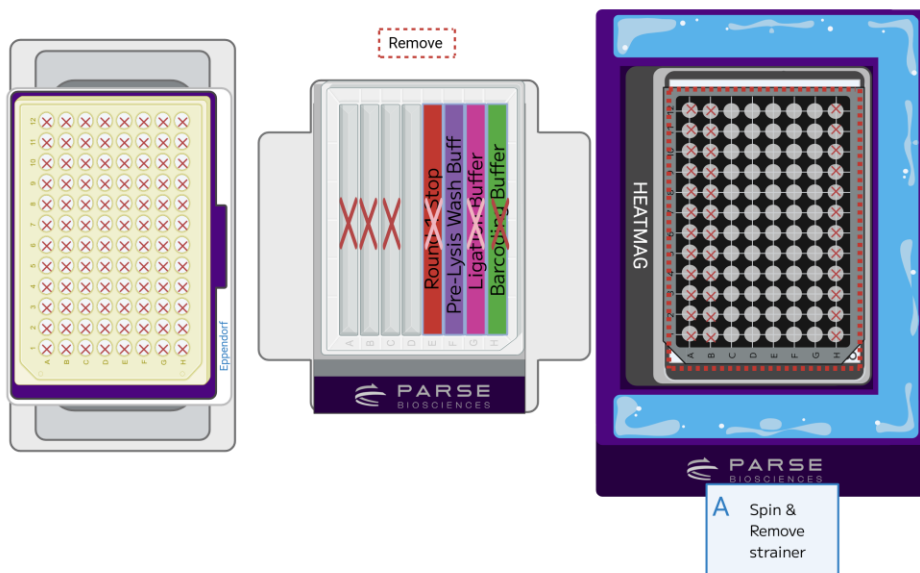


- a. Remove and seal the Round 3 Plate from Deck A using the Plate Seal Applicator. Place the Round 3 Plate into a thermocycler and run the following program.

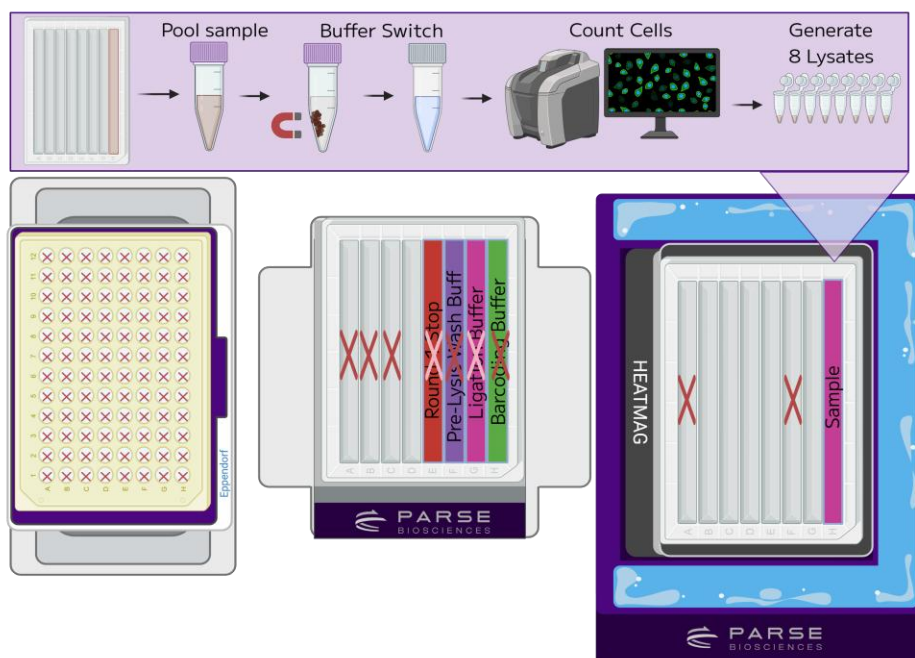
BARCODING ROUND 3		
Run Time	15 minutes	
Lid Temperature	50°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

- b. When the Round 3 Thermocycling program is complete, load the Round 3 Plate on the Thermochromic PCR Cold Block on Deck A and remove the seal. Ensure A1 is on the bottom left. Press "Run" to continue the program.

28. Follow the program prompts for manual intervention.



- Ensuring the strainer is not jostled, carefully remove **Reservoir 2** with the strainer on top from deck C. Centrifuge the strainer on **Reservoir 2** at 100g x **10 seconds**. Reload the **Reservoir 2** on Deck C. The strainer will no longer be used for this section and can be discarded.
29. Press "Run" to continue the program. Follow the program prompts for manual intervention.



- Immediately after the last supernatant removal, manually pool all of the beads from Row H of **Reservoir 2** on Deck C into a clean 1.5 mL tube using a P200 set to 200 μ L. If the remaining volume is less than 200 μ L in the 8 Row Reservoir, add 200 μ L of volume from Row D of **Reservoir 2**.
- Place the 1.5 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet (~2 minutes), remove and discard the supernatant, then fully resuspend the pellet in **80 μ L** of **Pre-Lysis Dilution Buffer**. Keep this tube on ice. **It contains your cells bound to the beads.**
- Count the number of cells/nuclei in the sample with a fluorescent (DAPI or equivalent) cell counting device. Record the cell/nuclei count.
- Decide how to divide cells/nuclei across the 8 sublibraries. See the “Sublibrary Loading” in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and **Pre-Lysis Dilution Buffer** that should be added to each sublibrary.



Note: The downstream Library Prep workflow on the INTEGRA ASSIST PLUS is compatible only with 8, 16 or 32 sublibraries. Different quantities of sublibraries may need to be run manually.



CRITICAL! Do not add more than 32,000 cells/nuclei to a sublibrary. Adding additional cells/nuclei will result in an increased multiplet rate.

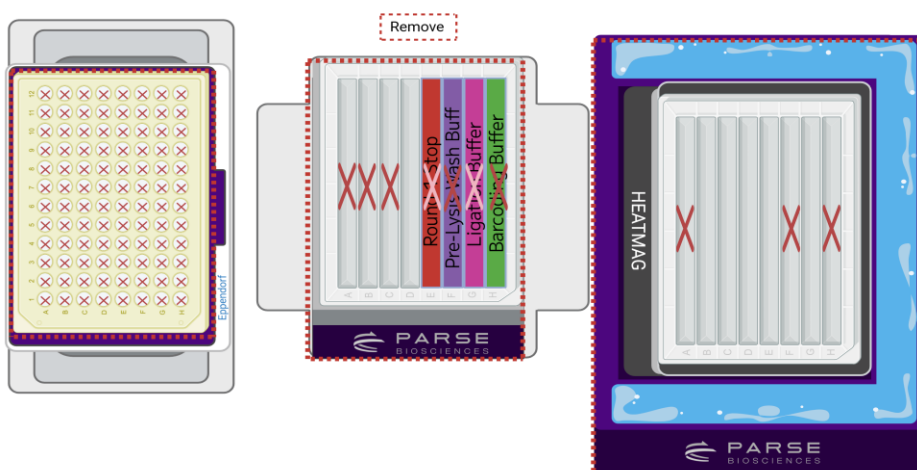
- e. Ensure the cells/nuclei are in suspension by **pipetting 5x with a P200 set to 50 μ L immediately before each transfer**. Add the appropriate volume of sample to the **different 0.2 mL PCR tubes**.



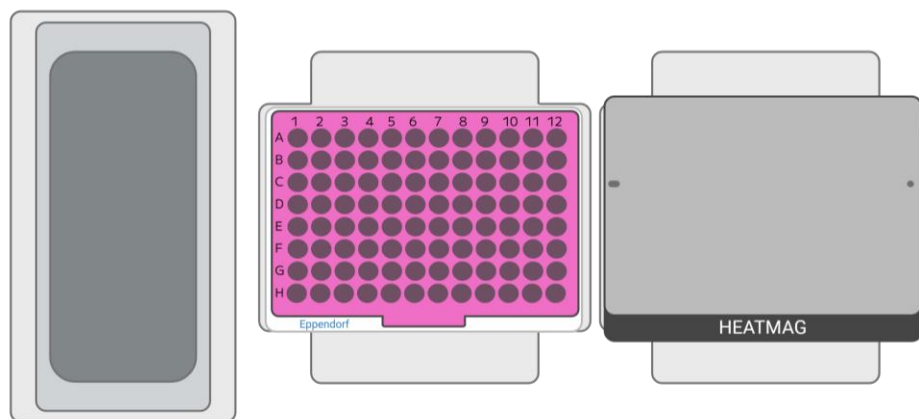
Note: If preparing 24 or 32 sublibraries, a low-bind 96-well PCR plate can be used.

- f. Keeping the samples on ice, add the appropriate volume of ● Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25 μ L** in each tube.

30. At the conclusion of the run:



- Remove all labware from Deck B. **Reservoir 1** can be discarded. Remove the Self-Standing 8 Row Reservoir Cooling Adapter and the Cooling Adapter Base.
- Discard the used Round 3 Plate on Deck A. Move the thawed Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser from Deck A to Deck B with A1 to the top left following the diagram below.
- Remove all the adapters on the HEATMAG. **Reservoir 2** can be discarded. Remove the HEATMAG 8 Row Reservoir Adapter and HEATMAG Cooling Accessory. Rotate the INTEGRA HEATMAG so the power button faces the front of the INTEGRA ASSIST PLUS base unit.



1.4. Lysate Creation

The following subsection is entirely manual. Lysis Master Mix is prepared the sublibraries are lysed, transferred into new tubes, and stored at -80°C.

The downstream Library Prep workflow on the INTEGRA ASSIST PLUS is compatible only with 8, 16 or 32 sublibraries. Different quantities of sublibraries may need to be run manually.

1. Gather the following reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Parse Biosciences Magnetic Rack	Equipment	1	
Permagen Bar Magnet	Equipment	1	Optional: if preparing 32 sublibraries.
PCR Plate 96 LoBind®	Consumables	1	
8-count PCR strip tube	Consumables	as needed	The quantity depends on the number of sublibraries desired.
1.5 mL tube	Consumables	1	
● Lysis Solution	-20°C Reagents	1	Thaw and store at room temperature.
● Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use

2. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x. Store at room temperature.

LYSIS MASTER MIX			
Number of Sublibraries	8	16	32
● Lysis Solution	220 µL	440 µL	880 µL
● Lysis Enzyme	44 µL	88 µL	176 µL
Total	264 µL	528 µL	1056 µL

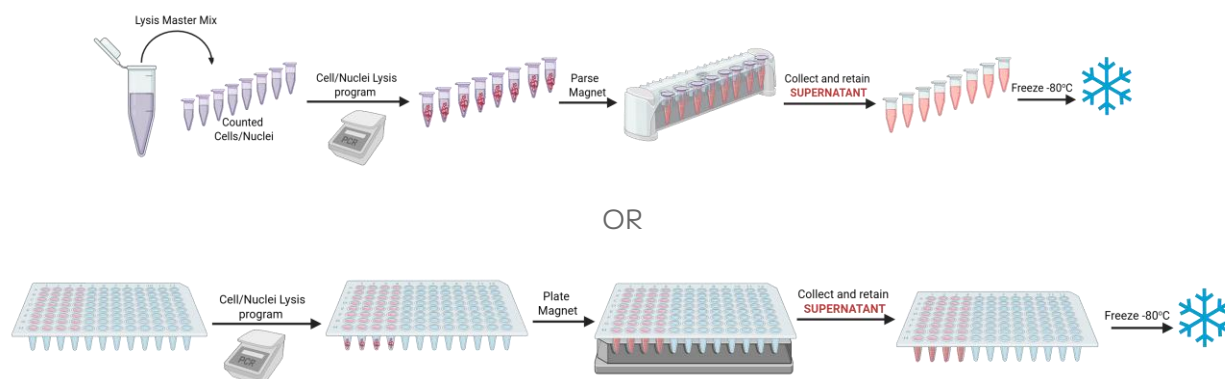


Note: Ensure that there is no precipitate before using the ● Lysis Solution. If there is a precipitate, place the Lysis Solution at 37°C until all crystals dissolve.



CRITICAL! Do not place Lysis Master Mix on ice, as a precipitate will form.

- For a visual representation of the next few steps see the workflow below. Add **30 μ L** of Lysis Master Mix to each 0.2 mL tube with diluted cells/nuclei. Store at room temperature.



Optional: You can use a plate magnet when preparing more than 16 sublibraries.

- Vortex the 0.2 mL tube(s) for **1 minute**. Briefly centrifuge.
- Place the tube(s) into a thermocycler and run the following program.

CELL/NUCLEI LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	55 μ L	
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C



CRITICAL! At this point, cells/nuclei are lysed, so the supernatant contains the barcoded transcripts. Do **NOT** discard the supernatant. The beads are no longer needed, and will be removed.

- Remove samples from the thermocycler and place the tubes on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).

7. While still on the magnetic rack, transfer **55 µL** of the **supernatant** containing lysed cells into new 0.2 mL tubes or a low-bind semi-skirted 96-well PCR plate.
8. Freeze the lysate(s) at -80°C or proceed to the appropriate Evercode MAX WT v4 Library Prep User Guide.



Note: If storing lysates in a plate, use foil plate seals as many clear plastic seals are not designed for storage at -80°C.



Safe stopping point: Sublibrary lysates can be stored at -80°C for up to 6 months.

This ends the Barcoding portion of the Evercode assay. To continue with the Sublibrary Preparation portion of the workflow, refer to the Evercode Max WT v4 Library Prep Reactions for INTEGRA ASSIST PLUS user manual.

Appendix A Sublibrary Gen Table

The Sublibrary Generation Table provides guidance for sample dilution in Section 1.3 to achieve a target sublibrary concentration.

Green text (top): Volume of cell suspension in μL to add to each sublibrary.

Purple text (bottom): Volume of Pre-Lysis Dilution Buffer in μL to add to each sublibrary.

Blue Shading: Serial dilution of cell stock is required to improve sublibrary cell count.

Red Shading: Insufficient cell stock concentration for target sublibrary cell count.

Cell Stock Conc. (cells/ μL)	Target Sublibrary Cell Count (cells/sublibrary)																
	200	500	1,000	2,000	5,000	8,000	10,000	12,000	12,500	15,000	20,000	25,000	30,000	31,250	32,000	62,500	64,000
50	4	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	21	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
100	Dilute	5	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	20	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
200	Dilute	2.5	5	10	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	22.5	20	15	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
400	Dilute	Dilute	2.5	5	12.5	20	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	22.5	20	12.5	5	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
600	Dilute	Dilute	Dilute	3.33	8.33	13.33	16.67	20	20.83	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	21.67	16.67	11.67	8.33	5	4.17	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
800	Dilute	Dilute	Dilute	2.5	6.25	10	12.5	15	15.63	18.75	25	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	22.5	18.75	15	12.5	10	9.38	6.25	0	N/A	N/A	N/A	N/A	N/A	N/A
1,000	Dilute	Dilute	Dilute	Dilute	5	8	10	12	12.5	15	20	25	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	20	17	15	13	12.5	10	5	0	N/A	N/A	N/A	N/A	N/A
1,200	Dilute	Dilute	Dilute	Dilute	4.17	6.67	8.33	10	10.42	12.5	16.67	20.83	25	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	20.83	18.33	16.67	15	14.58	12.5	8.33	4.17	0	N/A	N/A	N/A	N/A
1,400	Dilute	Dilute	Dilute	Dilute	3.57	5.71	7.14	8.57	8.93	10.71	14.29	17.86	21.43	22.32	22.86	N/A	N/A
	N/A	N/A	N/A	N/A	21.43	19.29	17.86	16.43	16.07	14.29	10.71	7.14	3.57	2.68	2.14	N/A	N/A
1,600	Dilute	Dilute	Dilute	Dilute	3.13	5	6.25	7.5	7.81	9.38	12.5	15.63	18.75	19.53	20	N/A	N/A
	N/A	N/A	N/A	N/A	21.88	20	18.75	17.5	17.19	15.63	12.5	9.38	6.25	5.47	5	N/A	N/A
1,800	Dilute	Dilute	Dilute	Dilute	2.78	4.44	5.56	6.67	6.94	8.33	11.11	13.89	16.67	17.36	17.78	N/A	N/A
	N/A	N/A	N/A	N/A	22.22	20.56	19.44	18.33	18.06	16.67	13.89	11.11	8.33	7.64	7.22	N/A	N/A
2,000	Dilute	Dilute	Dilute	Dilute	2.5	4	5	6	6.25	7.5	10	12.5	15	15.63	16	N/A	N/A
	N/A	N/A	N/A	N/A	22.5	21	20	19	18.75	17.5	15	12.5	10	9.38	9	N/A	N/A
2,500	Dilute	Dilute	Dilute	Dilute	Dilute	3.2	4	4.8	5	6	8	10	12	12.5	12.8	25	N/A
	N/A	N/A	N/A	N/A	N/A	21.8	21	20.2	20	19	17	15	13	12.5	12.2	0	N/A
3,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.67	3.33	4	4.17	5	6.67	8.33	10	10.42	10.67	20.83	21.33
	N/A	N/A	N/A	N/A	N/A	22.33	21.67	21	20.83	20	18.33	16.67	15	14.58	14.33	4.17	3.67
3,500	Dilute	Dilute	Dilute	Dilute	Dilute	2.86	3.43	3.57	4.29	5.71	7.14	8.57	8.93	9.14	17.86	18.29	
	N/A	N/A	N/A	N/A	N/A	22.14	21.57	21.43	20.71	19.29	17.86	16.43	16.07	15.86	7.14	6.71	
4,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	3.13	3.75	5	6.25	7.5	7.81	8	15.63	16	
	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21.88	21.25	20	18.75	17.5	17.19	17	9.38	9
4,500	Dilute	Dilute	Dilute	Dilute	Dilute	2.67	2.78	3.33	4.44	5.56	6.67	6.94	7.11	13.89	14.22		
	N/A	N/A	N/A	N/A	N/A	N/A	22.33	22.22	21.67	20.56	19.44	18.33	18.06	17.89	11.11	10.78	
5,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	4	5	6	6.25	6.4	12.5	12.8			
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21	20	19	18.75	18.6	12.5	12.2
5,500	Dilute	Dilute	Dilute	Dilute	Dilute	2.73	3.64	4.55	5.45	5.68	5.82	11.36	11.64				
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.27	21.36	20.45	19.55	19.32	19.18	13.64	13.36	
6,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3.33	4.17	5	5.21	5.33	10.42	10.67				
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	21.67	20.83	20	19.79	19.67	14.58	14.33	
7,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.86	3.57	4.29	4.46	4.57	8.93	9.14					
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.14	21.43	20.71	20.54	20.43	16.07	15.86
8,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3.13	3.75	3.91	4	7.81	8					
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	21.88	21.25	21.09	21	17.19	17
9,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.78	3.33	3.47	3.56	6.94	7.11						
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.22	21.67	21.53	21.44	18.06	17.89	
10,000	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3	3.13	3.2	6.25	6.4						
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	22	21.88	21.8	18.75	18.6	

Appendix B: Pipetting Programs

Section 1.1. Sample Normalization

M S1 St1 V4_0 DONE

STEPS	ACTION	DURATION
1	Initial Volumes	0
2	CombinedWTWorksheet	40 min 0 sec
3	Thaw R1 plate	0

Section 1.2. Reagents Preparation

M S1 St2 V4_0 DONE

STEPS	ACTION	DURATION
1	Initial Volumes	0
2	Prompt to add Barcoding Buffer	0
3	Prompt to add Ligation Buffer	0
4	Aliquot R1 Stop (900 µL/well x 2)	0 min 40 sec
5	Aliquot R2 Stop (1100 µL/well)	0 min 30 sec
6	Aliquot Final stop Buffer (1000 µL/well x 2)	0 min 41 sec
7	Aliquot Prelysis Wash Buffer (1100 µL/well x 4)	1 min 9 sec
8	Add Ligation Enz to Ligation Buffer (44 µL/well)	0 min 38 sec
9	Mix Ligation MM	0 min 35 sec
10	Mix Ligation MM	0 min 35 sec

STEPS	ACTION	DURATION
11	Mix Ligation MM	0 min 35 sec

Section 1.3. Round 1, Round 2, Round 3 Plate Loading and Pooling

M S1 St3 V4_0 VIAFLO_300

STEPS	ACTION	DURATION
1	Initial Volumes	0
2	Load the R1 Plate (15 µL/well)	2 min 13 sec
3	Prompt to start RT	0
4	Prompt to remove labware from Deck B	0
5	Prompt to load reservoir 1	0
6	Labware Change	0
7	Prompt to load R1 plate post RT	0
8	Prompt to thaw R2 Plate	0
9	Add R1 Stop to R1 plate (10 µL/well)	0 min 25 sec
10	R1 Plate 1st Pass Pool (50 µL/well)	1 min 28 sec
11	R1 Plate 2nd Pass Pool (10 µL/well)	0 min 27 sec
12	Pipette 192 µL CCMM in Row 1 on Deck C--	0
13	DELAY	5 min 0 sec
14	Bind beads	0 min 10 sec
15	DELAY	0 min 15 sec
16	Remove beads from pipetting locations	1 min 17 sec

STEPS	ACTION	DURATION
17	Bead Binding Incubation	4 min 0 sec
18	Remove beads from pipetting area. No offset	1 min 27 sec
19	Remove excess vol from tip	1 min 27 sec
20	Bead Binding Incubation	10 min 0 sec
21	Remove soup 1 (150 μ L/well)	2 min 26 sec
22	Remove Soup 2 (300 μ L/well)	0 min 33 sec
23	Remove Soup 3 (50 μ L/well)	0 min 33 sec
24	Add Binding Buffer 1 (208 μ L/well)	2 min 24 sec
25	Remove soup 1 (175 μ L/well)	0 min 33 sec
26	Remove Soup 2 (100 μ L/well)	0 min 33 sec
27	Remove Soup 3 (100 μ L/well)	0 min 33 sec
28	Add Binding Buffer 2 (208 μ L/well)	2 min 24 sec
29	Remove soup 1 (175 μ L/well)	0 min 33 sec
30	Remove Soup 2 (100 μ L/well)	0 min 33 sec
31	Remove Soup 3 (100 μ L/well)	0 min 33 sec
32	Release Magnet	0 min 10 sec
33	Add Ligation Mix (174 μ L/well x 2)	0 min 43 sec
34	Volume Change	0
35	Mix Middle	0 min 36 sec
36	Mix Odd	0 min 36 sec

STEPS	ACTION	DURATION
37	Mix Even	0 min 36 sec
38	Reminder to remove strainer film	0
39	Prompt to load strainer	0
40	Prompt to Load R2 Plate	0
41	Volume Change	0
42	Strain Cells- center (300 μ L/well)	0 min 43 sec
43	Strain Cells (100 μ L/well)	0 min 32 sec
44	Prompt to remove strainer	0
45	Row 1: Load Plate - Even (40 μ L/well)	0 min 38 sec
46	Row 2: Load Plate - Odd (40 μ L/well)	0 min 37 sec
47	Row 3: Load Plate - Even (40 μ L/well)	0 min 37 sec
48	Row 4: Load Plate - Odd (40 μ L/well)	0 min 36 sec
49	Row 5: Load Plate - Even (40 μ L/well)	0 min 36 sec
50	Row 6: Load Plate - Odd (40 μ L/well)	0 min 35 sec
51	Row 7: Load Plate - Even (40 μ L/well)	0 min 34 sec
52	Row 8: Load Plate - Odd (40 μ L/well)	0 min 36 sec
53	Prompt for R2 incubation	0
54	Prompt to thaw R3	0
55	Prompt to load R2 post incubation	0
56	R2 Plate 1st Pass Pool (50 μ L/well)	2 min 45 sec

STEPS	ACTION	DURATION
57	R2 Plate 2nd Pass Pool (20 µL/well)	0 min 43 sec
58	Manual intervention to mix Post R2 Pool	0
59	Bind beads	0 min 10 sec
60	DELAY	0 min 15 sec
61	Remove beads from pipette tip	1 min 17 sec
62	Bead Binding Incubation	2 min 0 sec
63	Remove beads from pipetting locations	1 min 27 sec
64	Remove excess volume from tip	1 min 31 sec
65	Bead Binding Incubation	6 min 0 sec
66	Remove soup 1 (300 µL/well)	0 min 33 sec
67	Remove Soup 2 (150 µL/well)	0 min 33 sec
68	Remove Soup 3 (100 µL/well)	0 min 33 sec
69	Add Binding Buffer 1 (208 µL/well)	2 min 24 sec
70	Remove soup 1 (100 µL/well)	0 min 33 sec
71	Remove Soup 2 (100 µL/well)	0 min 33 sec
72	Remove Soup 3 (100 µL/well)	0 min 33 sec
73	Add Binding Buffer 2 (208 µL/well)	2 min 24 sec
74	Remove soup 1 (100 µL/well)	0 min 33 sec
75	Remove Soup 2 (100 µL/well)	0 min 33 sec
76	Remove Soup 3 (100 µL/well)	0 min 33 sec

STEPS	ACTION	DURATION
77	Release Magnet	0 min 10 sec
78	Add Ligation Mix (175 µL/well x 2)	0 min 31 sec
79	Volume Change	0
80	Mix Middle	0 min 36 sec
81	Mix Odd	0 min 36 sec
82	Mix Even	0 min 36 sec
83	Prompt to load R3 Plate	0
84	Prompt to load strainer	0
85	Volume Change	0
86	Strain Cells- Center (300 µL/well)	0 min 43 sec
87	Strain Cells (100 µL/well)	0 min 49 sec
88	Prompt to move strainer	0
89	Row 1: Load Plate - Even (43 µL/well)	0 min 38 sec
90	Row 2: Load Plate - Odd (43 µL/well)	0 min 37 sec
91	Row 3: Load Plate - Even (43 µL/well)	0 min 37 sec
92	Row 4: Load Plate - Odd (43 µL/well)	0 min 36 sec
93	Row 5: Load Plate - Even (43 µL/well)	0 min 36 sec
94	Row 6: Load Plate - Odd (43 µL/well)	0 min 35 sec
95	Row 7: Load Plate - Even (43 µL/well)	0 min 34 sec
96	Row 8: Load Plate - Odd (43 µL/well)	0 min 36 sec

STEPS	ACTION	DURATION
97	Prompt for R3 incubation	0
98	Prompt to load R3 post incubation	0
99	R3 Plate 1st Pass Pool (50 µL/well)	2 min 45 sec
100	R3 Plate 2nd Pass Pool (20 µL/well)	0 min 43 sec
101	Strain Cells (300 µL/well)	0 min 44 sec
102	Strain Cells (300 µL/well)	0 min 37 sec
103	Prompt to remove strainer	0
104	Volume Change	0
105	Bind beads	0 min 10 sec
106	DELAY	0 min 15 sec
107	Remove beads from pipetting locations	1 min 17 sec
108	Bead Binding Incubation	2 min 0 sec
109	Remove excess liquid from tip	1 min 38 sec
110	Remove excess liquid from tip	1 min 31 sec
111	Bead Binding Incubation	6 min 0 sec
112	Remove soup 1 (300 µL/well)	0 min 33 sec
113	Remove Soup 2 (100 µL/well)	0 min 33 sec
114	Remove Soup 3 (100 µL/well)	0 min 33 sec
115	Add Pelyse Wash Buffer (166.5 µL/well x 2)	0 min 31 sec
116	Release Magnet	0 min 10 sec

STEPS	ACTION	DURATION
117	Mix Middle	0 min 45 sec
118	Mix Odd	0 min 45 sec
119	Mix Even	0 min 45 sec
120	Bind beads	0 min 10 sec
121	DELAY	0 min 30 sec
122	Remove beads from pipetting locations	1 min 24 sec
123	Bead Binding Incubation	2 min 0 sec
124	Remove excess liquid from tip	1 min 38 sec
125	Remove excess liquid from tip	1 min 31 sec
126	Bead Binding Incubation	7 min 0 sec
127	Remove soup 1 (300 µL/well)	0 min 33 sec
128	Remove Soup 2 (100 µL/well)	0 min 33 sec
129	Remove Soup 3 (100 µL/well)	0 min 33 sec
130	Prompt to pool samples	0
131	Prompt to count cells	0

Appendix C: Thermocycling Programs

Section 1: In situ Cell/Nuclei Barcoding

THAW ROUND 1 PLATE		
Run Time	3 minutes	
Lid Temperature	70°C	
Sample Volume	26 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

BARCODING ROUND 1			
Total Run Time			40 minutes
Lid Temperature			70°C
Sample Volume			40 µL
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 sec	8°C	3
3	45 sec	15°C	
4	45 sec	20°C	
5	30 sec	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1

THAW ROUND 2 PLATE		
Run Time	3 min	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

BARCODING ROUND 2		
Run Time	15 minutes	
Lid Temperature	50°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

THAW ROUND 3 PLATE		
Run Time	3 min	
Lid Temperature	70°C	
Sample Volume	10 µL	
Step	Time	Temperature
1	3 min	25°C
2	Hold	4°C

BARCODING ROUND 3		
Run Time	15 minutes	
Lid Temperature	50°C	
Sample Volume	50 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

CELL/NUCLEI LYSIS		
Run Time	15 minutes	
Lid Temperature	80°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	15 min	65°C
2	Hold	4°C

Appendix D: Troubleshooting

Error warning during the execution of a program

- In case of an error warning, abort the program. Do not press "RUN". Scroll down "Run from step". You can then continue from the error location. This allows to resume directly from the point of error.

Tip Pinching

- Tip pinching may occur when using a newly frozen PCR freezer block for a plate pooling step. Ensure that your PCR Freezer Block has been thawed at room temperature for at least 10 minutes.
- Ensure to apply sufficient pressure when placing the plate onto the frozen PCR freezer block.

Incorrect tip location inputted

- The D-ONE Pipette will check the pipette tips before the step starts. The ASSIST PLUS will display an error warning if a pipette tip is incorrectly detected. Abort and restart the program with the correct tip location input.
- The VIAFLO will check the pipette tip after the step is finished. The ASSIST PLUS will display an error warning if a pipette tip is incorrectly detected. Abort and restart the program with the correct tip location input.

Appendix E: Revision History

Version	Description	Date
1.0	Initial release	September 2026

Appendix F: Acknowledgements

All graphics were created with BioRender.com.



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