User Manual

Version 1.1 – UMWT3700INT

Evercode™ WT Penta v3 with INTEGRA ASSIST PLUS

For use with

ECWT3700

INTEGRA ASSIST PLUS

Support Suite

Legal Notices

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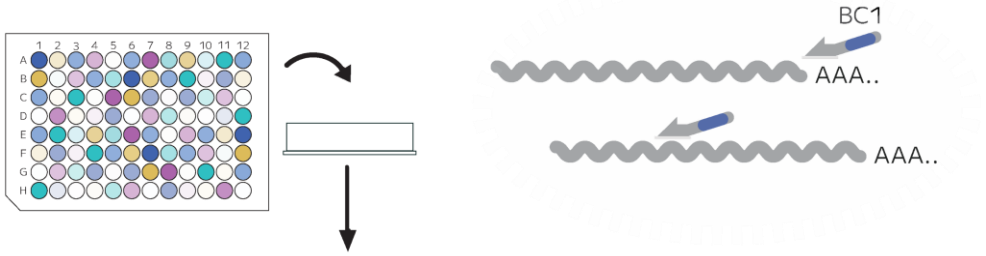
Overview

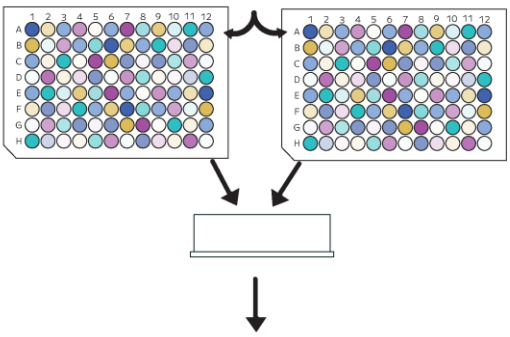
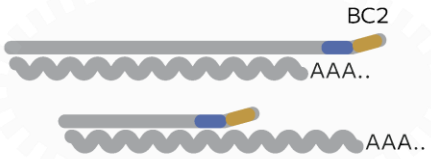
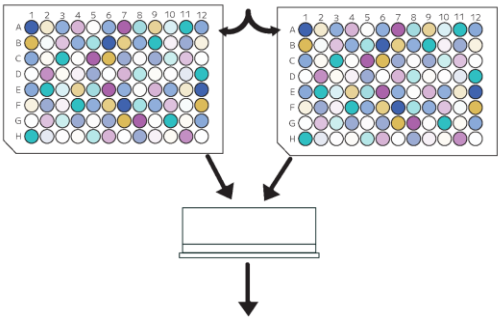
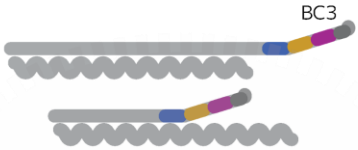
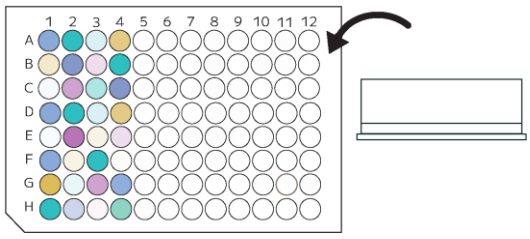
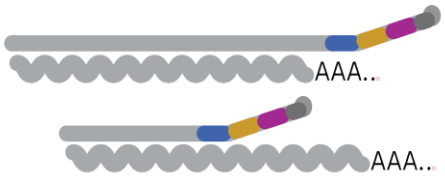
Workflow

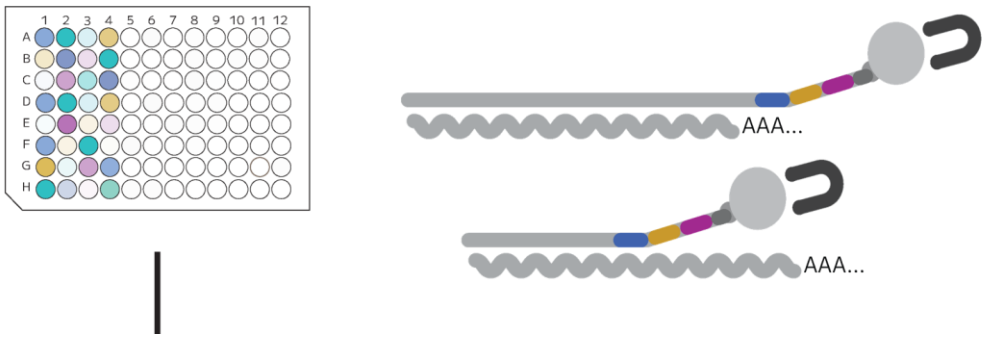
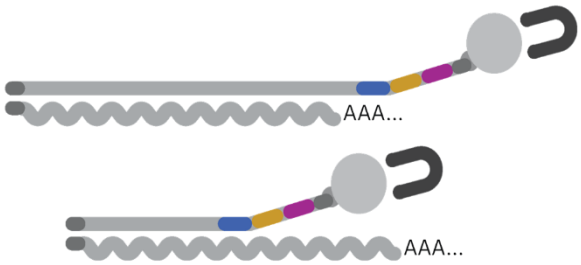
The Evercode combinatorial barcoding workflow is compatible with the INTEGRA ASSIST PLUS to enable large-scale single cell RNA-Seq experiments through a robust, semi-automated process.

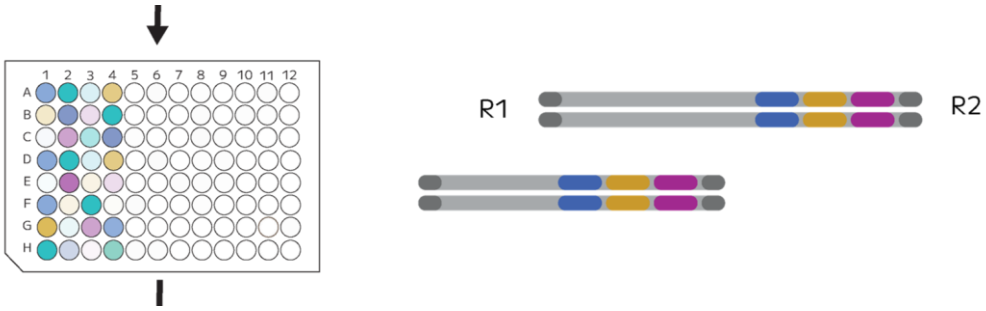
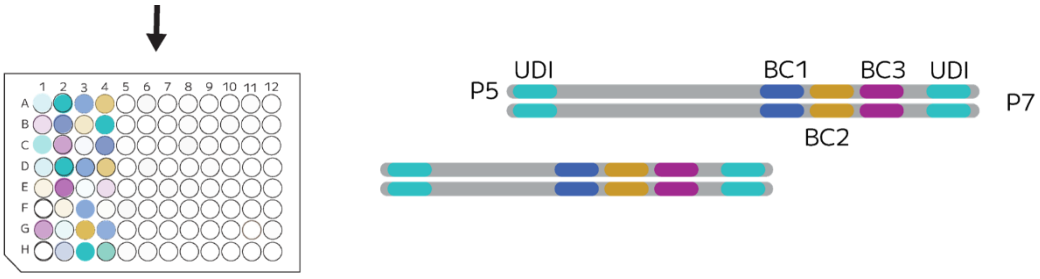
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Penta v3 kit can profile up to 5,000,000 cells/nuclei across up to 96 different biological samples or experimental conditions. Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 5,000,000 cells/nuclei while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same 4 barcode combination to a single cell/nuclei.

The tables below provide a high-level overview of the whole transcriptome workflow.

	Section 1: *In situ* Cell/Nuclei barcoding
1	 **Round 1 Barcoding:** Fixed and permeabilized cells are loaded into 96 wells of the Round 1 Plate. RNA is reverse transcribed via oligo dT and random hexamer primers with a well-specific barcode that are associated with specific samples.

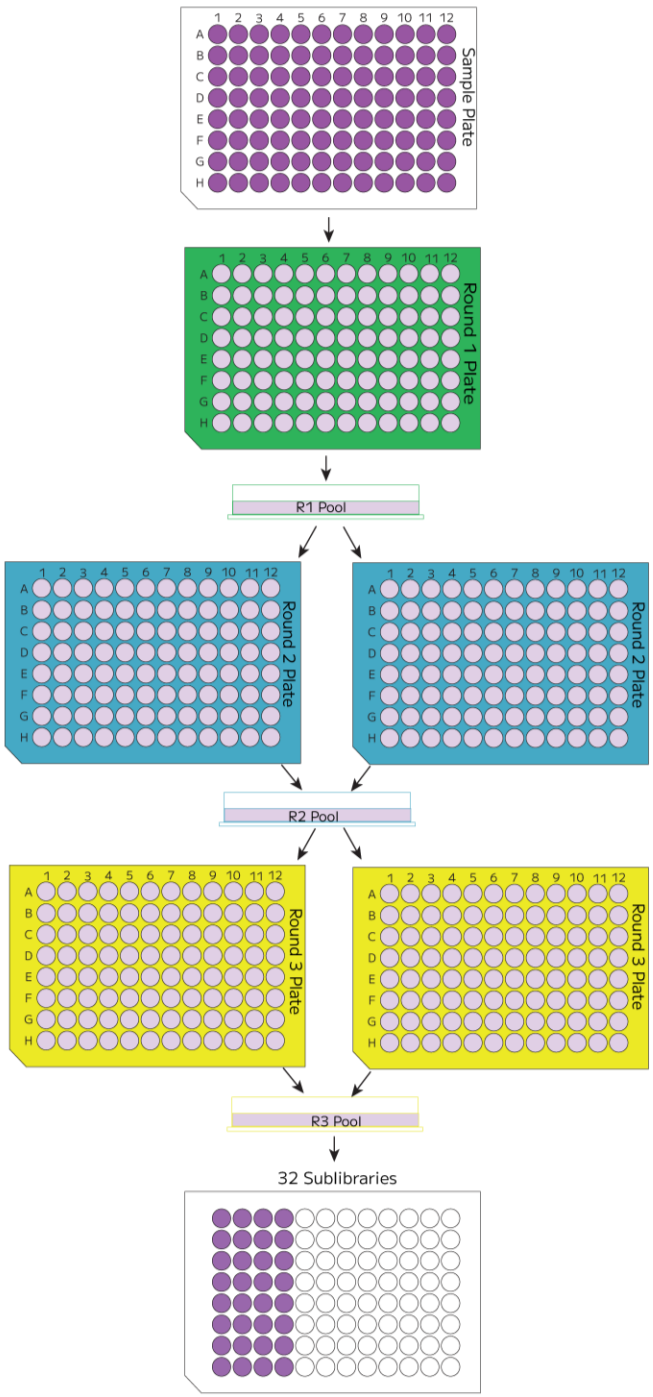
	Section 1: *In situ* Cell/Nuclei barcoding
2	  **Round 2 Barcoding:** The cells/nuclei are pooled and loaded into two distinct Round 2 Plates. An adapter with a well-specific barcode is ligated to the first barcode.
3	  **Round 3 Barcoding:** The cells/nuclei are pooled and loaded into two distinct Round 3 Plates. A third barcode is ligated to the cDNA, which also contains an Illumina Truseq Read 2 sequence, and biotin.
4	  **Lysis and Sublibrary Generation:** Cells are split across 32 sublibraries and lysed.

	Section 2: cDNA Capture and Amplification
1	 **cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.
2	 **cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA.
3	 **cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq Read 2.

	Section 3: Sequencing Library Preparation
1	 **Fragmentation and End Repair:** DNA is fragmented, ends are repaired, and A-tailed.
2	 **Adapter Ligation:** An Illumina Truseq R1 Adapter is ligated to the 5′ end of the DNA.
3	 **Round 4 Barcoding:** The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

The figure below shows the Evercode WT Penta workflow. This kit can barcode up to 5 million cells/nuclei across 96 samples in a single reaction. The WT Penta kit utilizes one Round 1 Plate holding 96 samples. Samples are pooled from the Round 1 Plate and randomly distributed across two Round 2 Plates, each carrying different barcode sets. Cells are pooled from both Round 2 Plates, and randomly split again across two Round 3 Plates, also each carrying different sets of barcodes.

Cells/nuclei are then pooled and subsequently split to generate 32 sublibraries.



Evercode WT Penta workflow pools 96 samples from a single Round 1 Barcoding Plate into two Round two and Round 3 Barcoding Plates respectively, generating 32 Sequencing Libraries.

Important Guidelines

The following guidelines provide additional information to obtain optimal performance. 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

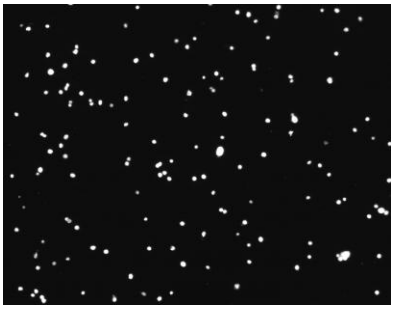
- This protocol begins with cells or nuclei fixed with an Evercode Cell Fixation v3 or Evercode Nuclei Fixation v3 kit.
- Even if samples were counted before freezing, and if you have a set aside a counting aliquot, we strongly recommend counting cells/nuclei again after thawing to account for any changes during storage and freeze thaw. Typically, a 5-15% decrease after thawing should be expected. These counts are used to determine how cells/nuclei are loaded in the Round 1 Plate, and their accuracy is critical to recover the desired number of cells/nuclei.
- When processing many fixed samples, we recommend aliquoting samples after fixation and counting the aliquots the day before using an Evercode Whole Transcriptome kit. The Evercode Fixation User Manuals 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 obtained immediately after fixation.
- Counting Aliquot plates should be thawed at 37°C and counted with an automated cell counter or alternative counting device. Counts should be recorded in the Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm, and any remaining counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- Due to the scale of high-throughput experiments, we recommend an automated cell counter for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices to a hemocytometer when using Evercode Whole Transcriptome kits for the first time.
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.

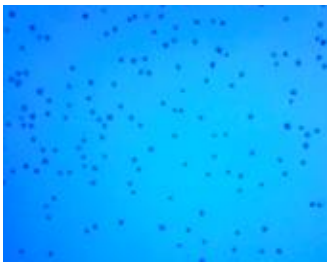
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).

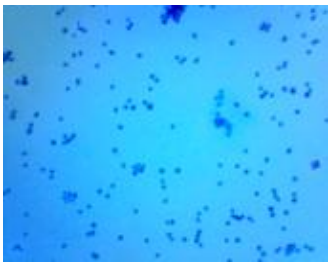
- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells/nuclei.

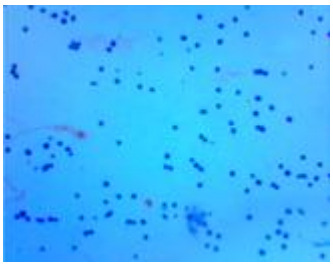


Example of AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample Aggregation Debris







Example trypan blue stained fixed cells.

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 (Thermo Fisher Scientific®). These can be sprayed on benchtops and pipettes.

- Low retention, filtered pipette tips should be used to reduce RNase contamination from pipettes.

Centrifugation

- A range of centrifugation speeds and durations are provided in this workflow in order to optimize and balance high sample retention with resuspension efficiency. Comprehensive information to optimize centrifugation conditions for each sample type is provided in the Cells and Nuclei Fixation User Guides.

- Use a swinging bucket rotor for all high-speed centrifugation steps in this workflow. A fixed-angle rotor will lead to substantial cell/nuclei loss.

Sample Loading Table

- Ensure that the Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm 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 Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm 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), choose to either:

    - 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.

Indexing Primers

- The Evercode v3 kits require the UDI Plate - WT. The UDI Plate - WT box is included in the INTEGRA bundles but will need to be separately purchased if necessary for subsequent orders.

- The UDI Plate - WT is a 96-well plate containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one Barcoding Round 4 PCR reaction for a single sublibrary. Thus, the UDI Plate - WT can be used for multiple Evercode kits. Ensure you have at least 32 unused wells in your UDI-WT Plate before beginning the

Penta 384 workflow. If combining samples that were prepared with different UDI plates, ensure you use a unique well position to avoid duplicate barcodes.

- The UDI Plates are sealed with a pierceable foil to minimize cross-contamination. The plate seal should be wiped with 70% ethanol and pierced with a new pipette tip immediately prior to use. Avoid splashing or mixing the liquid between individual wells. Once a well has been used, it should not be resealed or reused.

- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix A.

Plate Sealing

- 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.

Basin Liners Usage

- Basin liners are used throughout the procedure for reagent storage. Validated basins are listed in the Consumables and Reagents section.

- We recommend using the 8 Row Polypropylene Reservoir for its lower cell adherence qualities. However, the 8 Row Polystyrene Reservoir (PN6373) is an acceptable substitute.

Sample Concentrations

- For an Evercode Penta kit, samples can be pre-diluted with Sample Dilution Buffer to a more appropriate working concentration of >4,114 cells/nuclei per µL prior to Sample Normalization on the ASSIST PLUS.

- Samples will be diluted with Sample Dilution Buffer to the desired concentration based on the Sample Loading Table.

- For each sample we recommend preparing a 20 µL post-fixation counting aliquot and two 40-50 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 Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm accordingly.

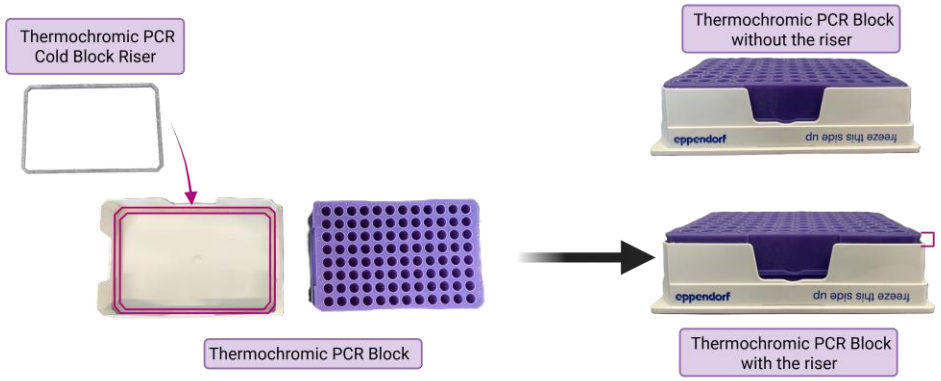
- If the sample stock concentrations are too high the Sample Loading Table may indicate that more than 1 tube of Sample Dilution Buffer is needed. Additional Sample Dilution Buffer can be purchased in the Dilution Accessory Box (Parse PN ECAC3901) before starting the barcoding workflow.

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.



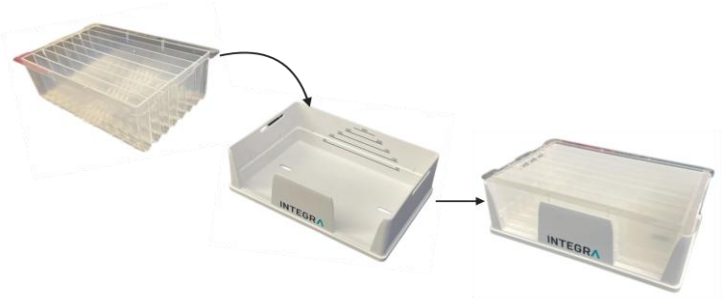
Note: Uneven freezing of the liquid inside the Thermochromic PCR Block can cause slanting of the cooling position. The Thermochromic PCR Cold Block Riser reduces the variability caused by the freezing process. If the PCR Cold Block riser is not used, there will be extra liquid left in the wells of the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

- Tip pinching may occur when using a fully frozen Thermochromic PCR Cold Block 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.

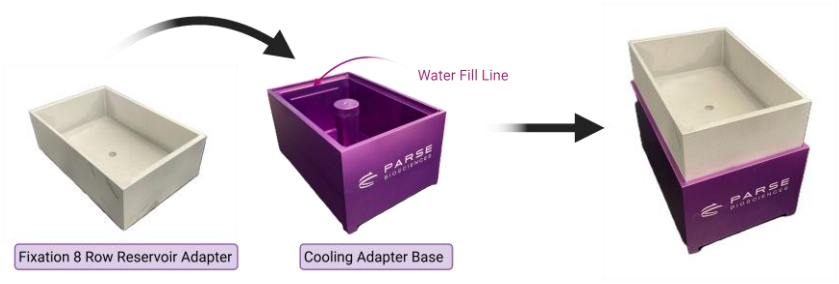
Bases and Reservoirs

- Both the 300 mL Reservoir Base and the 8-Row Polystyrene Reservoir are consumables that must be purchased from INTEGRA. The 300 mL Reservoir Base is designed to securely hold the 8-Row Reservoir in place during use.

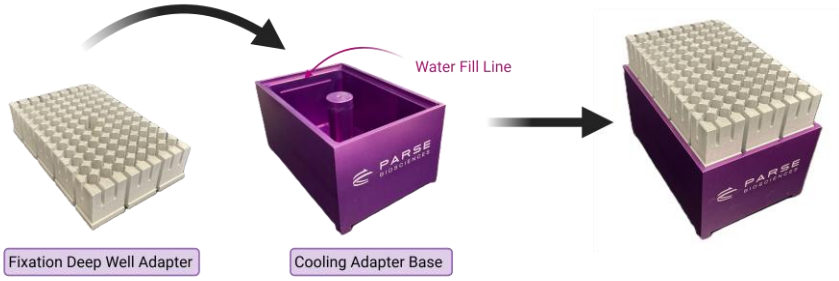


Cooling Adapter Bases

- 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 D-ONE 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 with INTEGRA ASSIST PLUS Precheck Scripts available on the Customer Support Suite.
- Ensure that the .csv worklists generated via the Parse Evercode Penta INTEGRA Sample Loading Table ("CombinedPentaWorksheet_YYYYMMDD_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. 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 WT Penta v3 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store at -20°C, PT100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate P	PT101	Green semi-skirted 96 well plate	1
	Round 2 Plate	MG102	Blue semi-skirted 96 well plate	1
	Round 2 Plate B	PT102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Round 3 Plate B	PT103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	MG131	5 mL tube	1
	Sample Dilution Buffer	MG132	5 mL tube	4
	Round 2 Ligation Buffer	MG133	5 mL tube	1
	Round 2 Ligation Enzyme	PT104	1.5 mL tube	1
	Round 2 Stop Buffer	PT105	5 mL tube	1
	Round 3 Ligation Enzyme	PT106	1.5 mL tube	1
	Final Stop Buffer	PT107	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Wash Buffer	MG110	5 mL tube	2
	Pre-Lysis Dilution Buffer	PT108	5 mL tube	1
	Lysis Enzyme	PT109	1.5 mL tube	1
	Penta Lysis Buffer	PT126	2 mL tube	1
	Capture Enhancer	PT110	1.5 mL tube	1
	Bead Wash Buffer	PT111	5 mL tube	2
	Binding Buffer	PT112	5 mL tube	1
	Wash Buffer 1	PT113	5 mL tube	3
	Wash Buffer 2	PT114	5 mL tube	3
	Wash Buffer 3	PT115	5 mL tube	3
	Template Switch Buffer	PT116	5 mL tube	1
	Template Switch Enzyme	PT117	1.5 mL tube	1
	Template Switch Primer	PT118	1.5 mL tube	1
	cDNA Amp Mix	PT119	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	cDNA Amp Primers	PT120	5 mL tube	1
	Fragm/End Prep Buffer	PT121	1.5 mL tube	1
	Fragm/End Prep Enzymes	PT122	1.5 mL tube	1
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	PT123	1.5 mL tube	1
	Adapter Ligation Enzyme	PT124	1.5 mL tube	1
	Library Amp Mix	PT125	1.5 mL tube	1

4°C Reagents Store at 4°C, PT200

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	MG204	1.5 mL tube	1
	Streptavidin Beads	PT201	2 mL tube	2

-20°C Sample Dilution Accessory Kit* Store at -20°C, PN ECAC3901

LABEL	ITEM	PN	FORMAT	QTY
	Sample Dilution Buffer	MG105	2 mL tube	3

***The -20°C Sample Dilution Accessory Kit is optional and can be purchased separately if needed to dilute concentrated samples.

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
Thermochromic PCR Cold Block	NTAC1102	5
Thermochromic PCR Cold Block Riser	NTAC1103	5
Parse Metal Cold Block	NTAC1107	1
Fixation Cooling Adapter	NTAC1106	1
Fixation 8 Row Reservoir Adapter	NTAC1105	1

INTEGRA Components

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

ITEM	ITEM TYPE	PN	QTY
Pipette Communication Module for VIAFLO / VOYAGER Pipettes	Accessory	4222	3
ASSIST PLUS Slanted Plate Holder (0°-30°)	Adapter	4510	1
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 µL	Pipette	4532	1
VIAFLO Pipette 12-Ch, 5-125 µL	Pipette	4632	1
VOYAGER Pipette 8-Channel, 5 - 125 µL	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
8 Row Polypropylene Reservoir*	6374	4
300 mL Reservoir Bases	6305	1
1250 µL Pipette Tips	6545	as needed
125 µL Pipette Tips	6565	

* We recommend using the 8 Row Polypropylene Reservoir (PN6374) for its lower cell adherence qualities. However, the 8 Row Polystyrene Reservoir (PN6373) is an acceptable substitute.

Other Consumables

ITEM	SUPPLIER	PN	QTY
Sterilized 25 µm, 40 µm, or 70 µm Mini Cell Strainer	DiagnoCine	FNK-HT-AMS-12502 FNK-HT-AMS-14002 FNK-HT-AMS-17002	2
10 mL Transport Tube	GlobeScientific™	6102S	3
1.5 mL Microtube	Genesee Scientific	21-257	6
5 mL Screw cap Tubes	Genesee Scientific	21-398	1
PCR Strip Tubes	USA Scientific®	1402-4700	8
Strip of 8 Domed Caps	Fisherbrand	14230231	as needed
2 mL Microtubes	Genesee Scientific	21-255	4
Semi-skirted 96 well plates	Eppendorf®	E951020362	8
SealPlate®	Excel Scientific	100-SEAL-PLT	as needed
TempPlate® EXT Sealing Foil	USA Scientific®	2998-0100	as needed
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	as needed

Reagents

ITEM	SUPPLIER	PN	NOTES
AMPure® XP Reagent	Beckman Coulter®	A63880 (5 mL) A63881 (60 mL)	Choose one. We do not recommend substituting other magnetic beads. **About 10 mL SPRI beads will be needed throughput the protocol.**
SPRIselect Reagent	Beckman Coulter	B23317 (5mL) B23318 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific®	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich®	459844	Or equivalent 100% non-denatured ethanol.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent DNA quantifier.
High Sensitivity DNA Kit	Agilent®	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or Tapestation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	

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
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 96 well plates and with 10 mL or 15 mL tubes. Capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting devices. We recommend validating alternatives relative to a hemocytometer.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.
2 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.
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath 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.
6-inch Platform	Scientific Industries	146-6005-00	
Microplate Foam Insert	Scientific Industries	504-0235-00	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific	Q33327	Or an equivalent fluorometer.
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	

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.

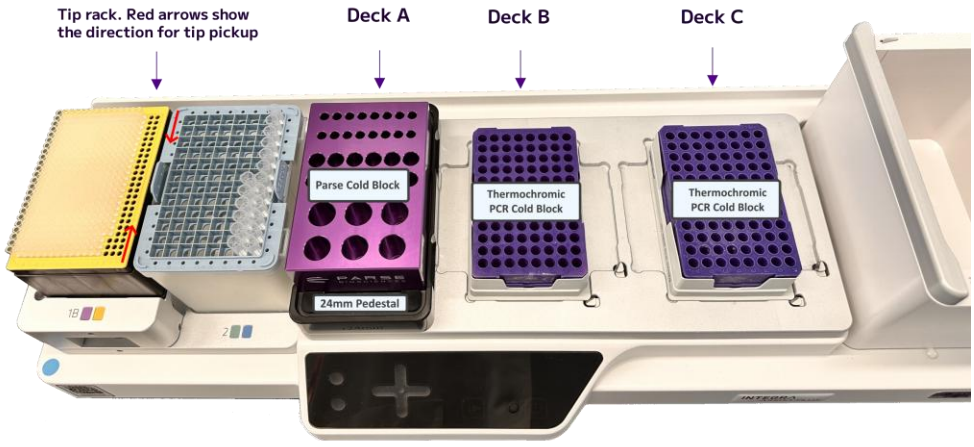
There are three types of figures in this manual.

- 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 Pedestals 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

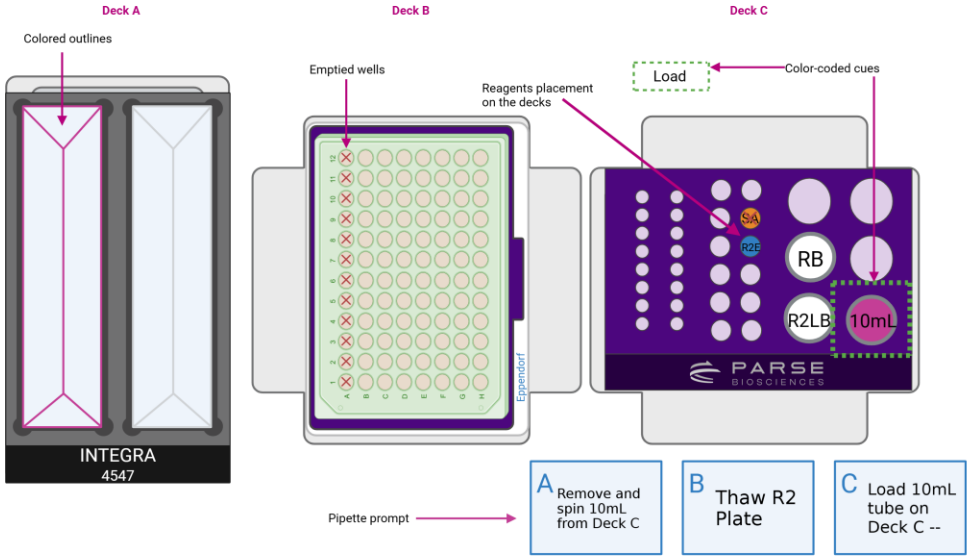
decks, list the consumables required at each step, and highlight pipette prompts for manual intervention.

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.

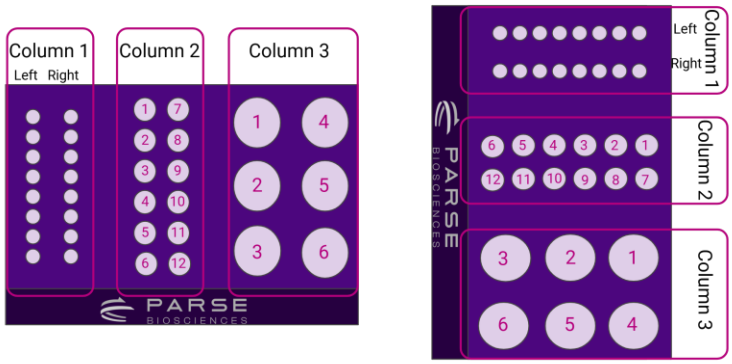
The lowercase letters in the text (e.g. a, b, c…) are lists for the robot's individual actions. These same letters also appear in the diagrams, so you can match the text to the visuals.



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.

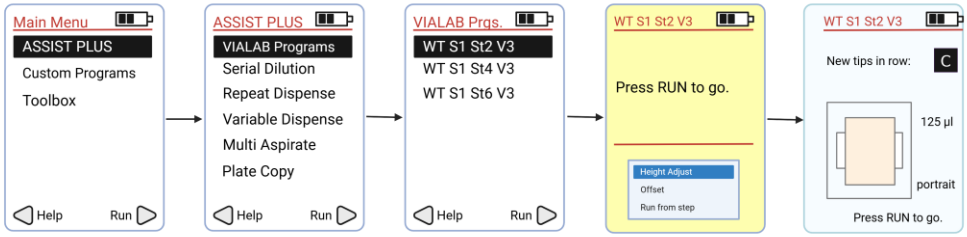
- **Load**: Indicates labware and/or reagents that need to be placed or reloaded onto the deck.
- **Remove**: Identifies labware that should be taken off the deck, either for incubation, disposal, or because it is not needed in the upcoming step.
- **Replace**: Highlights labware that has been used and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

Penta requires a minimum concentration of 4,114 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.

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. 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 P.

1. Cool a centrifuge with swinging bucket rotors to 4°C.
2. Set a water bath to 37°C.
3. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	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 Blocks with risers 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 Sample Dilution Buffer	Consumables	4	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Sample Dilution Buffer	-20°C Reagents or ECAC3901	4	Thaw at room temperature then store on ice. Mix by inverting 3x.
Round 1 Plate P	-20°C Reagents	1	Place directly on ice.

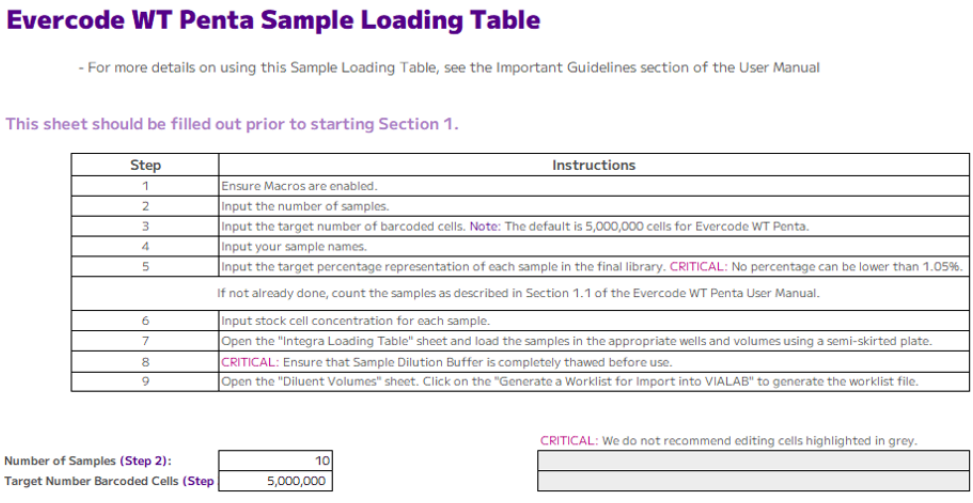
4. Download the Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm. The most current version of the Sample Loading Table can be found on the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.

5. 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.

6. 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.

7. Fill the Sample Loading Table tab of the worksheet.

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



Evercode WT Penta Sample Loading Table

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

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 5,000,000 cells for Evercode WT Penta.
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 1.05%.
	If not already done, count the samples as described in Section 1.1 of the Evercode WT Penta User Manual.
6	Input stock cell concentration for each sample.
7	Open the "Integra Loading Table" sheet and load the samples in the appropriate wells and volumes using a semi-skirted plate.
8	CRITICAL: Ensure that Sample Dilution Buffer is completely thawed before use.
9	Open the "Diluent Volumes" sheet. Click on the "Generate a Worklist for Import into VIALAB" to generate the worklist file.

Number of Samples (Step 2):	10
Target Number Barcoded Cells (Step	5,000,000

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

Figure 1: Parse Biosciences Evercode WT Penta INTEGRA Sample Loading Table v2.1.xlsm instructions.

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 C (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.

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

Number of Samples (Step 2): 10
Target Number Barcoded Cells (Step 5,000,000

Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/uL) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/uL)	
1	Sample 1	10.00%	3,500	10	500000	4114	CRITICAL: This cell stock concentration is too low. No Dilution Buffer will be added to this well.
2	Sample 2	10.00%	5,374	10	500000	4114	
3	Sample 3	10.00%	5,374	10	500000	4114	
4	Sample 4	10.00%	5,374	10	500000	4114	
5	Sample 5	10.00%	5,374	10	500000	4114	
6	Sample 6	10.00%	5,374	10	500000	4114	
7	Sample 7	10.00%	5,374	9	500000	4114	
8	Sample 8	10.00%	5,374	9	500000	4114	
9	Sample 9	10.00%	5,374	9	500000	4114	
10	Sample 10	10.00%	5,374	9	500000	4114	
TOTALS:		100.00%		96	5,000,000		

Figure 2: Example error message, noting that the sample stock concentration is too low. If the sample stock concentration is too low, no dilution buffer will be added to the sample and a lower proportion of the sample will be present. However, a worklist can still be generated if the concentration is out of the lower range.

c. Navigate to the "INTEGRA Loading Table" tab. If the "Required Number of Sample Dilution Tubes" is greater than 4, samples need to be serially diluted (see the introductory paragraph in this section) (Figure 3).

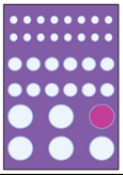
Sample Name	Sample Location	Min Sample Stock Needed for Dilution (uL)	*Note: due to semi-skirted plate volumes, multiple wells might be needed for the same samples.	Min Diluent Needed (uL)	Required Number of Sample Dilution Tubes	Sample Dilution Tube Locations
Sample 1	A1	207.2		781.0	1	
Sample 1	A2	207.2				
Sample 1	A3	103.6				
Sample 2	A4	198.3				
Sample 2	A5	198.3				
Sample 3	A6	198.3				
Sample 3	A7	198.3				
Sample 4	A8	198.3				
Sample 4	A9	198.3				
Sample 5	A10	198.3				
Sample 5	A11	198.3				
Sample 6	A12	198.3				
Sample 6	B1	198.3				
Sample 7	B2	198.3				
Sample 7	B3	198.3				
Sample 8	B4	198.3				
Sample 8	B5	198.3				
Sample 9	B6	198.3				
Sample 9	B7	198.3				
Sample 10	B8	198.3				
Sample 10	B9	198.3				
	B10					

Figure 3: INTEGRA Loading Table tab notes the minimum Diluent needed, Sample Dilution tube deck loading locations, and Sample Locations.

d. Referring to the "INTEGRA Loading Table" tab, manually load fixed samples into the directed "Sample Location" well into a new Eppendorf semi-skirted plate. This is the sample stock plate. Store the sample stock plate on ice for later use.

e. 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 "CombinedPentaWorksheet_YYYYMMDD_HHMMSS.csv”) for later use (Figure 4).

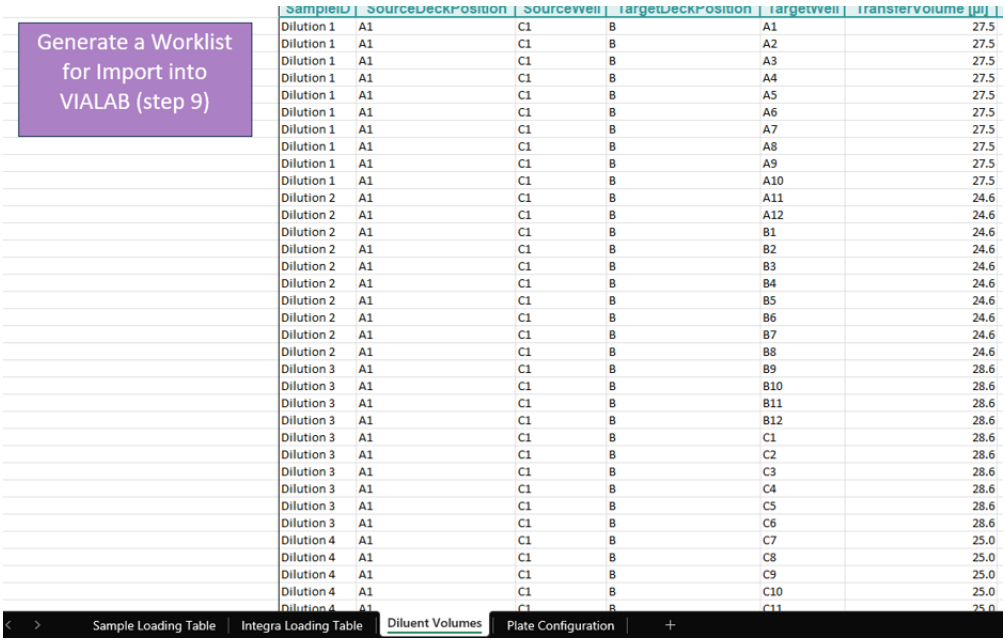
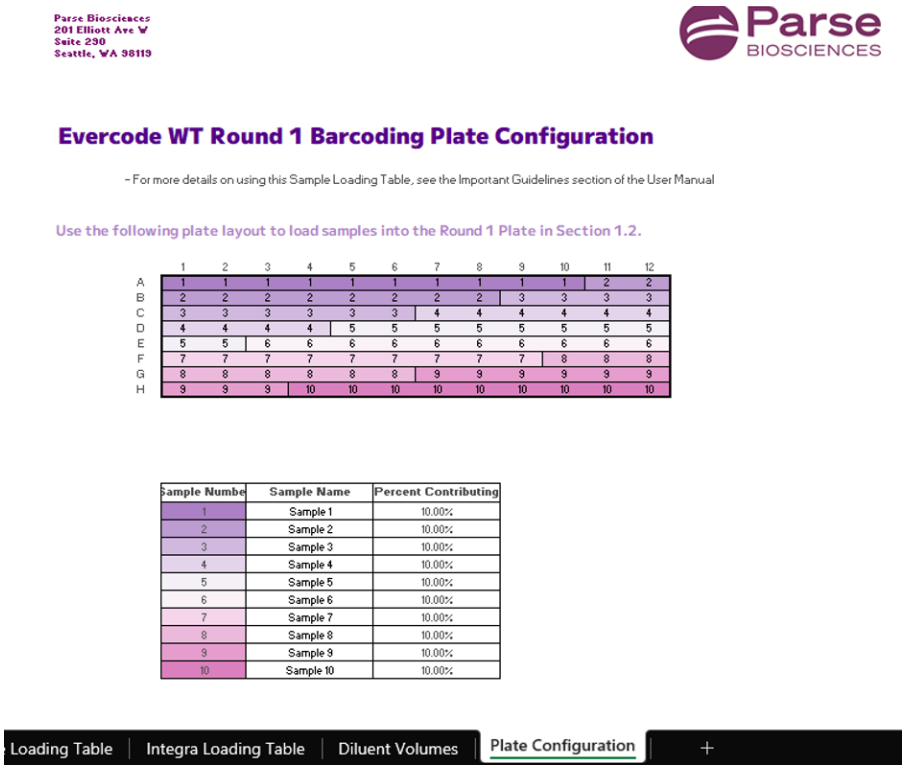


Figure 4: Diluent Buffer Volumes tab for generating a VIALAB worklist.

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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Parse BIOSCIENCES

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	2	2
B	2	2	2	2	2	2	2	2	3	3	3	3
C	3	3	3	3	3	3	4	4	4	4	4	4
D	4	4	4	4	5	5	5	5	5	5	5	5
E	5	5	6	6	6	6	6	6	6	6	6	6
F	7	7	7	7	7	7	7	7	7	8	8	8
G	8	8	8	8	8	8	9	9	9	9	9	9
H	9	9	9	10	10	10	10	10	10	10	10	10

Sample Numbe	Sample Name	Percent Contributing
1	Sample 1	10.00%
2	Sample 2	10.00%
3	Sample 3	10.00%
4	Sample 4	10.00%
5	Sample 5	10.00%
6	Sample 6	10.00%
7	Sample 7	10.00%
8	Sample 8	10.00%
9	Sample 9	10.00%
10	Sample 10	10.00%

Loading Table | Integra Loading Table | Diluent Volumes | Plate Configuration | +

Figure 5: Plate Configuration tab visualizes the sample locations.

8. Import the generated CSV file into VIALAB to be uploaded on the pipettes.

    a. Open the VIALAB program **PT S1 St1 DONE V3_5** and navigate to the “Method” section.

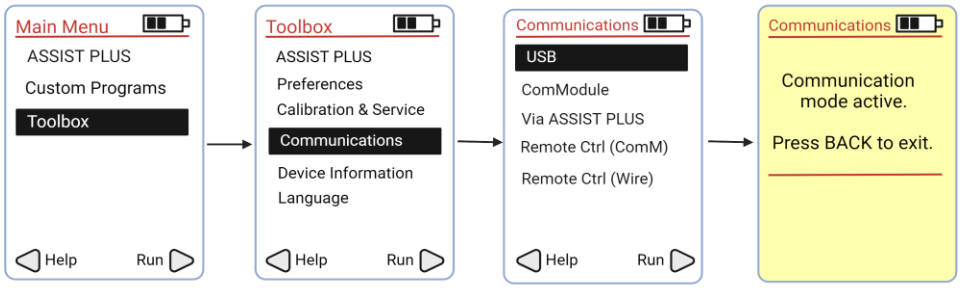
    b. In the “02 Worklist”, under the “Worklist and Volumes” tab, upload the "CombinedPentaWorksheet_YYYYMMDD_HHMMSS.csv" worklist file generated in Step 4e using the “Import” button (Figure 6).

Figure 6: VIALAB worklist generation for diluent volumes using 02 Worklist.

9. 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:

    a. Connect your computer with D-ONE Pipette using Communication/Charging Cable for VIAFLO.

    b. Follow the instructions on the pipette menu, as shown in the diagram below:

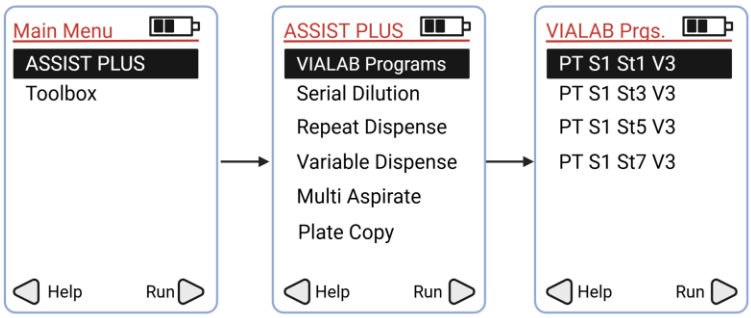


    c. Select the “Transfer” tab in the opened **PT S1 St1 DONE V3_5** VIALAB program.

    d. Confirm that the pipette has been recognized by the software. Click "Send to pipette". An audible signal will indicate the successful upload of the **PT S1 St1 V3_5** program to the D-ONE Pipette.

    e. Disconnect the cable and proceed with the protocol on the INTEGRA ASSIST PLUS platform.

    f. If done correctly, a program named **PT S1 St1 V3_5** will be found on your pipette as shown in the diagram below:



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

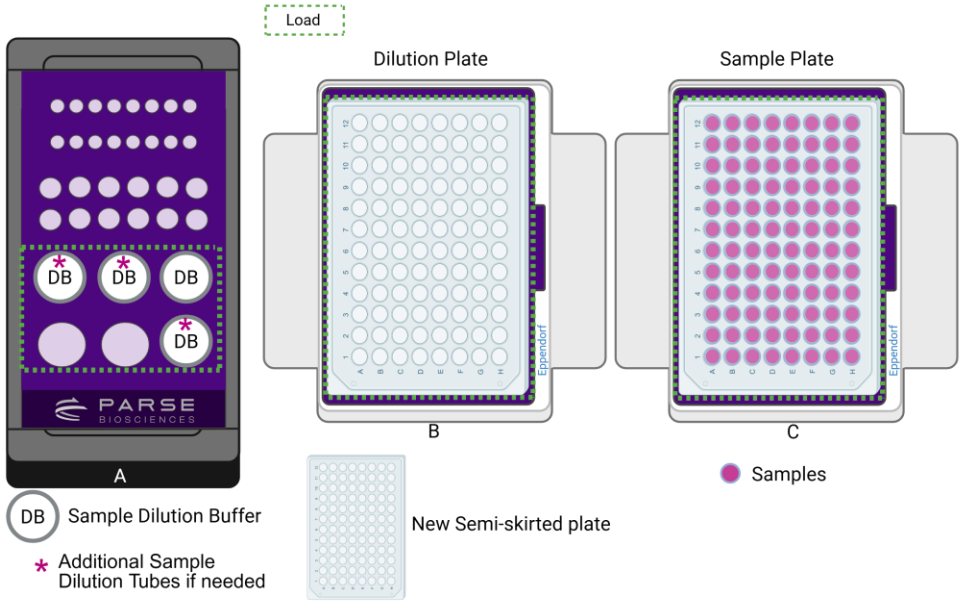
11. Load the following reagents and consumables to their respective positions on Decks A, B, and C:

    a. Deck A, column 3

        i. Pos 1-4: ○ Sample Dilution Buffer. The sample loading table indicates the tubes position (Figure 3).

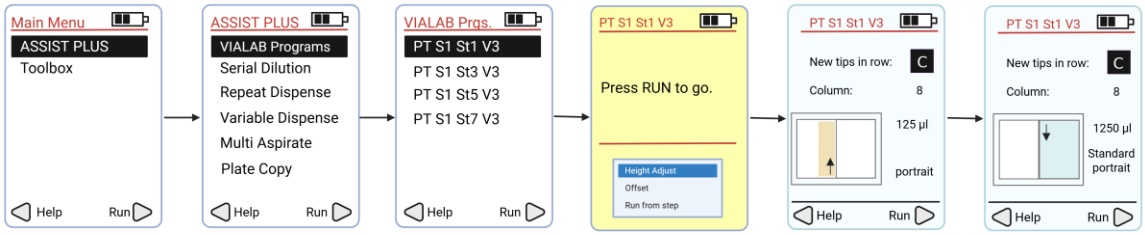
    b. Deck B: a clean semi-skirted plate on Deck B with A1 corner in the bottom left.

    c. Deck C: Sample plate with A1 corner in the bottom left.



12. Attach D-ONE Pipetting Module 1-Ch, 5-1250 µL and the corresponding Tip Deck.

    a. Remove the reagent caps, then select and run the program **PT S1 St1 V3_5**. Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.



13. During the run, but after the Sample Dilution Buffer has been dispensed:

    a. Thaw the Round 1 Plate P using the following thermocycling program. Remove a Thermochromic PCR Cold Block to thaw at room temperature during the following thermocycling program.

THAW ROUND 1 PLATE P		
Total Run Time		10 minutes
Lid Temperature		70°C
Sample Volume		65 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

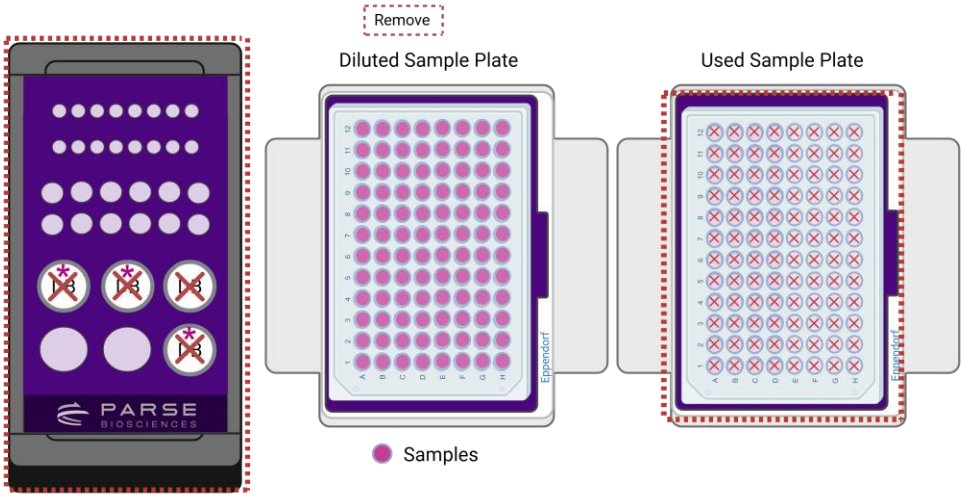
    b. At the completion of the thermocycler program, centrifuge the Round 1 Plate P for **1 minute** at 100 x g at 4°C

14. At the conclusion of the run:

    a. Discard any remaining Sample Dilution Buffer from Deck A. Remove all labware from Deck A.

    b. Keep all the labware, including the Diluted Sample Plate on Deck B. This will be used in the next step.

    c. Remove all the hardware on Deck C. Discard the used Sample Plate on Deck C.



1.2. Loading and Pooling the Round 1 Plate

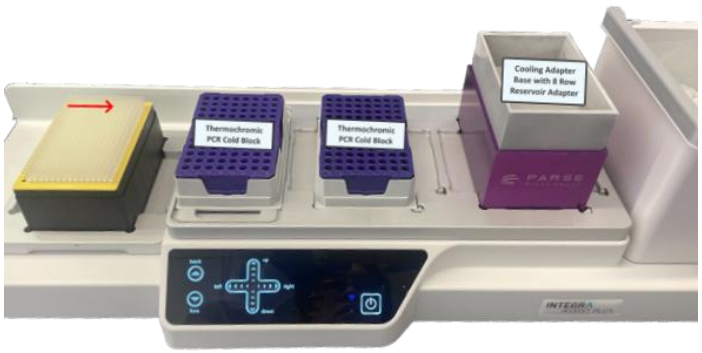
The program loads the normalized cells/nuclei on Deck B into the Round 1 Plate on Deck C. After Barcoding Round 1 incubation, move the Round 1 Plate onto Deck B.

The program then pools all the samples in the Round 1 Plate into row 4 of the 8 row reservoir.

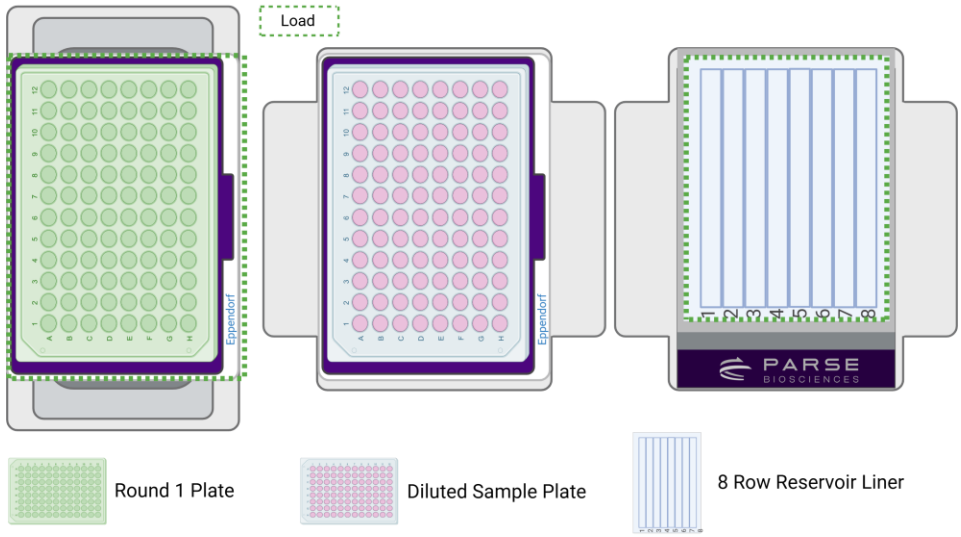
1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	1	
Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	1	
5-125 µL Tip Rack	INTEGRA	1	
8 Row Reservoir	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	2	If not done earlier, 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	
Round 1 Plate	-20°C Reagents	1	Place directly on ice.
Fixation Cooling Adapter Base	Parse	1	
Fixation 8 Row Reservoir adapter	Parse	1	

2. Place the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser that was thawed during the previous step on Deck A following the configuration below.



3. 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. The diluted sample plate is still on Deck B with A1 at the bottom left.

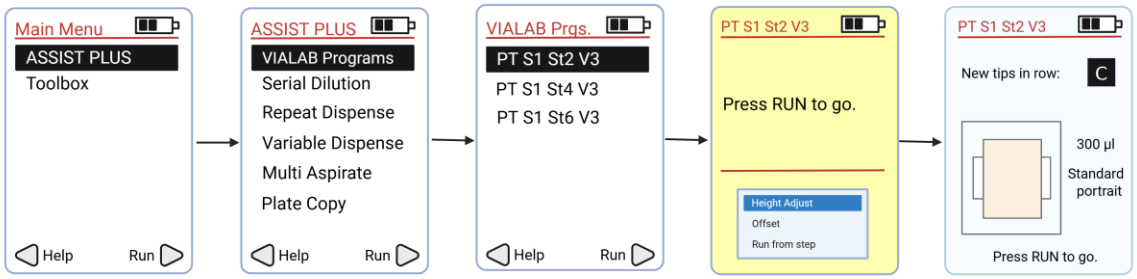


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

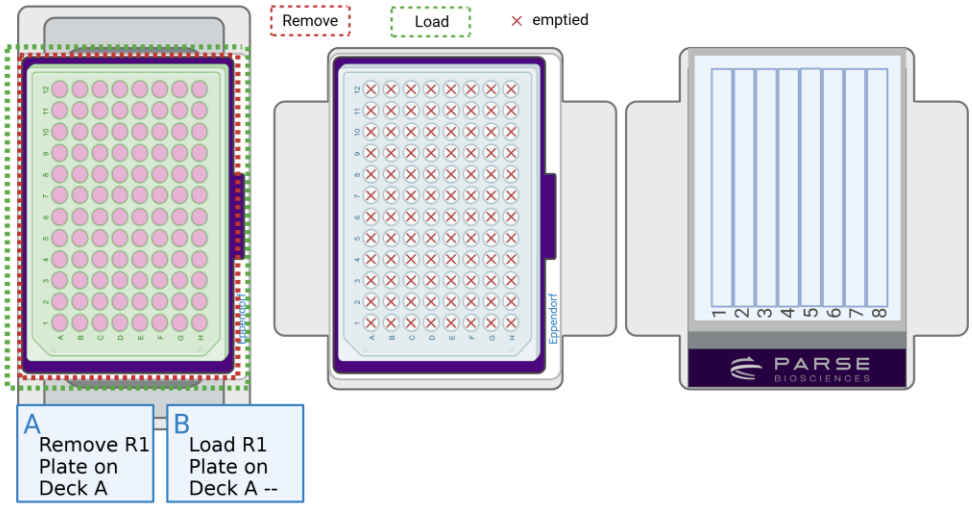
    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VIAFLO 12-Ch 5-125 µL 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.

5. Run the program **PT S1 St2 V3_5** following the diagram below.



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



a. Remove the Round 1 Plate P from Deck A and use a Plate Seal Applicator to seal the plate. Place the Round 1 Plate P into a thermocycler and run the following program.

BARCODING ROUND 1			
Total Run Time			40 minutes
Lid Temperature			70°C
Sample Volume			100 µL
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	1

b. When the Barcoding Round 1 Thermocycling program is complete, remove the seal and load the Round 1 plate on the Thermochromic PCR Cold Block on Deck A. Press “Run” to continue the program.

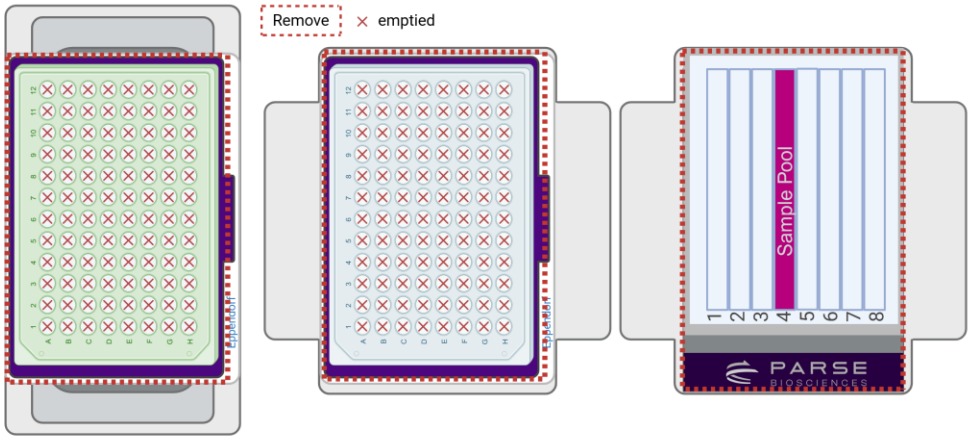
7. At the conclusion of the run:

   a. Remove all labware from Deck A. Store the Thermochromic PCR Cold Block in the freezer. Discard the used Round 1 Plate.

   b. Remove all the labware from Deck B. Store the Thermochromic PCR Cold Block in the freezer. Discard the used semi-skirted plate.

   c. Remove the Fixation Cooling Adapter Base, Fixation 8 Row Reservoir Cooling Adapter and the 8 row reservoir containing the pooled samples from Deck C. Do not discard the 8 row reservoir.

Note: The 8 row reservoir will be used in the next step. Keep it cold either in the cooling adapter base or in the fridge until the next step.



1.3. Round 2 Ligation Preparation

This program transfers the pooled samples from Section 1.2 into a 10 mL tube to be centrifuged. While the samples are being centrifuged, the Round 2 Ligation Master Mix is created in a row of the 8 Row Reservoir. After centrifugation, the supernatant is removed from the samples and the cell pellet is resuspended using the Round 2 Ligation Master Mix. The sample pool is then strained and transferred to the wells of the semi skirted plate on Deck A.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	1	Pull the freezer 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	
ASSIST PLUS Slanted Plate Holder	INTEGRA Components	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate	Consumables	1	
10 mL transport tube	Consumables	2	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Round 2 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Round 2 Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Resuspension Buffer	-20°C Reagents	1	
Round 2 Plate A	-20°C Reagents	1	Place directly on ice.
Round 2 Plate B	-20°C Reagents	1	
Spin Additive	4°C Reagents	1	Briefly centrifuge. Keep at room temperature.
25 µm, 40 µm, or 70 µm cell strainer	Consumables	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
300 mL Reservoir Base	INTEGRA	1	

2. Prepare the deck hardware:

   a. Place the Thermochromic PCR Cold Block on Deck A with A1 orientated towards the bottom left.

   b. Add the ASSIST PLUS Slanted Plate Holder on Deck B, incorporating a 10° tilt.

Note: Ensure that the lowest side of the Slanted Plate Holder is positioned adjacent to Deck A

   c. Place the Integra 8 Row Reservoir Plastic Base on the Slanted Plate Holder.

CRITICAL! Ensure the blue reservoir locks are removed from the Plastic Base.

   d. Place the Parse Metal Cold Block on Deck C.

3. Load the consumables, reagents, and samples on Deck:

   a. Load a clean Semi-Skirted 96 Well PCR Plate on the Thermochromic PCR Cold Block on Deck A with A1 orientated towards the bottom left.

   b. Load the reagents and consumables on the Parse Metal Cold Block

      i. Column 2:

         1. Pos 8: ● Spin Additive

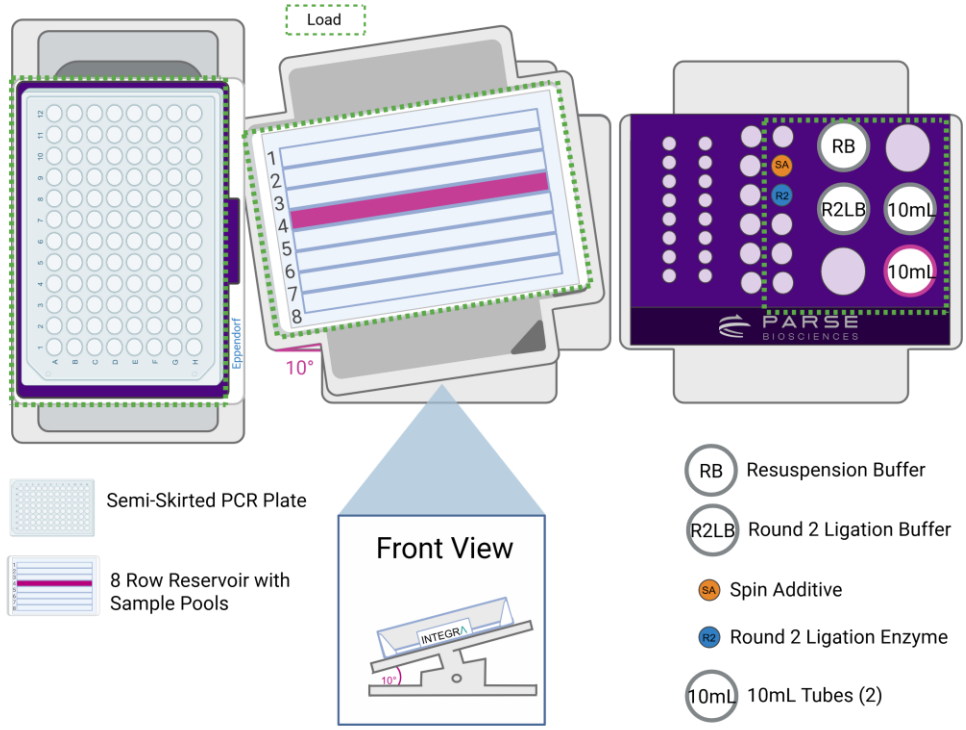
         2. Pos 9: ● Round 2 Ligation Enzyme

      ii. Column 3:

1. Pos 1: O Resuspension Buffer
2. Pos 2: O Round 2 Ligation Buffer
3. Pos 5: Clean 10mL transport tube
4. Pos 6: Clean 10mL transport tube

4. Carefully Load the 8 Row Reservoir filled with the Round 1 Plate pool from Section 1.2 onto the Integra 8 Row Reservoir Plastic Base with row 1 on the back left corner.

Note: When moving the 8 Row Reservoir onto the slanted plate holder on Deck B, take extra care when moving the sample to avoid spills.

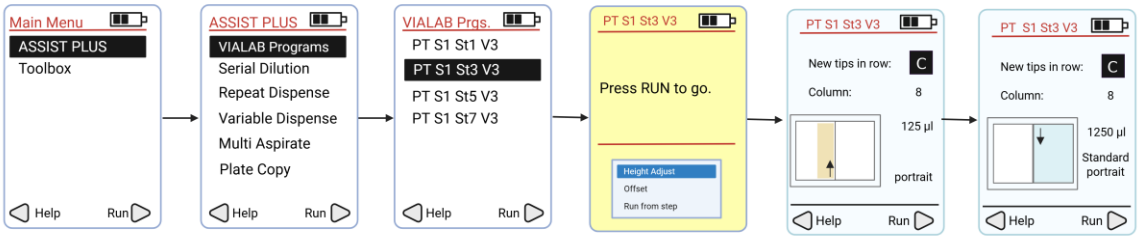


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

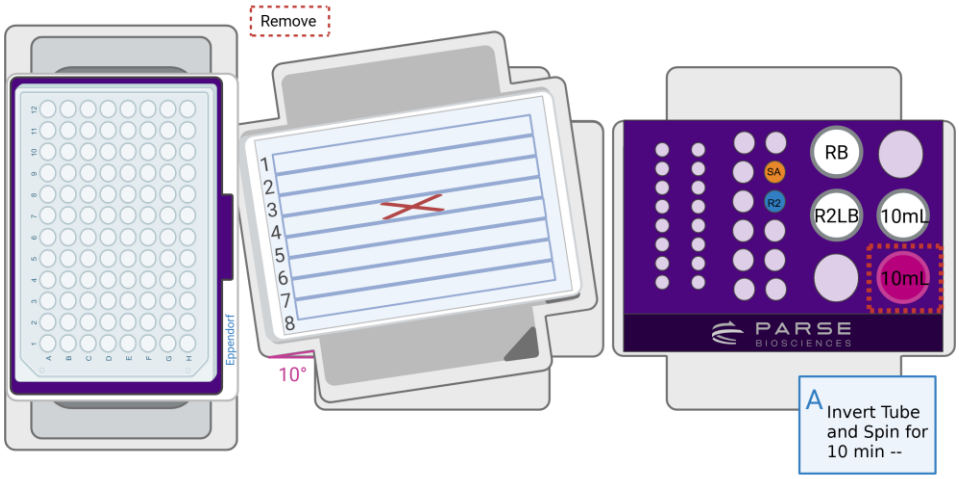
    a. Remove the VIAFLO 12-Ch-125 µL Pipette and corresponding Tip Deck A. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

6. Remove the reagent caps, select and run the program **PT S1 St3 V3_5** following the diagram below.



7. When prompted, cap and invert the 10 mL tube in Column 3 position 6 of the Parse Metal Cold Block once. Centrifuge the 10 mL tube using a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step during centrifugation.



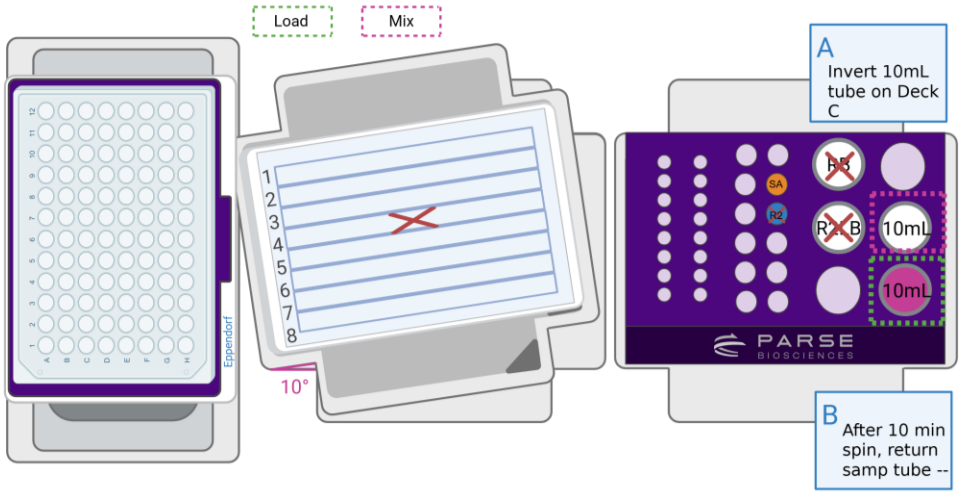
8. While the samples are centrifuging press “Run” to continue. The Round 2 Ligation Master Mix will be created in row 8 of the 8 Row Reservoir. Proceed immediately to the next step while the program is running.

    a. While the Round 2 Ligation Master Mix is being made, thaw the two Round 2 Plates—Round 2 Plate A and Round 2 Plate B—into two thermocyclers and run the following program for later use. While the thermocycling program is running, remove two new Thermochromic PCR Cold Blocks with Risers from the -20°C freezer and thaw at room temperature and continue to the next step.

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

Note: The Round 2 Plates can stay in the thermocycler or at 4C until prompted for use.

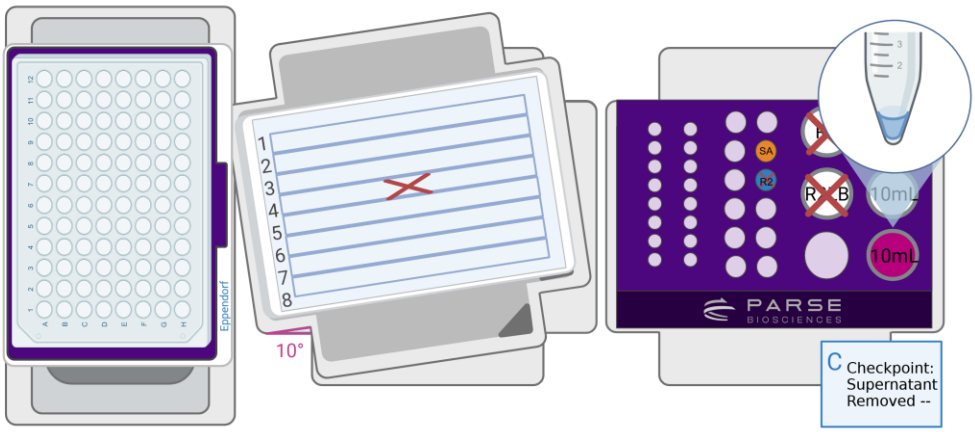
9. Follow the prompt instructions and do the following:



a. Cap and invert the 10mL tube in Column 3 position 5 of the Parse Metal Cold Block three times. This contains the Round 2 Ligation Master Mix. The volume is too large to be mixed with the provided pipette tip and must be done manually. Return the tube to Column 3 position 5 of the Parse Metal Cold Block and remove the cap before pressing "Run" to continue.

b. Immediately after the centrifugation is complete, load the sample tube on Deck C in Column 3 position 6 of the Parse Metal Cold Block. Remove the cap, and press "Run" to continue the program.

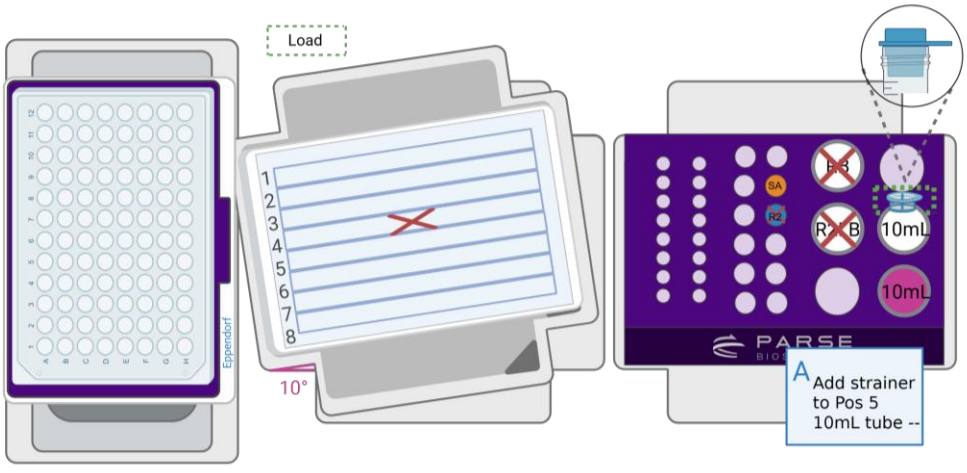
CRITICAL! Move quickly and handle the sample tubes gently to avoid dislodging the pellet, which will have a significant impact on data quality.



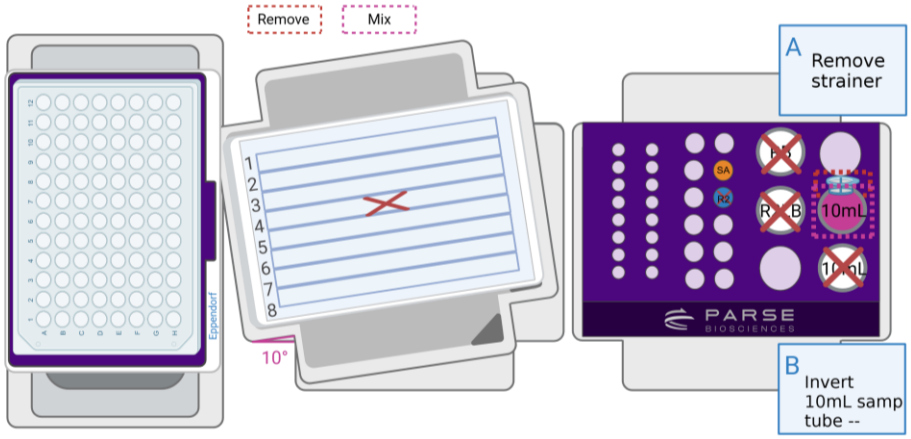
c. After removing the supernatant, inspect the 10 mL tube and visually confirm that the supernatant has been removed, leaving only a small volume at the bottom. If there is more than ~100ul of volume left in the tube, manually remove supernatant until about ~100 µL of liquid remains above the pellet. A pellet may not be visible. Avoid disturbing the pellet—do not insert the pipette tip into the pellet area to measure the supernatant. Press "Run" to continue the program.

Note: The supernatant waste is dispensed in Row 4 of the 8 Row Reservoir on Deck B.

10. When prompted, add a strainer on the 10mL tube in Column 3 position 5 of the Parse Metal Cold Block. Press "Run" to continue the program.



11. Follow the prompt instructions and do the following:



a. When prompted, remove the strainer. Press “Run” to continue the program.

b. Cap the 10 mL tube in Column 3, position 5 of the Parse Metal Cold Block. Invert this tube three times. Remove the cap and press “Run” to continue the program.

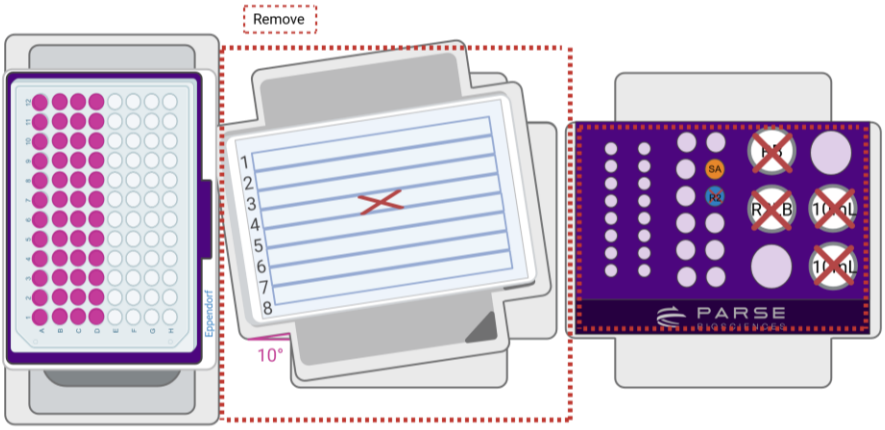
12. At the conclusion of the run:

a. Remove the Spin Additive from Column 2 position 8 of the Parse Metal Cold Block and set aside at room temperature for later use.

b. Remove all labware on Deck B. The 8 Row Reservoir should be sealed with a plate seal and stored on ice as it will be used at later steps.

c. Remove all labware on Deck C. Discard the used tubes on the Parse Metal Cold Block in Deck C including the empty reagent tubes.

d. The semi skirted plate on Deck A contains the samples and will be used for the next section. Do NOT remove the contents from Deck A.



1.4. Round 2 Ligation

This program loads the Round 2 Plate A on Deck B and Round 2 Plate B on Deck C with the sample pools from Deck A. After Round 2 incubation, Round 2 Stop Mix is mixed into the Round 2 Plates from the reservoir on Deck C. Once Round 2 Stop incubation is completed, the sample is then pooled into row 6 of the reservoir on Deck C.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 125 µL	INTEGRA Components	1	
Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	1	
5-125 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	3	Pull the freezer blocks with risers from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	3	
8 Row Reservoir	INTEGRA	1	
○ Round 2 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Round 2 Plate A	-20°C Reagents	1	Previously thawed and in the thermocycler
Round 2 Plate B	-20°C Reagents	1	
Fixation Cooling Adapter Base	Parse	1	
Fixation 8 Row Reservoir Adapter	Parse	1	

2. Remove the thawed Round 2 Plates from the thermocycler and centrifuge for **1 minute** at 100 x g at 4°C.

3. While secured in a PCR plate rack on a flat surface, remove the plate seal from the Round 2 Plates and store on ice.

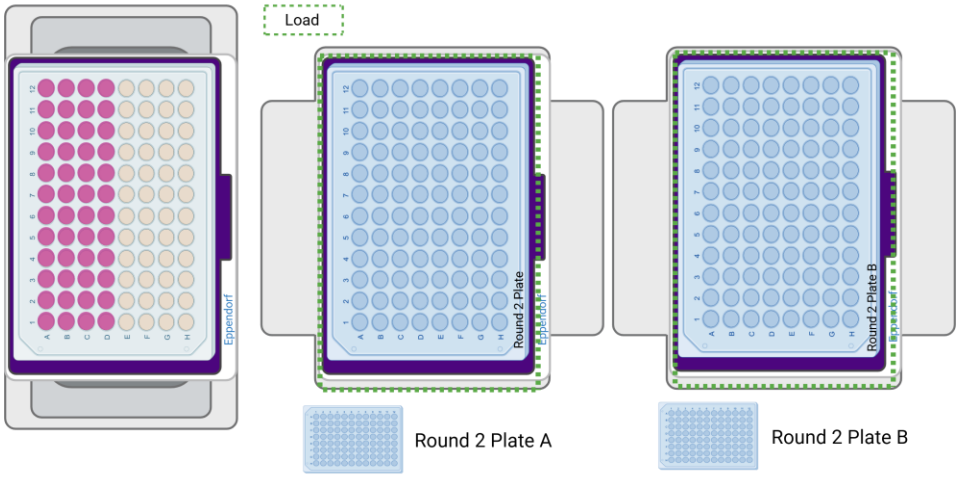
4. Prepare and load the hardware:

    a. Place Thermochromic PCR Cold Blocks on Decks A, B, and C with A1 oriented towards the bottom left. Ensure the Cold Blocks have thawed at room temperature for at least 10 minutes.

5. Load the consumables and reagents on Deck:

    a. Load Round 2 Plate A on Deck B with A1 orientated towards the bottom left.

    b. Load Round 2 Plate B on Deck C with A1 orientated towards the bottom left.

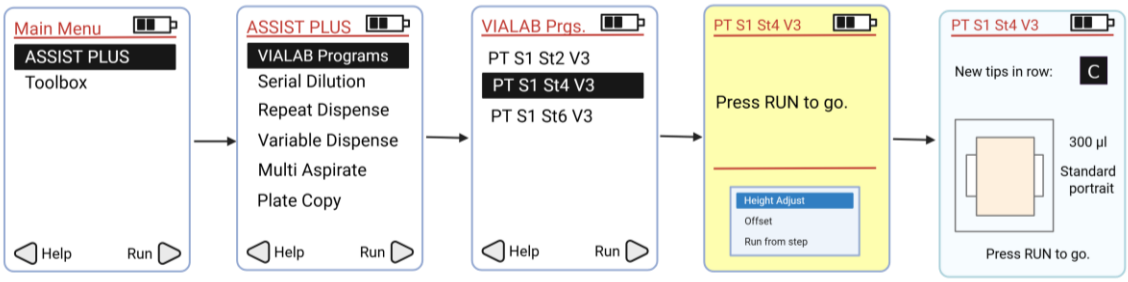


6. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

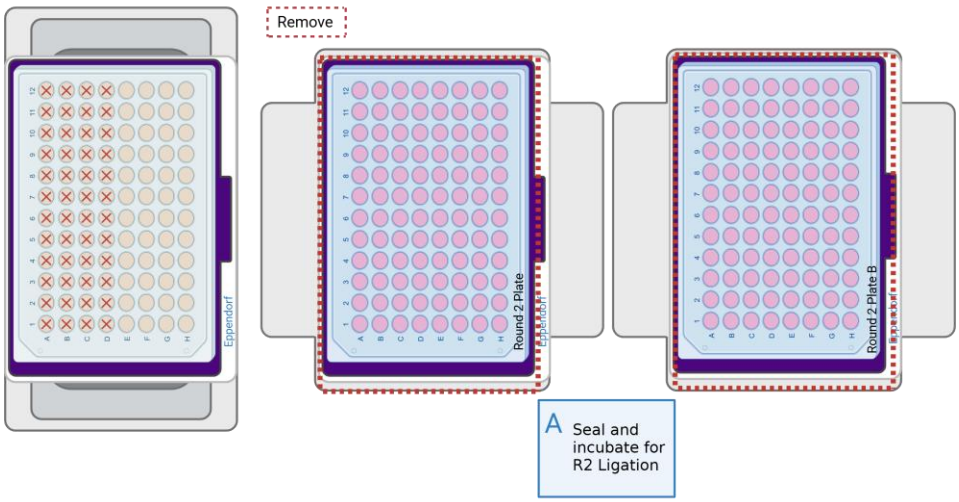
    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VIAFLO 12-Ch 5-125 µL 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 **PT S1 St4 V3_5** following the diagram below.



8. When prompted:



a. Remove the Round 2 Plates from Decks B and C. Reseal the Round 2 Plates with an adhesive seal, place them into separate thermocyclers, and run the following program. Press "Run" to continue the program.

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

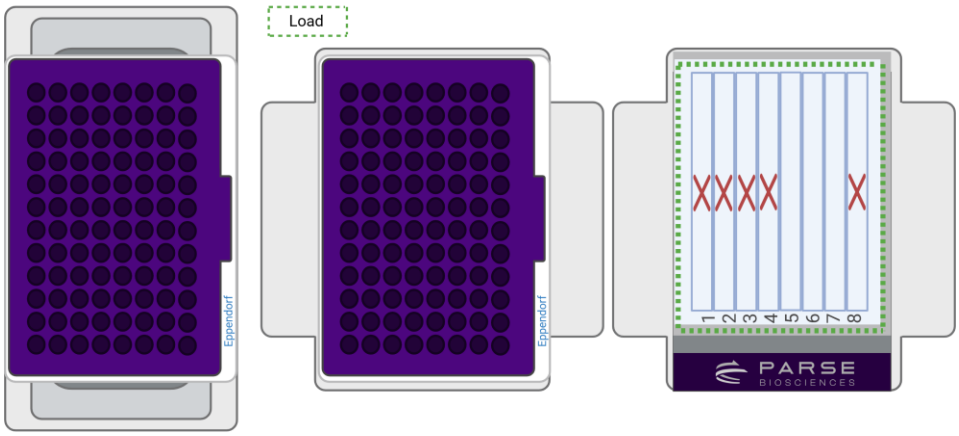
9. When prompted for a labware change:

a. Remove the Thermochromic PCR Cold Block from Deck A and store upside down in the freezer. The used Semi-skirted plate can be discarded.

b. Move the Thermochromic PCR Cold Block from Deck C to Deck A.

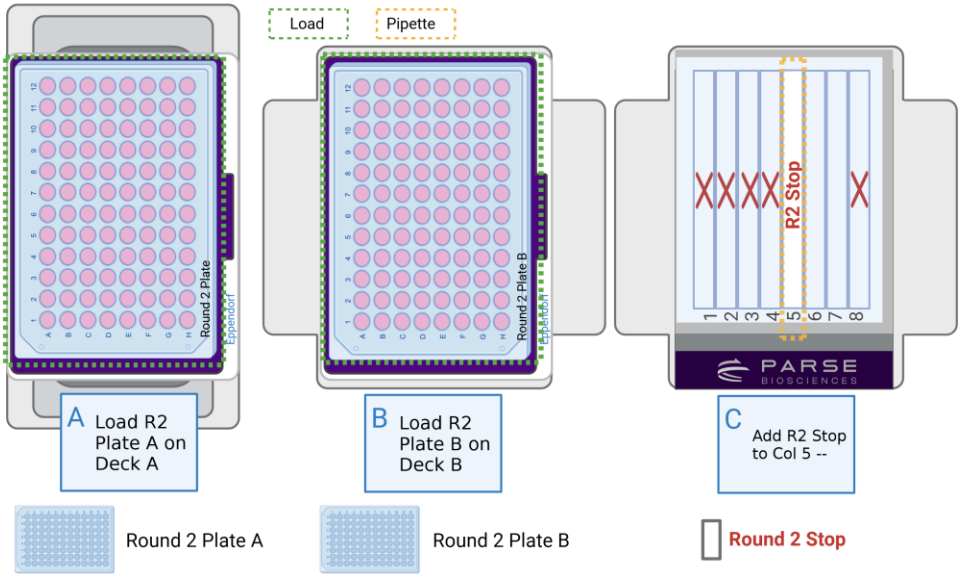
c. Fill a Cooling Adapter Base with pebble ice and place on Deck C. If the Cooling Adapter Base was filled with water and frozen the night before, ensure that the Cooling Adapter Base is thawed at room temperature for at least **10 minutes** before use.

d. Place the 8 Row Reservoir Cooling Adapter on the Fixation Cooling Adapter Base on Deck C.



e. Load the 8 Row Reservoir stored in Section 1.3 on Deck C with Row 1 facing the left. Remove the seal and press “Run” to continue.

10. When prompted and after the Barcoding Round 2 thermocycler program has completed:

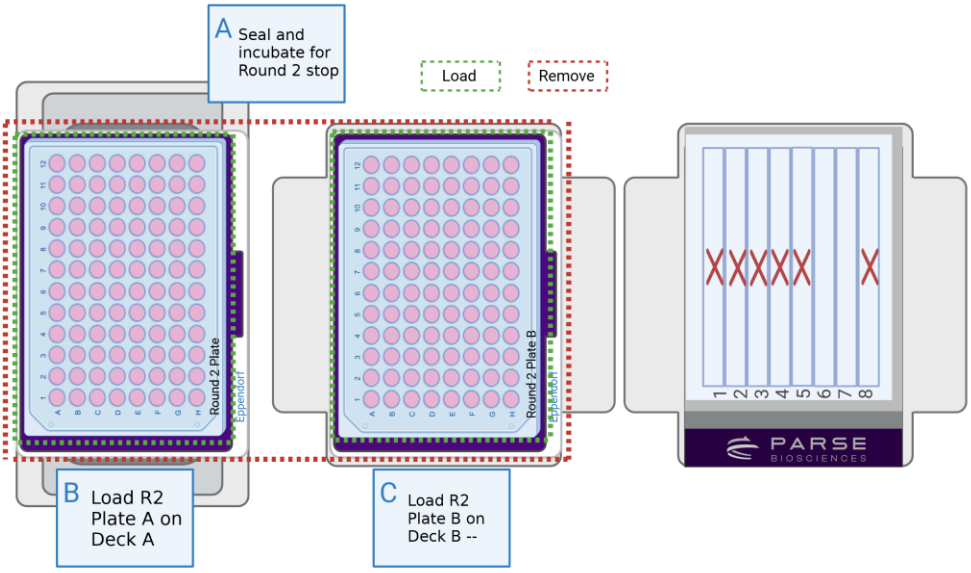


a. Remove the plate seal and load the Round 2 Plate A on the Thermochromic PCR Cold Block on Deck A with A1 oriented towards the lower left corner.

b. Remove the plate seal and load the Round 2 Plate B on the Thermochromic PCR Cold Block on Deck B with A1 oriented towards the lower left corner.

c. Briefly vortex (2-3 seconds) the Round 2 Stop Buffer and ensure there is no precipitate. Pipette the total volume (~2.5 mL) to the row 5 of the 8 row reservoir on Deck C with a P1000 set to 1000 µL. Disperse the liquid to ensure it is even across the basin. Press “Run” to continue the program.

11. When prompted:



a. Remove the Round 2 Plates from Decks A and B. Reseal the Round 2 Plates with an adhesive seal, and incubate them in separate thermocyclers using the following protocol.

ROUND 2 STOP (2)		
Total Run Time		5 minutes
Lid Temperature		50°C
Sample Volume		60 µL
Step	Time	Temperature
1	5 min	16°C
2	Hold	4°C

b. When the Barcoding Round 2 Stop Thermocycling program is complete, load the Round 2 Plate A on the Thermochromic PCR Cold Block on Deck A with A1 oriented towards the lower left corner. Remove the plate seal.

c. Load the Round 2 Plate B on the Thermochromic PCR Cold Block on Deck B with A1 oriented towards the lower left corner. Remove the plate seal. Press “Run” to continue the program.

12. At the conclusion of the run:

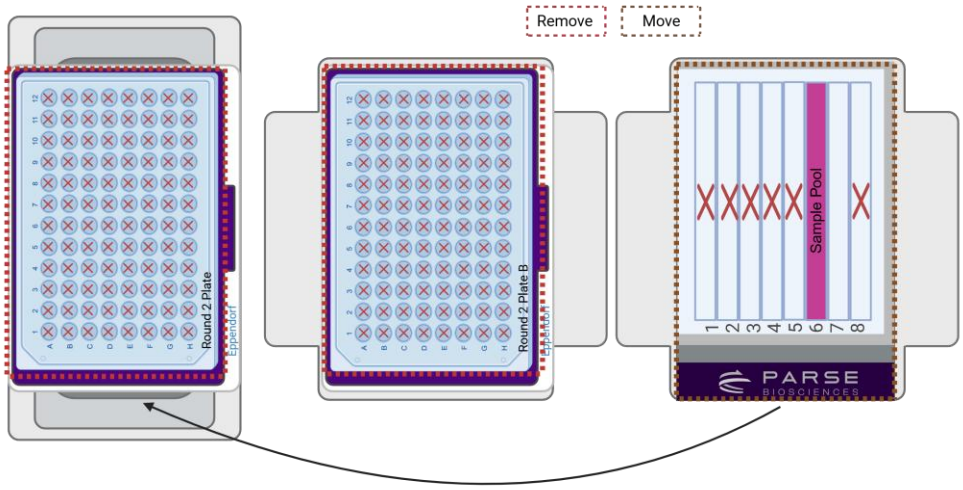
    a. Remove all labware from Deck A. Discard the empty Round 2 plate A. Store the Thermochromic PCR Cold Block upside down in the freezer.

    b. Remove all labware from Deck B. Discard the empty Round 2 plate B. Store the Thermochromic PCR Cold Block upside down in the freezer.

    c. Keep all the labware on Deck C. The sample is in Row 6 of the 8 Row reservoir.

    d. Move all the labware from Deck C to Deck A with Row 1 on the left.

Note: The Sample Pool is in row 6 of the 8 Row Reservoir. When moving the 8 Row Reservoir from Deck C to Deck A, take extra care when moving the sample to avoid spills.



1.5. Round 3 Ligation Preparation

The pooled cell suspension in row 6 of the 8 Row reservoir on Deck A is strained into two 10 mL transport tubes on Deck C. The program adds Round 3 Ligation Enzyme into the cells and mixes. It then transfers the cell/nuclei suspension mix to Row 7 of the 8 Row reservoir on Deck A.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
ASSIST PLUS Slanted Plate Holder	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
Round 3 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
Round 3 Plate A	-20°C Reagents	1	Place directly on ice.
Round 3 Plate B	-20°C Reagents	1	
25 µm, 40 µm, or 70 µm cell strainer	Consumables	2	
300 mL Reservoir Base	INTEGRA	1	
10 mL transport tube	Consumables	2	
Fixation Cooling Adapter Base	Parse	1	
Fixation 8 Row Reservoir Adapter	Parse	1	
8 Row Reservoir	INTEGRA	1	

2. Place the ASSIST PLUS Slanted Plate Holder set to 10° on Deck B. The lower end should be on the left.

3. Place the Integra 300 mL Reservoir Base on the Slanted Plate Holder.

    a. Ensure the blue reservoir locks are removed from the Plastic Base

4. Place the Parse Metal Cold Block on Deck C. Deck layout should correspond to the configuration below.

5. Load the following reagents to their respective positions on the Parse Metal Cold Block on Deck C.

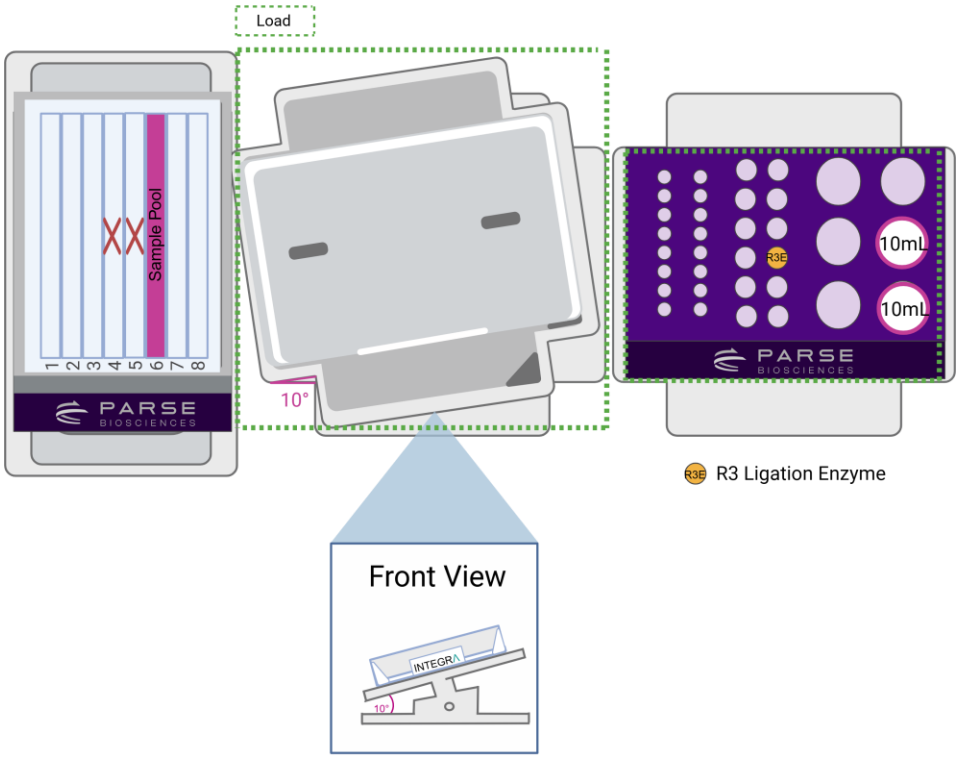
    a. Column 2:

        i. pos 10: ● Round 3 Ligation Enzyme.

    b. Column 3:

        i. pos 5: Clean 10 mL transport tube.

        ii. pos 6: Clean 10 mL transport tube.

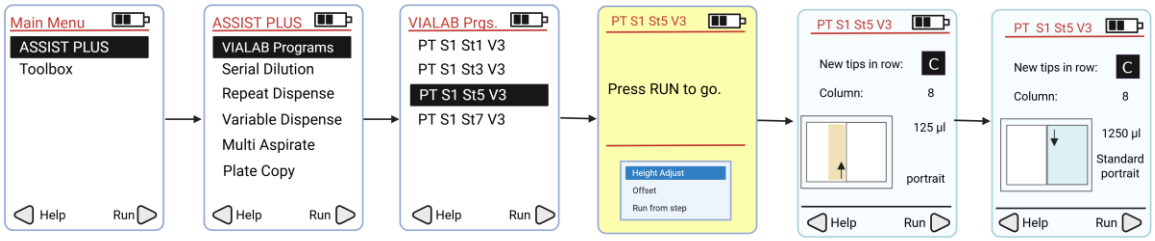


6. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

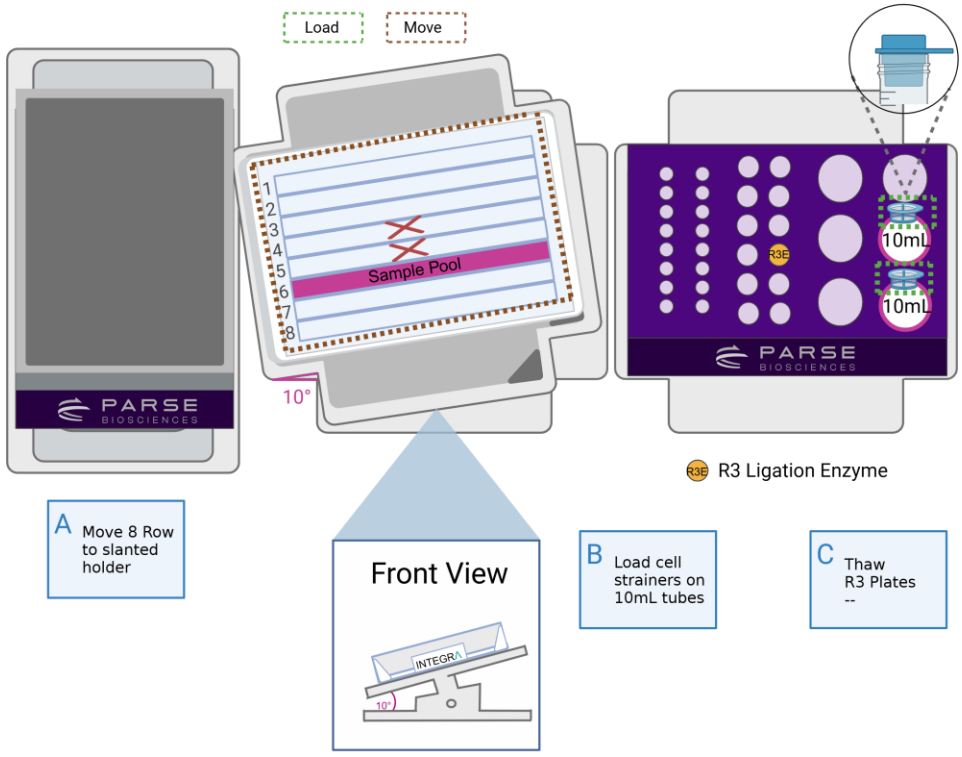
    a. Remove the VIAFLO 12-Ch 5-125 µL pipette and corresponding tip deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with the D-ONE Pipetting Module 1-Ch, 5-1250 µL pipette and corresponding tip deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

7. Remove the reagent caps, then select and run the program **PT S1 St5 V3_5** following the diagram below.



8. Press “Run” to continue the program. Follow the program prompts for manual intervention to move the 8 Row Reservoir as indicated below:



a. Move the 8 Row Reservoir filled with the Round 2 Plate Pool from the 8 Row Reservoir Cooling Adapter on the Fixation Cooling Adapter Base on Deck A to the Integra 8 Row Reservoir Plastic Base on the Slanted Plate Holder on Deck B. Ensure that row 1 is on the back left corner.

Note: When moving the 8 Row Reservoir onto the slanted plate holder on Deck B, take extra care when moving the sample to avoid spills.

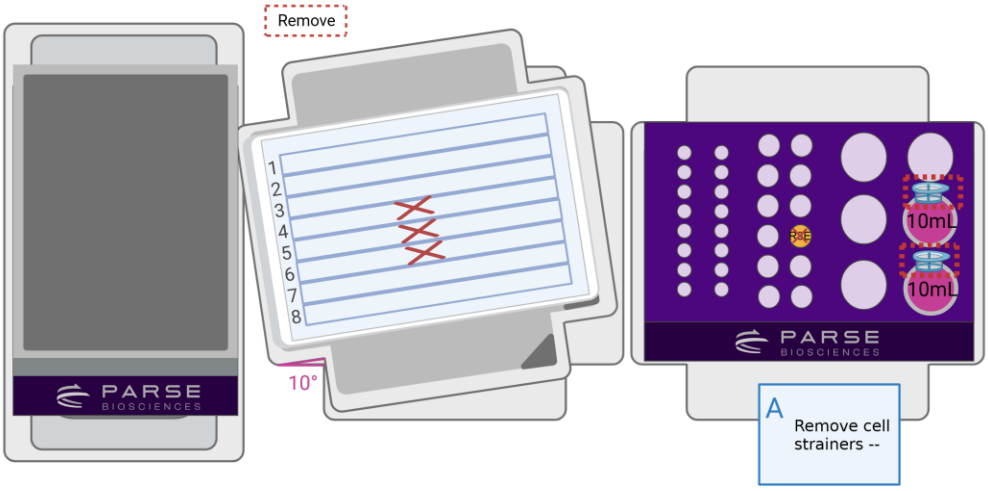
b. Load cell strainers on both 10 mL transport tubes on Deck C Column 3, positions 5 and 6.

c. Thaw the Parse Round 3 Plates A and B using the program below for later use. While the thermocycling program is running, remove two new Thermochromic PCR Cold Blocks with Risers from the -20°C freezer and thaw at room temperature and continue to the next step. Press "Run" to continue the program.

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

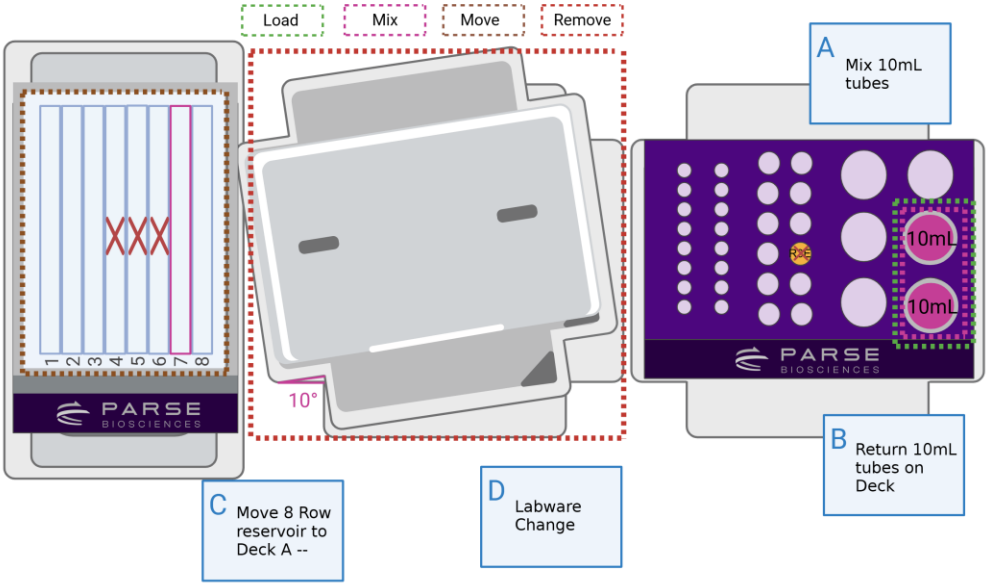
Note: The Round 3 Plates can stay in the thermocycler or at 4°C until prompted for use.

9. Follow the program prompts for manual intervention:



a. Remove the cell strainers from the 10 mL transport tubes on Deck C, column 3, positions 5 and 6 Press “Run” to continue the program.

10. Follow the prompts for manual intervention:



a. Mix both 10 mL transport tubes on Deck C, column 3, positions 5 and 6 by capping and inverting the tubes three times.

b. Return both 10 mL transport tubes on Deck C, column 3, positions 5 and 6. Remove the caps. These tubes are interchangeable.

c. Move the 8 Row Reservoir from the Integra 300 mL Reservoir Plastic Base on the Slanted Plate Holder on Deck B to the 8 Row Reservoir Cooling Adapter on the

Fixation Cooling Adapter Base on Deck A with Row 1 on the left. The sample will be transferred to Row 7 of the 8 Row Reservoir.

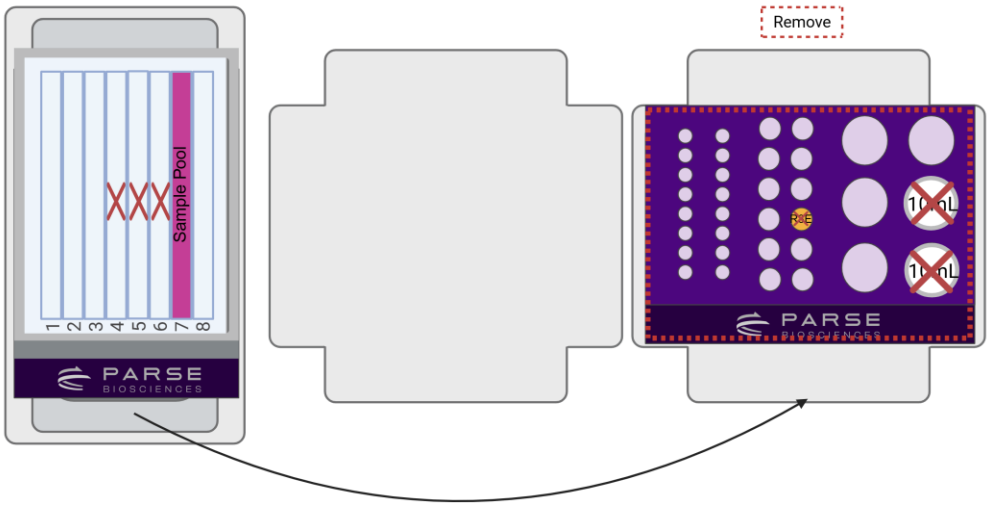
d. Remove the Integra 8 Row Reservoir Plastic Base and the Slanted Plate Holder from Deck B. There should be no labware on Deck B. Press "Run" to continue the program.

11. At the conclusion of the run:

a. Remove all labware from Deck C. The used tubes can be discarded. Remove the Parse Metal Cold Block from Deck C.

b. Move all labware from Deck A to Deck C. The sample pool will be in Row 7 of the 8 Row Reservoir. Height restrictions for Round 3 Plate loading do not allow the 8 Row Reservoir Cooling Adapter on the Fixation Cooling Adapter Base to be on Deck A.

c. Centrifuge Round 3 Plates A and B for **1 minute** at 100 x g at 4°C.



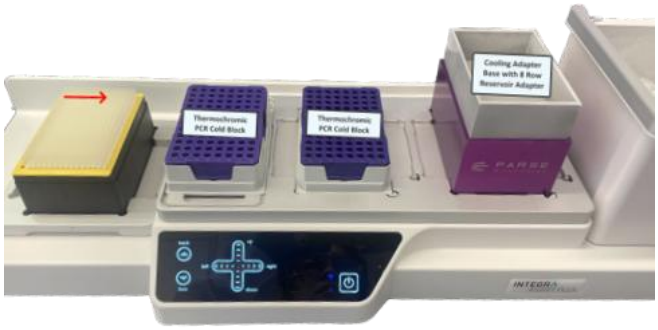
1.6. Round 3 Ligation

The program loads the Cell Suspension Mix on Deck C into the Round 3 Plates on Decks A and B. After the Round 3 incubation, the program will then load Final Stop Buffer into all the wells and pool all the samples together on Deck C.

1. Gather the following components and reagents:

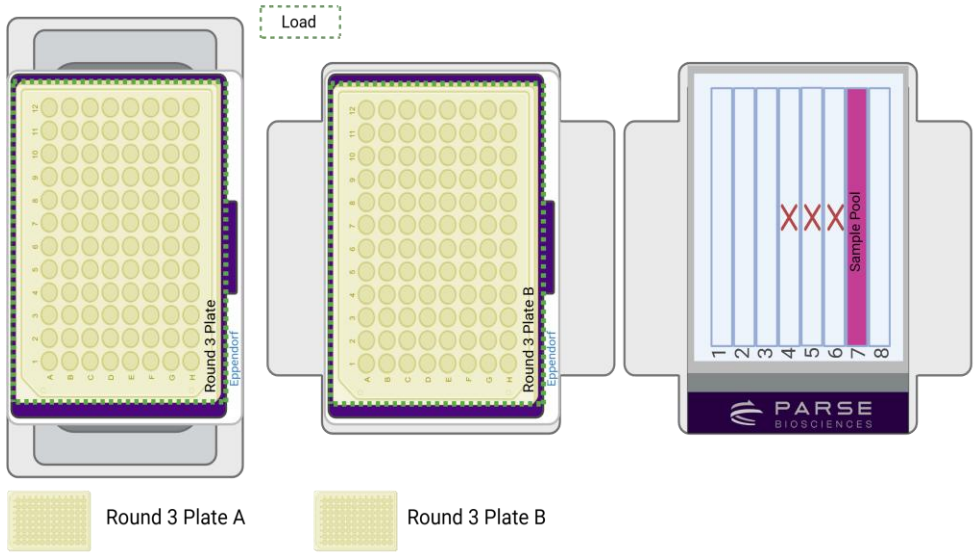
ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	1	
Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	1	
5-125 µL Tip Rack	Consumables	1	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with risers from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	2	
○ Final Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
Round 3 Plate A	-20°C Reagents	1	Place directly in ice
Round 3 Plate B	-20°C Reagents	1	
Fixation Cooling Adapter Base	Parse	1	
Fixation 8 Row Reservoir Adapter	Parse	1	
8 Row Reservoir	INTEGRA	1	

2. Place the two Thermochromic PCR Cold Blocks with Risers removed from the freezer in step 1.5.8c on Decks A and B. Deck layout should correspond to the configuration below.



3. While secured in a PCR plate rack on a flat surface, remove the plate seal from the Round 3 Plate A and place it on Deck A with A1 at the bottom left.

4. While secured in a PCR plate rack on a flat surface, remove the plate seal from the Round 3 Plate B and place it on Deck B with A1 at the bottom left.

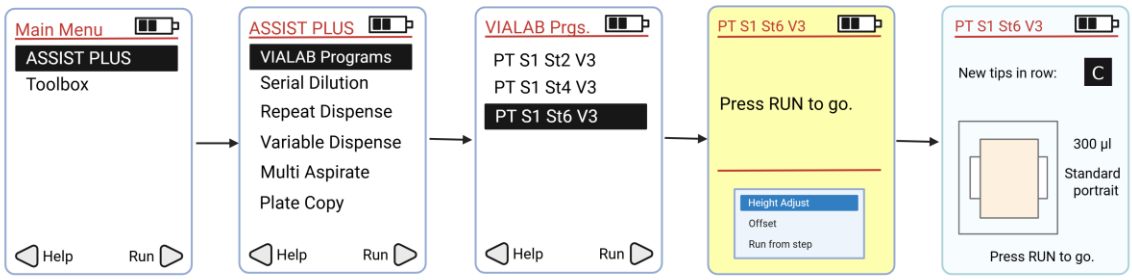


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

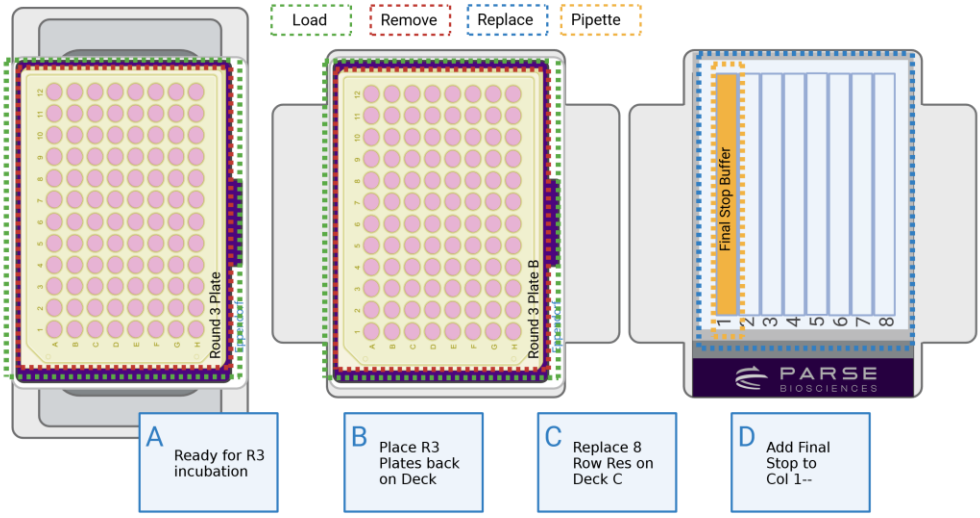
    a. Remove the D-ONE Pipetting Module 1-Ch, 5-1250 µL pipette and corresponding tip deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with the VIAFLO 12-Ch 5-125 µL 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.

6. Run the program **PT S1 St6 V3_5** following the diagram below.



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



a. Remove the Round 3 Plates from Decks A and B. Reseal the Round 3 Plates with an adhesive seal, place them into two thermocyclers, and run the following program.

BARCODING ROUND 3		
Run Time	15 minutes	
Lid Temperature	50°C	
Sample Volume	60 µL	
Step	Time	Temperature
1	15 min	16°C
2	Hold	4°C

b. When the Barcoding Round 3 Thermocycling program is complete, load the Round 3 Plate A on the Thermochromic PCR Cold Block on Deck A with A1 oriented towards the lower left corner. Load the Round 3 Plate B on the Thermochromic PCR Cold Block on Deck B with A1 oriented towards the lower left corner. Remove the plate seals.

c. Replace the used 8 Row Reservoir on Deck C with a new 8 Row Reservoir, with row 1 on the left.

d. Briefly vortex (2-3 seconds) the O Final Stop Buffer and ensure there is no precipitate. Pipette the total volume (~5.5 mL) to row 1 of the 8 Row Reservoir on Deck C. Press "Run" to continue the program.

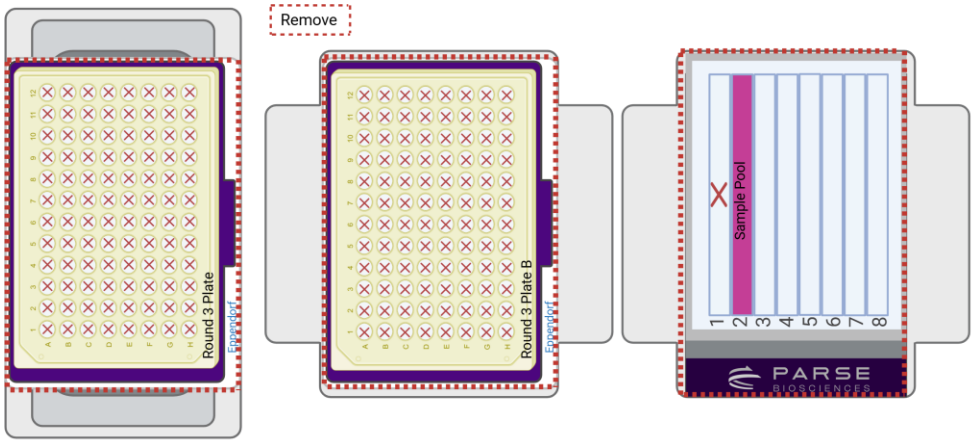
8. At the conclusion of the run:

   a. Remove all labware from Deck A. Discard the used Round 3 Plate A.

   b. Remove all labware from Deck B. Discard the used Round 3 Plate B. Store the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser at room temperature. A fully thawed block is required in Section 1.8.

   c. Remove the Fixation Cooling Adapter Base, Fixation 8 Row Reservoir Cooling Adapter and the 8 row reservoir containing the pooled samples from Deck C. Do NOT discard the 8 row reservoir.

Note: The 8 row reservoir will be used in the next step. Keep it cold either in the cooling adapter base or in the fridge until the next step.



1.7. Pre-Lysis

The pooled cell suspension on Deck B is strained into the 10 mL transport tube on Deck C. The Spin Additive is then added into the cells and centrifuged. Supernatant is removed; the cells are resuspended in Pre Lysis Wash Buffer, and centrifuged again. The supernatant is then removed and the cells/nuclei are counted for sublibrary generation.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
ASSIST PLUS Slanted Plate Holder	INTEGRA Components	1	
10 mL transport tube	Consumables	2	
25 µm, 40 µm, or 70 µm cell strainer	Consumables	2	
300 mL Reservoir Base	INTEGRA	1	
Spin Additive	4°C Reagents	1	Keep at room temperature.
Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by pipetting 3x.
Pre-Lysis Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice until use in Section 1.8. Mix by pipetting 3x before use.
Penta Lysis Buffer	4°C Reagents	1	Place in a 37°C water bath until use.
Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
8-count PCR strip tube	Consumables	2	
1.5 mL tube	Consumables	2	

2. Place the Parse Metal Cold Block on Deck C.

3. Place the ASSIST PLUS Slanted Plate Holder set to 10° on Deck B. The lower end should be on the left. Deck layout should correspond to the configuration below.

4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove the VIAFLO 12-Ch 5-125 µL pipette and corresponding tip deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with the D-ONE Pipetting Module 1-Ch, 5-1250 µL pipette and corresponding tip deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

5. Load the following reagents to their respective positions on the Parse Metal Cold Block on Deck C.

    a. Column 1:

        i. Left and Right: (2) 8-count PCR strip tube

    b. Column 2:

        i. Pos 6: 1.5 mL transport tube

        ii. Pos 8: ● Spin Additive

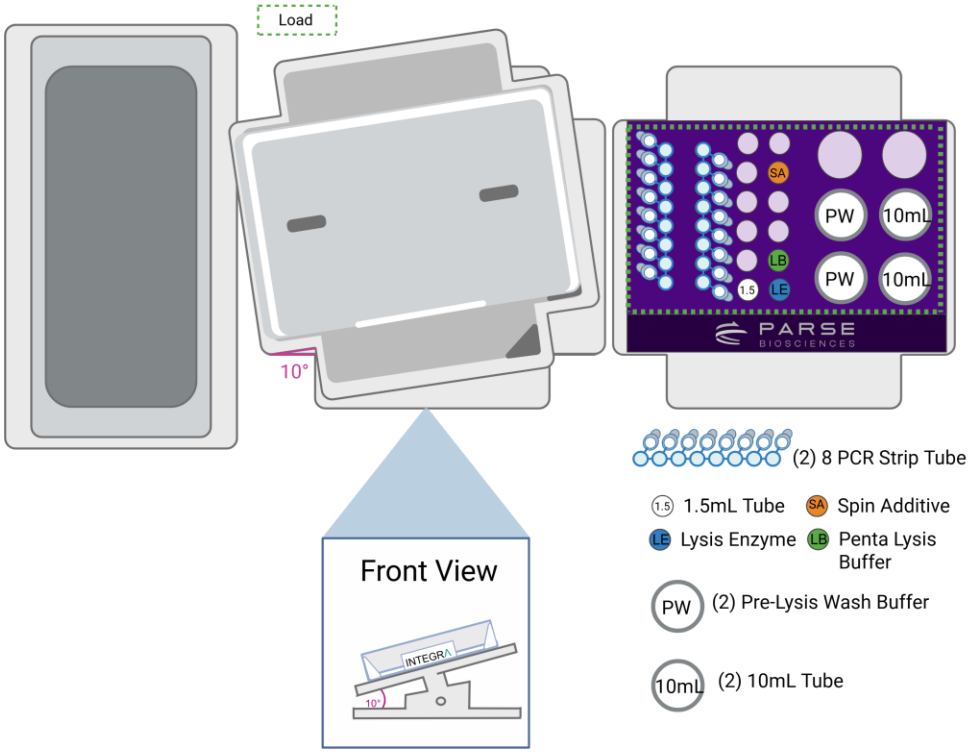
        iii. Pos 11: ● Penta Lysis Buffer

        iv. Pos 12: ● Lysis Enzyme

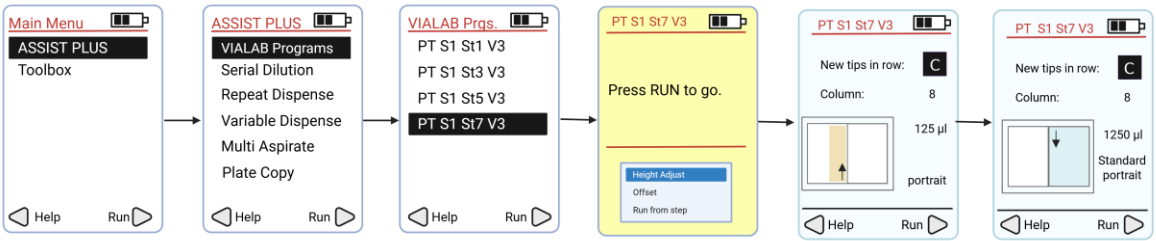
    c. Column 3:

        i. Pos 2 and 3: (2) ○ Pre-Lysis Wash Buffer

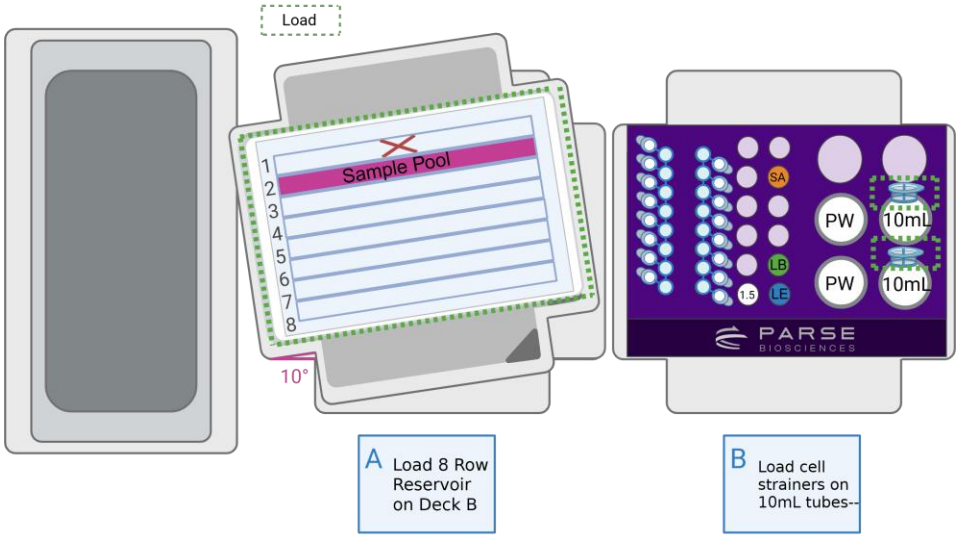
        ii. Pos 5 and 6: (2)10 mL transport tubes



6. Remove reagent caps, select and run the program **PT S1 St7 V3_5** following the diagram below.



7. Follow the program prompts for manual intervention:

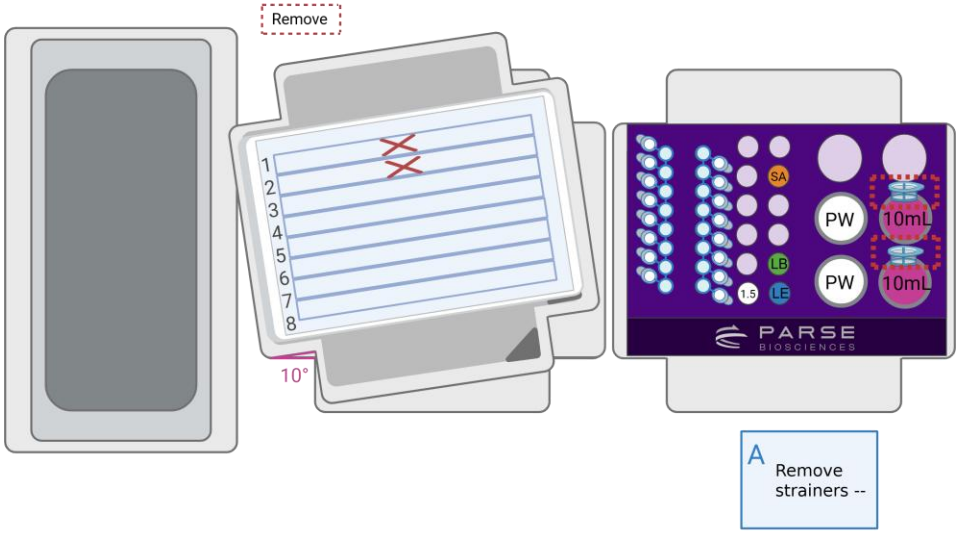


a. Carefully load the 8 Row Reservoir filled with the sample pool from Section 1.6 onto the Integra 8 Row Reservoir Plastic Base with row 1 on the back left corner on Deck B.

Note: When moving the 8 Row Reservoir onto the slanted plate holder on Deck B, take extra care when moving the sample to avoid spills.

b. Load cell strainers on both 10 mL transport tubes on Deck C, Column 3, Positions 5 & 6. Press “Run” to continue the program.

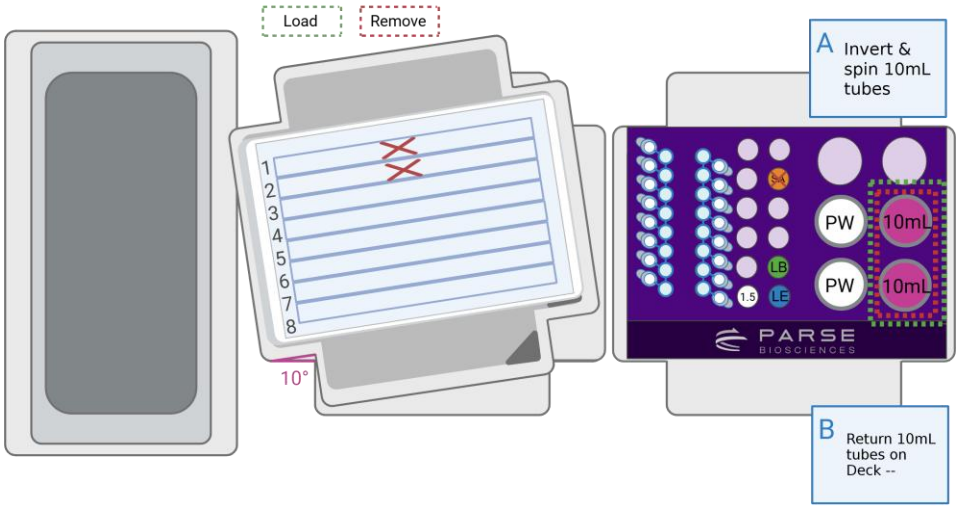
8. Follow the program prompts for manual intervention:



a. Remove the used cell strainers on both 10mL transport tubes on Deck C, Column 3, positions 5 and 6. Press “Run” to continue the program.

Note: There may be bubbles left on the strainer. This will not affect the results.

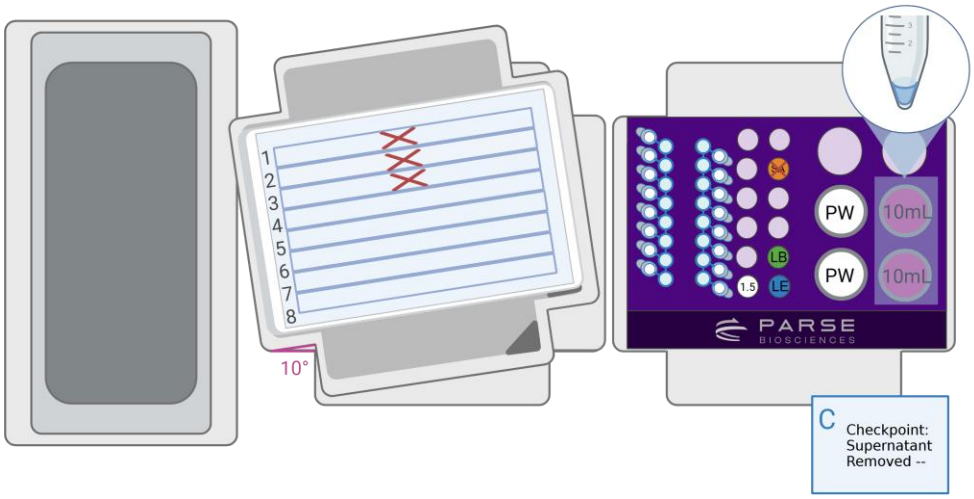
9. Follow the program prompts for manual intervention:



a. Cap and invert the 10 mL transport tubes containing the pooled cells/nuclei once. Centrifuge the sample in a swinging bucket centrifuge cooled to 4°C for **5-10 minutes** at 200-500 x g.

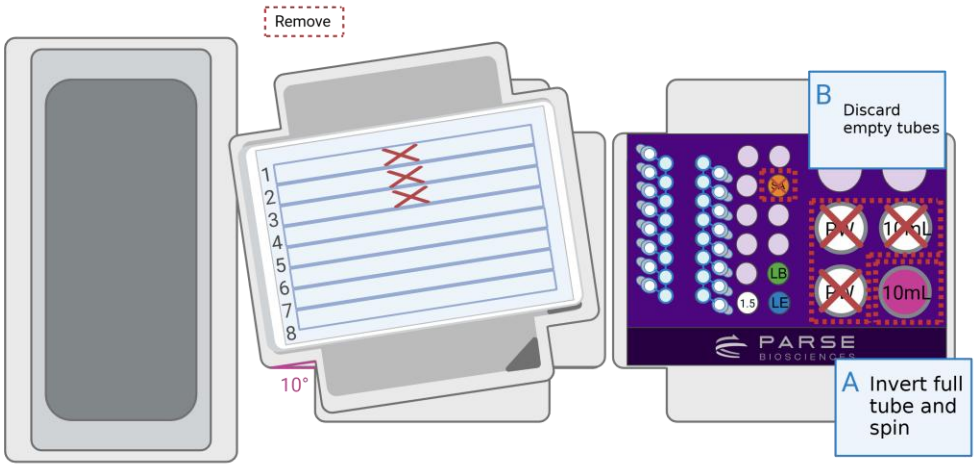
b. Once centrifugation is complete, remove the cap from the 10 mL transport tubes and place them back in their original positions within the Parse Metal Cold Block on Deck C, Column 3, positions 5 and 6. The 10 mL transport tubes are interchangeable.

CRITICAL! Return the 10 mL transport tube to the Parse Metal Cold Block **immediately** after centrifugation. Handle the tube with care to avoid jostling or disturbing the pellet. If the pellet becomes resuspended prior to supernatant removal, it may lead to loss of cells/nuclei.



c. After removing the supernatant, inspect the 10 mL tube and visually confirm that the supernatant has been removed, leaving only a small volume at the bottom. If there is more than ~100ul of volume left in the tube, manually remove supernatant until about ~100 µL of liquid remains above the pellet. Avoid disturbing the pellet—do not insert the pipette tip into the pellet area to measure the supernatant. Press "Run" to continue the program.

10. Follow the program prompts for manual intervention:



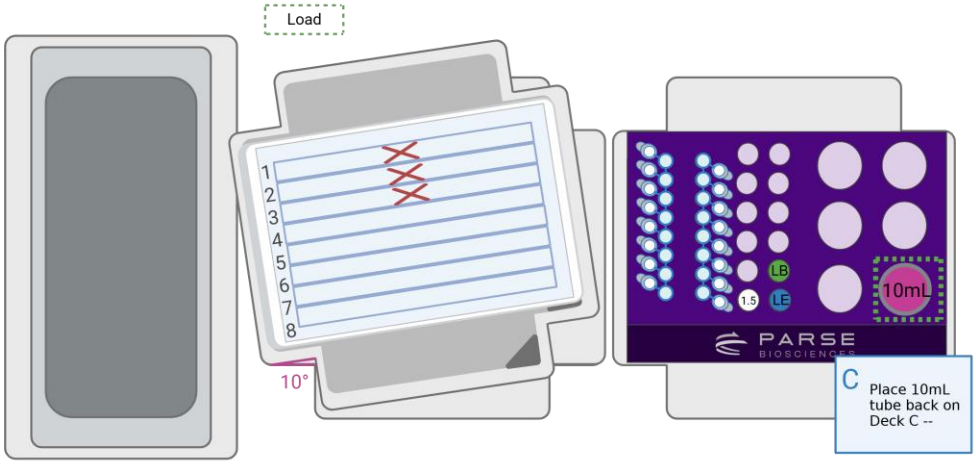
a. Cap and invert the 10 mL transport tube in Column 3 position 6 once. This tube contains the pooled cells/nuclei. Centrifuge the sample in a swinging bucket centrifuge cooled to 4°C for **5 -10 minutes** at 200-500 x g.

b. Discard the following used tubes from the Deck C:

   i. Col 2, Pos 8: ● Spin Additive

ii. Col 3, Pos 3 and 4: ○ Pre-Lysis Wash Buffer

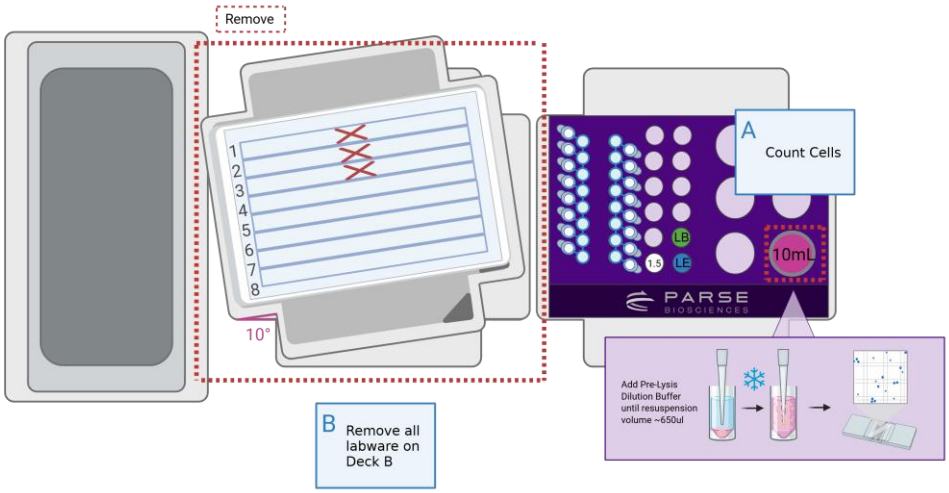
iii. Col 3, Pos 5: Used and emptied 10 mL tube



c. Once centrifugation is complete, remove the cap from the 10 mL transport tube and place it back in its original position within the Parse Metal Cold Block on Deck C, Column 3, position 6.

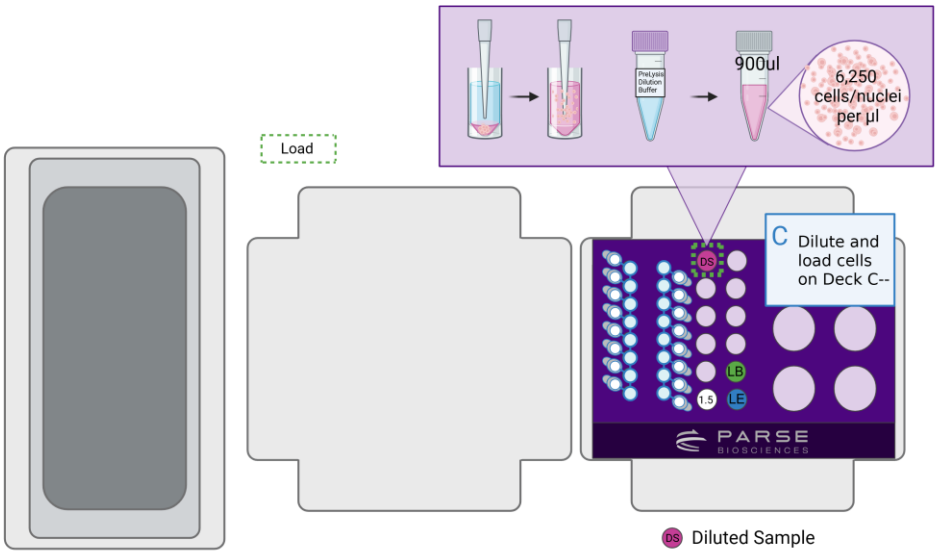
CRITICAL! Return the 10 mL transport tube to the Parse Metal Cold Block **immediately** after centrifugation. Handle the tube with care to avoid jostling or disturbing the pellet. If the pellet becomes resuspended prior to supernatant removal, it may lead to loss of cells/nuclei.

11. Follow the program prompts for manual intervention:



a. Store the 10 mL transport tube on Deck C on ice. Manually add ○ Pre-Lysis Dilution Buffer for a final total volume of **650 µL**. Minimizing time on ice, mix and count the number of cells/nuclei in the sample from the 10 mL transport tube on Deck C with a hemocytometer or alternative counting device. Record the cell/nuclei count.

b. Remove the Integra 8 Row Reservoir with the Plastic Base on the Slanted Plate Holder on Deck B.



c. Dilute the cells to a concentration of 6,250 cells/nuclei per µL for a total volume of at least **900 µL** using the Pre-Lysis Dilution Buffer in a 1.5 mL transport tube. Store this dilution on ice until ready to use.

Note: If you do not have at least 5,000,000 cells/nuclei, dilute the remaining cells to 900 µL and record the amount of cells per sublibrary.

Note: The robot uses 25 µL of this sample dilution to generate the lysates. If lysates of another size is required, dilute the sample suspension to a different concentration. (Example: A sample suspension with the concentration of 300 cells per µL will create lysates with 7,500 cells/nuclei).

Note: Automated dispensing of 5,000,000 cells/nuclei across 32 sublibraries requires a minimum input of 5,500,000 cells/nuclei. If fewer than 5,500,000 cells/nuclei are available, the dispensing can be performed manually to ensure maximum utilization of all available cells/nuclei.

d. Load the following reagents to their respective positions on the Parse Metal Cold Block on Deck C. Press “Run” to continue the program.

    a. Column 2:

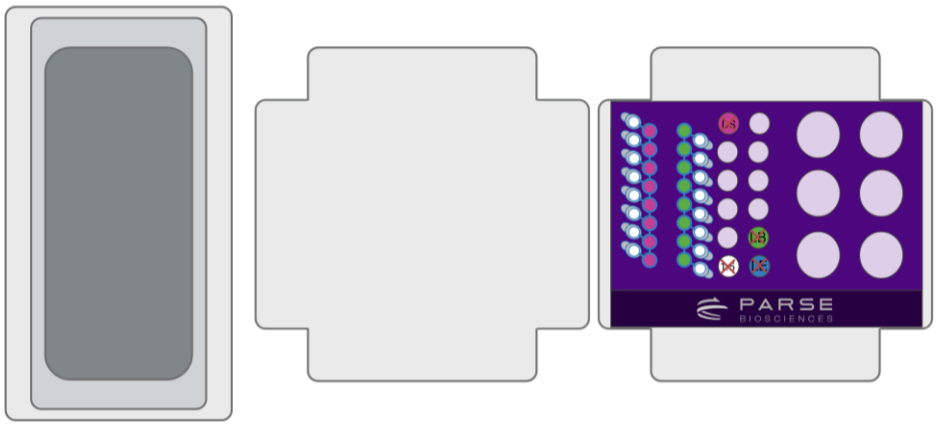
        i. Pos 1: diluted sample in a 1.5 mL tube.

12. At the conclusion of the run:

    a. Leave all labware on Deck C.

    b. Each tube in the left 8-strip tube on the Parse Metal Cold Block contains 110 µL of the diluted sample. This will be multidispensed in the next step.

c. Lysis Master Mix is in the right strip tube of Column 1 of the Parse Metal Cold Block. This will be multidispensed in the next step.



1.8. Lysis and Sublibrary Generation

The program will create thirty-two lysates with 156,250 cells/nuclei each. It then mixes the Lysis Master Mix and aliquots the master mix into the generated lysates.

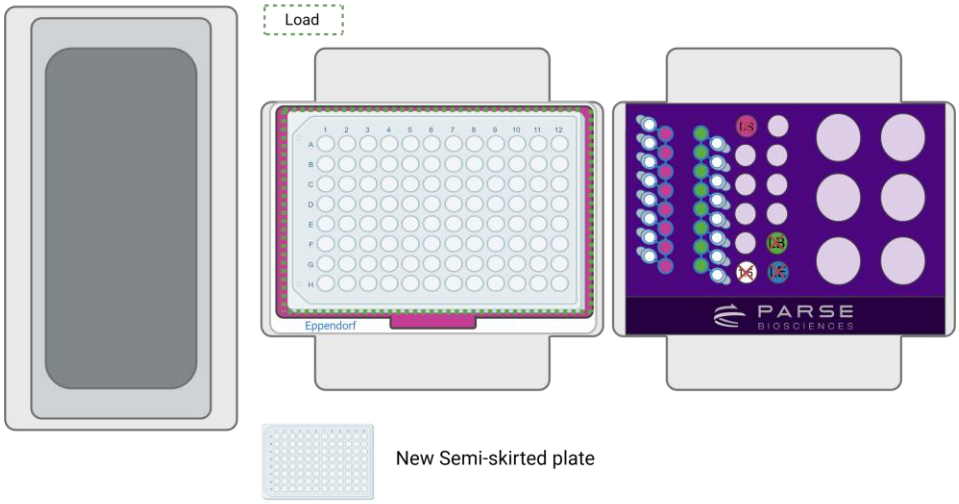
1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	1	Previously thawed pink block.
Thermochromic PCR Cold Block Riser	Parse	1	
8-count PCR strip tube	Consumables	2	
Parse Metal Cold Block	Parse	1	
Foil seal	Consumables	1	
Clear plastic seal	Consumables	1	

2. Load the thawed Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser from Section 1.6.8b on Deck B with A1 on the top left. Ensure it is fully thawed and pink.

3. The deck layout should correspond to the configuration below.

4. Load an Eppendorf Semi-Skirted plate on Deck B with A1 on the top left.

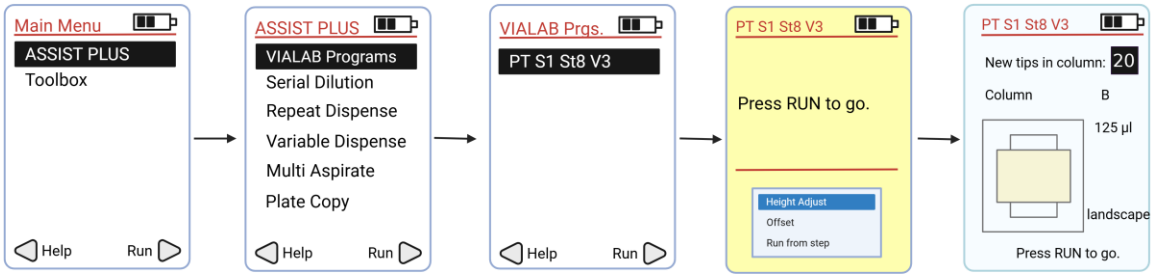


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VOYAGER 8-Ch 5-125 µL 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.

6. Ensure that all strip tubes are open, select and run the program **PT S1 St8 V3_5** following the diagram below. The deck layout should correspond to the configuration below.



7. At the conclusion of the run:

    a. Remove the Semi-skirted plate from Deck B. The lysates are in Columns 1-4.

b. While secured in a PCR tube rack on a flat surface, seal the plate with a foil seal and layer with a plastic seal to prevent punctures.

c. While secured in the PCR tube rack, vortex the PCR plate at 100% power for 1 minute.

d. Centrifuge the lysate plate for 1 minute at 100 x g at 4°C.

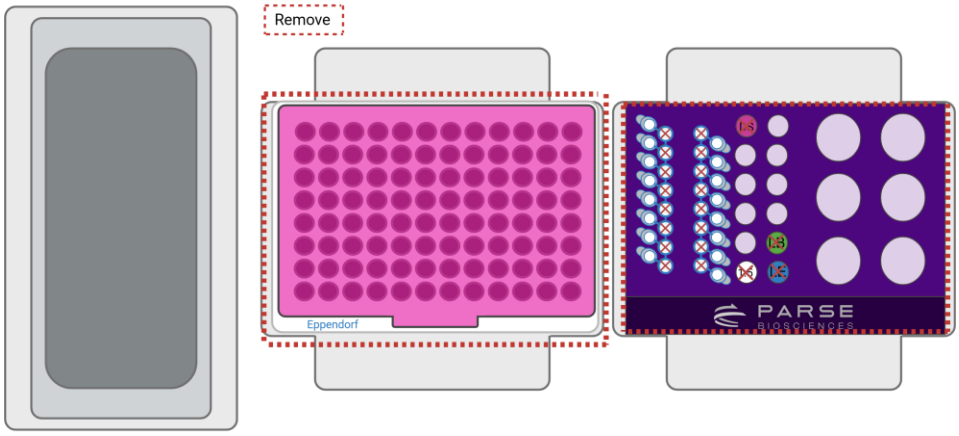
e. Place the plate into a thermocycler and run the following program. If continuing to Section 2 without freezing the sample, proceed to Section 2 while the program is still running.

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

f. Freeze the lysate(s) at -80°C or proceed to Section 2.

g. Remove all labware from Decks B and C. The Thermochromic Cold Block can be left at room temperature as it will be used throughout Section 2.

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



Section 2: cDNA Capture and Amplification

2.1. Reagents Plating

SPRI beads, Streptavidin Beads, and Binding Buffer are dispensed into a semi-skirted PCR plate. The Bead Wash, Wash 1, Wash 2, and Wash 3 Buffers are dispensed into the INTEGRA 8 Row Reservoir on Deck A. Capture enhancer is then added to the lysates created in Section 1.

CRITICAL! In section 2, you will need one thawed Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser. The Thermochromic PCR Cold Block should be at room temperature. We recommend either leaving the Thermochromic PCR Cold Block at room temperature overnight to thaw before starting, or running it under warm water to thaw quickly on the morning of Section 2.

CRITICAL! In section 2, **Sample and Reagent Plates** should be loaded with A1 on the upper left corner.

1. Fill an ice bucket.

2. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Parse Metal Cold Block	Parse	1	Keep at room temperature.
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
8 Row Reservoir	INTEGRA	1	Individually wrapped consumable
300 mL Reservoir Base	INTEGRA	1	
Strip of 8 Domed PCR Caps	Consumables	16	
8-count PCR strip tubes	Consumables	2	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Streptavidin Beads	4°C Reagents	2	Keep at room temperature.
○ Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Capture Enhancer	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
○ Bead Wash Buffer	-20°C Reagents	2	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Wash Buffer 1	-20°C Reagents	3	
○ Wash Buffer 2	-20°C Reagents	3	
○ Wash Buffer 3	-20°C Reagents	3	

3. Place the 24 mm Labware Pedestal on Deck A.

4. Place the Parse Metal Cold Block on Deck B.

5. Place the HEATMAG with 96 Well PCR Adapter (INTEGRA logo oriented to the front) on Deck C. Ensure HEATMAG power is on for the entire duration of Section 2.

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

7. Vortex the ○ Streptavidin Beads.

Note: Ensure that the Streptavidin Beads are thoroughly mixed before placing them on the deck. Beads can settle if the program isn't started immediately after loading all reagents.

8. Briefly centrifuge and load the following reagents and consumables to their respective positions on Decks A, B and C:

    a. Deck A: clean 8 Row Reservoir on 300 mL Reservoir Base. Label this as the **Wash Buffer Plate**.

    b. Deck B, column 2:

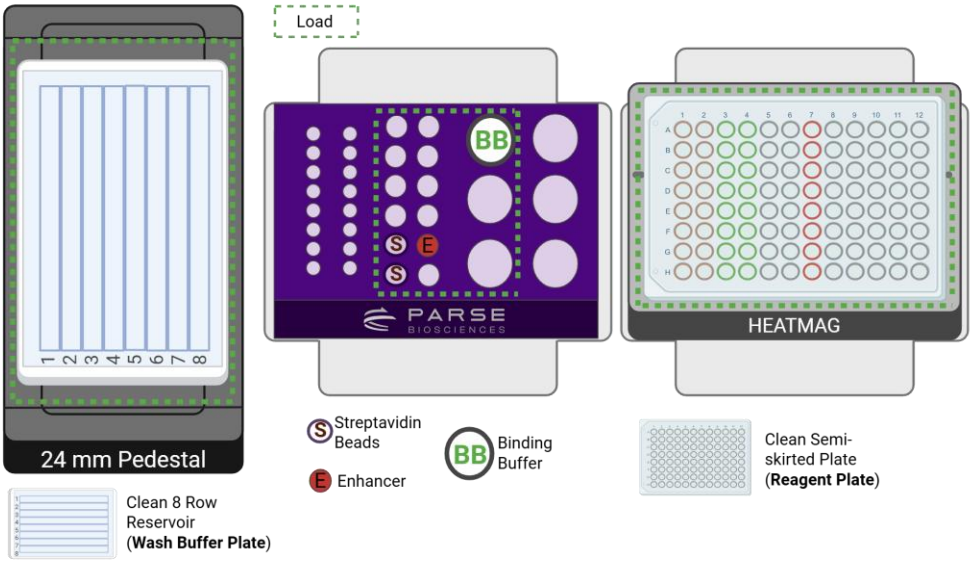
        i. Pos 5 and 6: Fully resuspended O Streptavidin Beads.

        ii. Pos 11: ● Capture Enhancer.

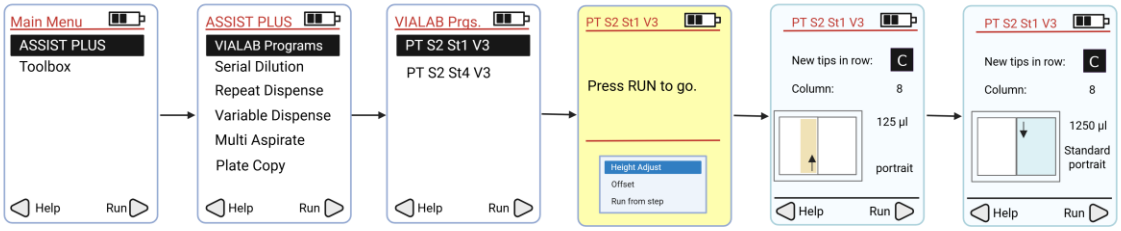
    c. Deck B, column 3:

        i. Pos 1: O Binding Buffer.

    d. Deck C: A clean semi-skirted PCR plate. Label this as the **Reagent Plate**.

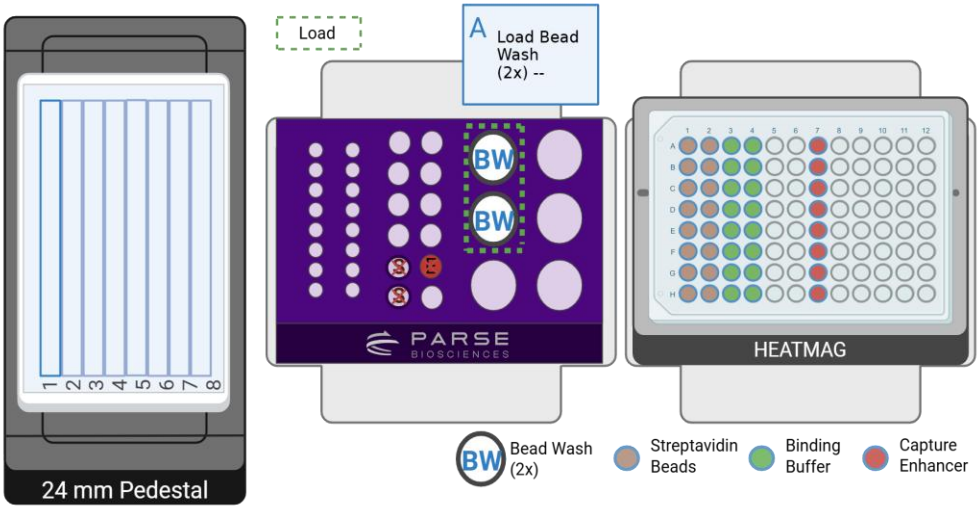


9. On the D-ONE Pipette select and run the program **PT S2 St1 V3_5** following the diagram below.

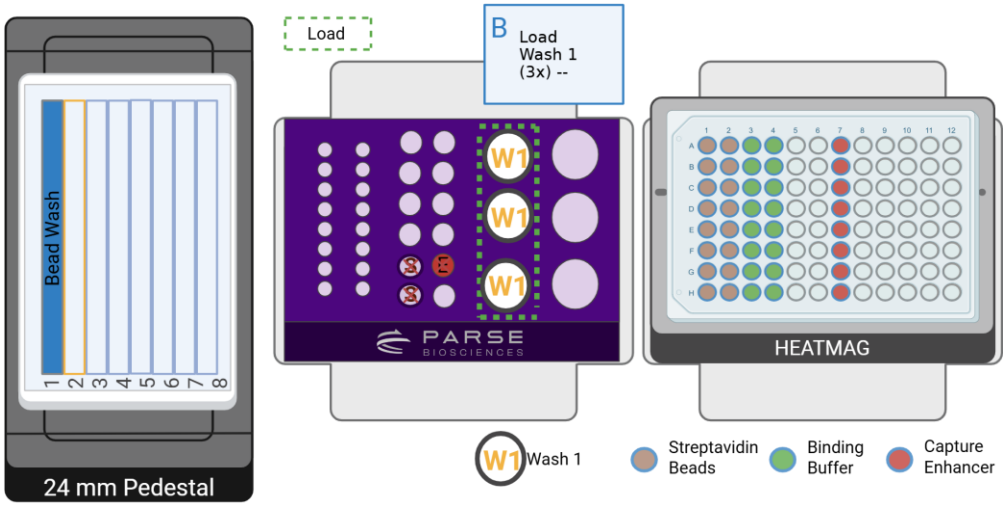


10. Press “Run” to continue the program.

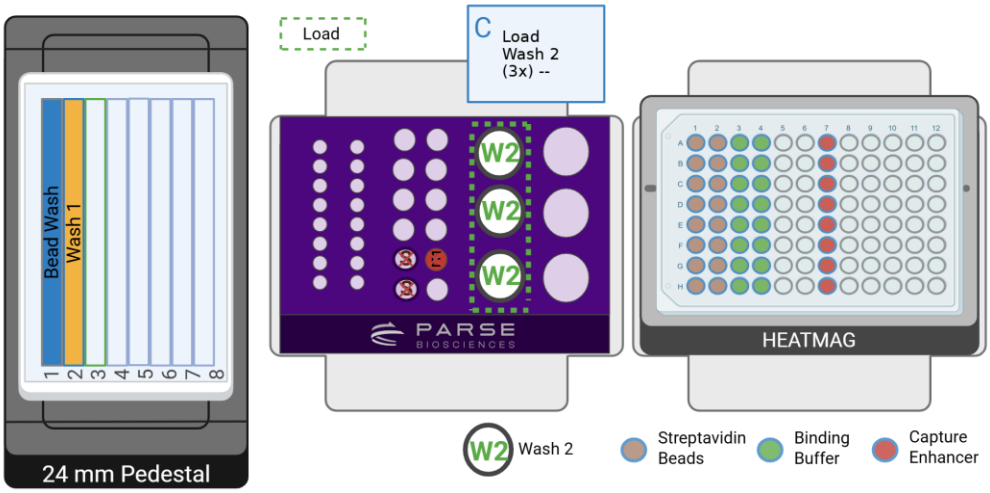
11. Follow the program prompts for manual interventions:



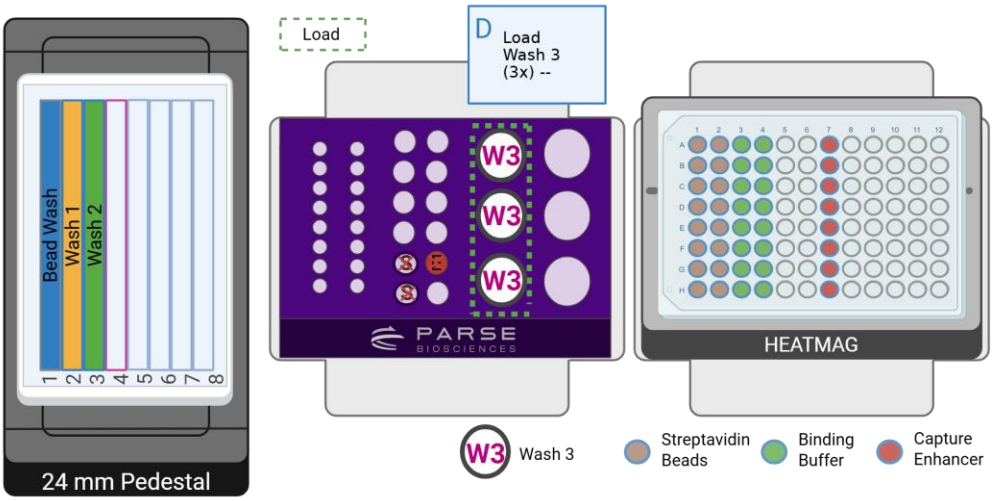
a. Replace the O Binding Buffer with O Bead Wash Buffer on Deck B, column 3, position 1 and 2. Press “Run” to continue.



b. Replace the OBead Wash Buffer with OWash Buffer 1 on Deck B, column 3, position 1, 2, and 3. Press “Run” to continue.



c. Replace the Wash Buffer 2 with Wash Buffer 3 on Deck B, column 3, position 1, 2, and 3. Press "Run" to continue.



d. Replace the Wash Buffer 2 with Wash Buffer 3 on Deck B, column 3, position 1, 2, and 3. Press "Run" to continue.

12. At the conclusion of the run:

    a. Verify that Streptavidin Bead volumes in columns 1 and 2 of Deck C are even.

    b. Verify that the Binding Buffer volumes in columns 3 and 4 of Deck C are even.

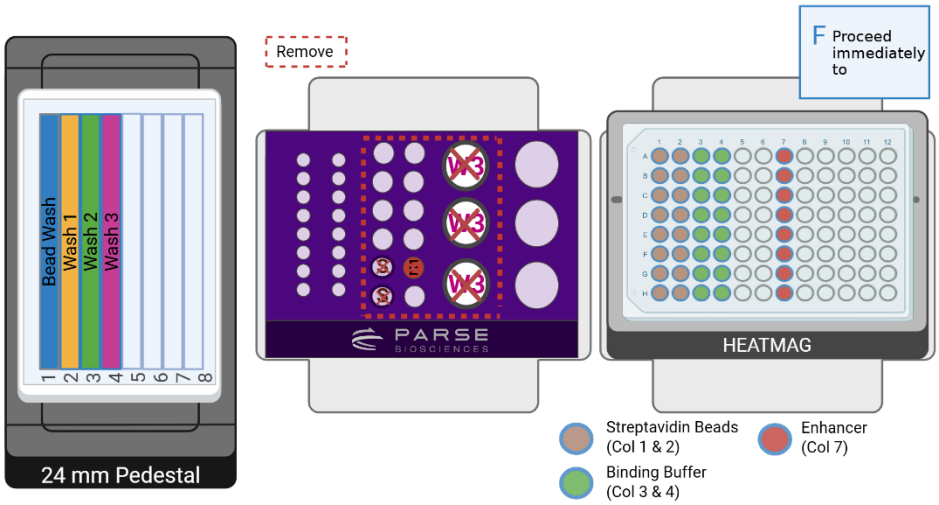
    c. Verify that the Capture Enhancer volumes in column 7 of Deck C are even.

d. Verify that all wash buffer tubes on Deck B are empty and buffers have been transferred to the **Wash Buffer Plate** on Deck A.

Note: Refer to Appendix B for specific volumes.

e. Remove all labware on Deck B and discard the empty tubes.

f. Proceed immediately to Section 2.2.



2.2. cDNA Capture

Streptavidin Beads are washed, then cDNA is captured with streptavidin-coated magnetic beads. The program controls the temperature and strength of the HEATMAG with 96 Well Adapter on Deck C.

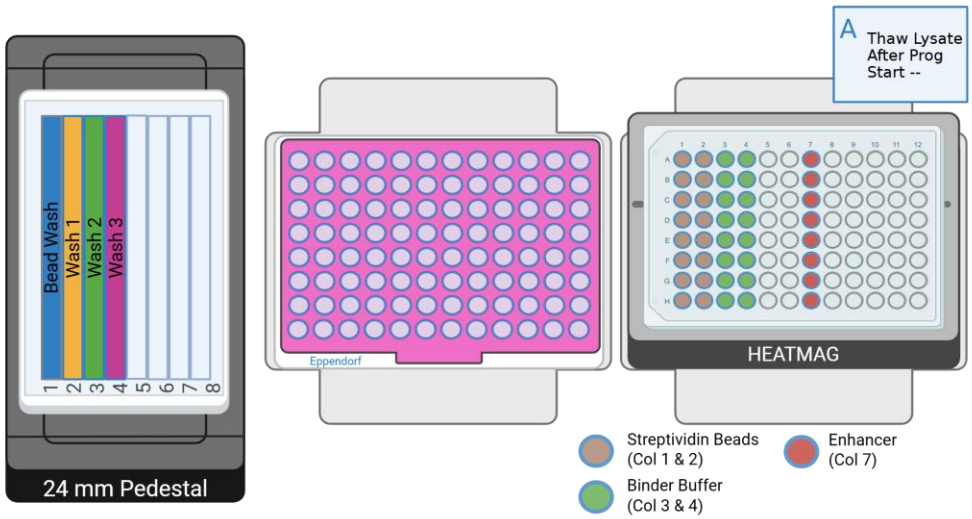
1. Gather the following items and set up the deck as shown.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	1	Keep at room temperature
Thermochromic PCR Cold Block Riser	Parse	1	

2. Place a previously thawed thermochromic PCR Cold Block with a Thermochromic PCR Cold Block Riser on Deck B with A1 on the top left. The deck layout should correspond to the figure below.

3. Load the **Reagent Plate** (if removed from previous steps) on the HEATMAG with 96 Well PCR Adapter.

4. Load **Wash Buffers Plate** on Deck A on the 24 mm Pedestal with row 1 on the left side.

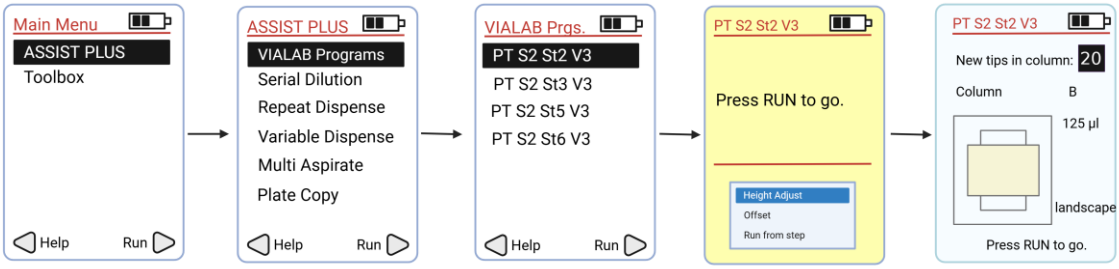


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VOYAGER 8-Ch 5-125 µL 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.

6. Select and run the program **PT S2 St2 V3_5** following the diagram below.



7. If the lysates were previously frozen, when prompted, incubate the lysates in a thermocycler at 37°C for **5 minutes** to thaw. Press "Run" on the pipette to continue the program while the lysates are thawing.

Note: The program will remind you to thaw the lysate. Press "Run" to continue the program if continuing directly from Section 1.

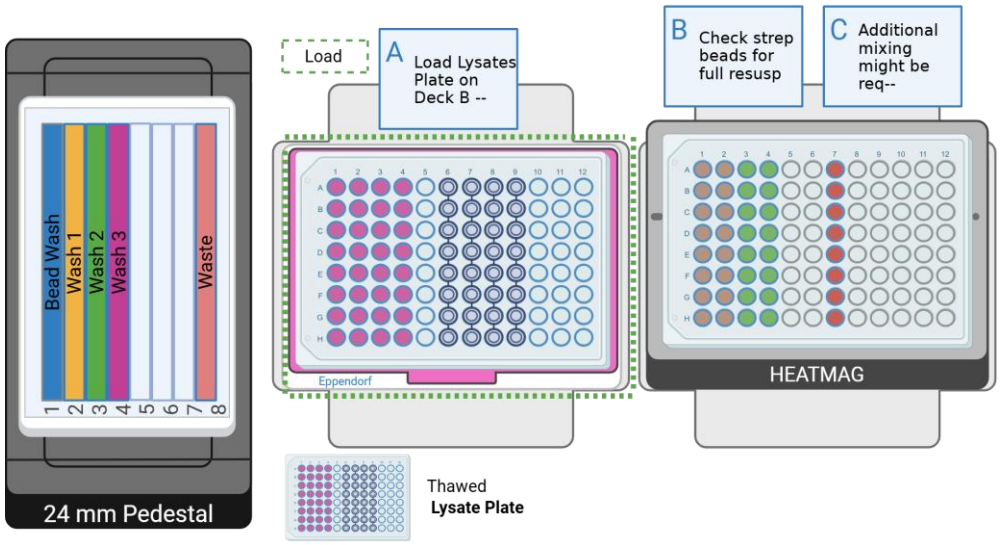
Note: When thawing the lysate, ensure there is no precipitate before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

8. When the lysates finish thawing:

    a. Label the plate as the **Lysate Plate**.

    b. Briefly centrifuge **1 minute** at 100 x g at 4°C and store at room temperature.

9. Follow the program prompts for manual intervention:



    a. When prompted, unseal and load the **Lysate Plate** on Deck B. Press "Run" to continue the program.

    b. Cap columns 6-9 with domed strip caps to prevent contamination. Do not remove until instructed.

    c. Verify that Streptavidin Beads are fully resuspended in columns 1 and 2 of the **Reagent Plate** located on the HEATMAG with a 96-Well PCR Adapter positioned on Deck C.

Note: If any bead pellet is visible, set a P200 pipette to 70 µL and manually pipette up and down to ensure complete resuspension.

d. Press "Run" to continue.

10. At the conclusion of the run:

a. Cap samples on columns 1 - 4 on the **Lysate Plate** on the Deck B.

b. Caps should be present on columns 6-9 on the **Lysate Plate**. Do not remove the caps.

c. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex on 100% power for **1 minute**.

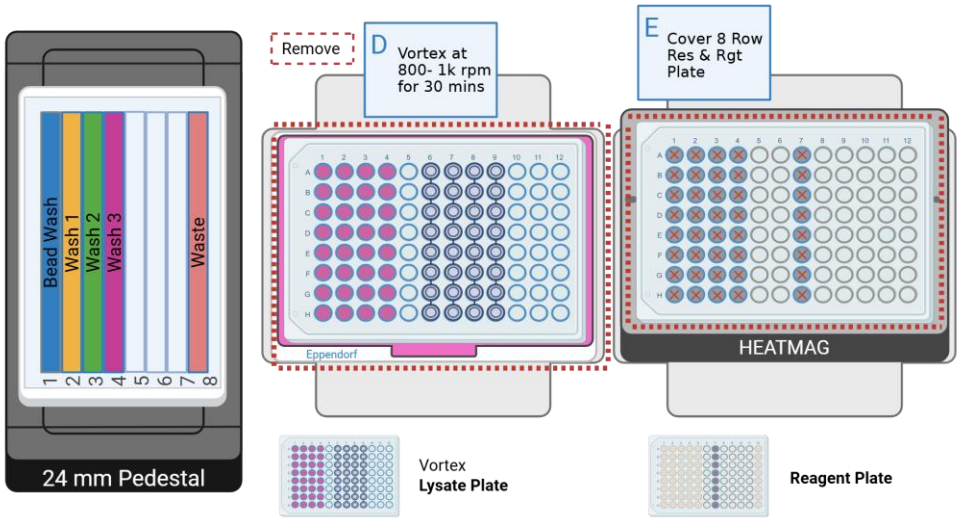
d. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature.

Note: To ensure the beads are being mixed sufficiently, check that the beads are in solution 10 minutes into the incubation. If settled, increase the vortex mixing speed to keep the beads in solution.

e. While the samples are vortexing, press "Run" to complete the program.

f. Seal the **Wash Buffer Plate** on the 24 mm Pedestal on Deck A with a plastic plate seal.

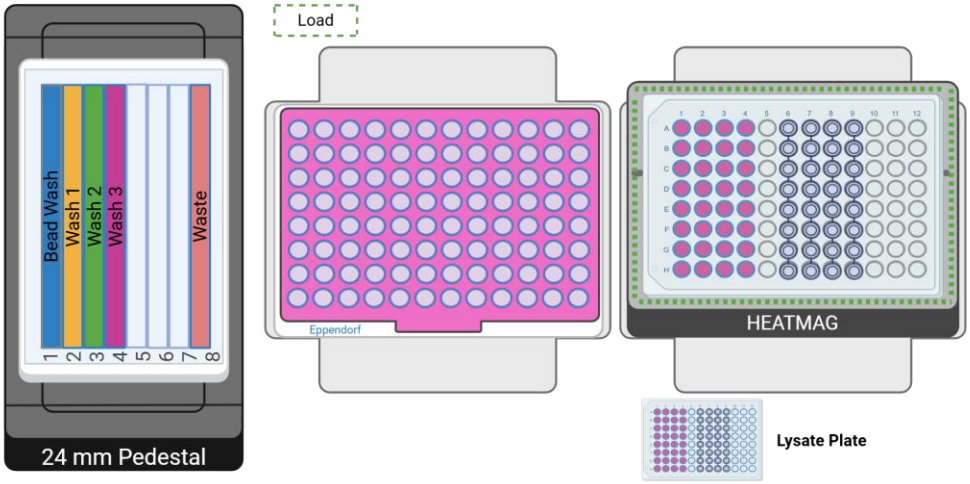
g. Seal and remove the **Reagent Plate** from Deck C. Store the **Reagent Plate** at room temperature to be used in a later step.



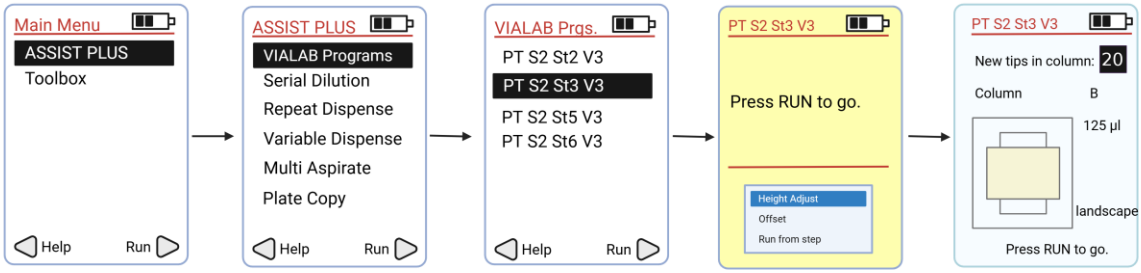
2.3. Streptavidin Beads Wash

Captured cDNA samples are washed to remove cellular debris.

1. Briefly centrifuge the **Lysate Plate** tubes for **30 seconds** at 100 x g at 4°C.
2. Load the **Lysate Plate** on the HEATMAG with 96 Well Adapter on Deck C with A1 on the top left.
3. Uncap columns 1-4 of the **Lysate Plate**. Discard the used caps.
4. Unseal the **Wash Buffer Plate** on the 24 mm Pedestal on Deck A.
5. The deck layout should correspond to the configuration below.

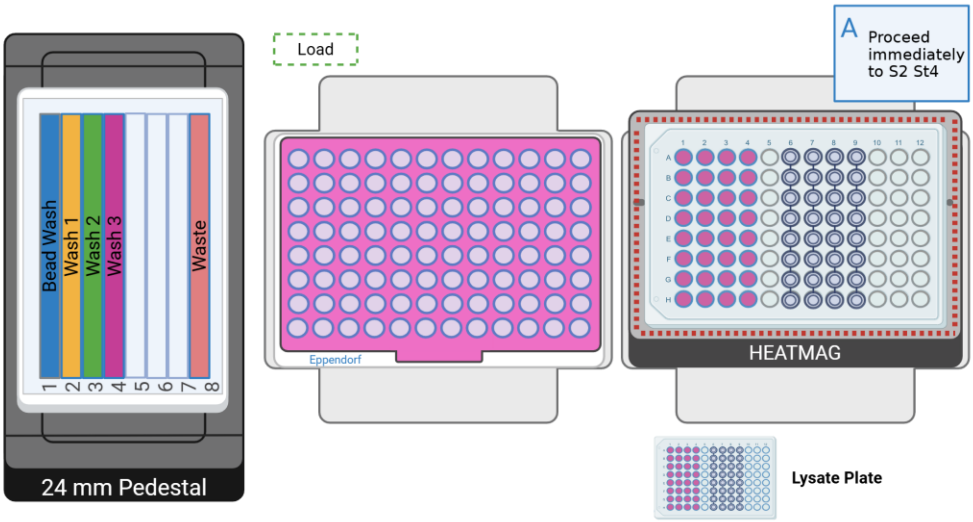


6. Select and run the program **PT S2 St3 V3_5** following the diagram below.



7. At the conclusion of the run:
    a. Remove the **Lysate Plate** on the HEATMAG with 96 Well Adapter on Deck C, and seal columns 1-4 with new caps.
    b. Store **Lysate Plate** at room temperature.

c. Proceed immediately to section 2.4.



2.4. Master Mixes Preparation

The program prepares the Template Switch Master Mix and the cDNA Amplification Master Mix for the template switch and the cDNA amplification reactions.

1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Components	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
300 mL Reservoir Base	INTEGRA Components	1	
8-count PCR strip tubes	Consumables	2	
5 mL tube	Consumables	1	
2 mL tubes	Consumables	4	
○ Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
● Template Switch Primer	-20°C Reagents	1	
● Template Switch Enzyme	-20°C Reagents	1	Keep on ice. Briefly centrifuge before use.
○ cDNA Amp Mix	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
○ cDNA Amp Primers	-20°C Reagents	1	
SPRI Beads	Room Temperature	2.7 mL	Ensure the beads are vortexed and fully resuspended before use.

Note: Ensure that there is no precipitate in the ○ Template Switch Buffer before proceeding.

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

3. Load the following reagents to their respective positions on the Parse Metal Cold Block on Deck B:

    a. Column 1 left and right: two clean 8-count PCR strip tubes. Label these as **cDNA Amp Mix**.

    b. Column 2:

        i. Pos 5, 6, 11, 12: clean 2 mL tubes.

    c. Column 3:

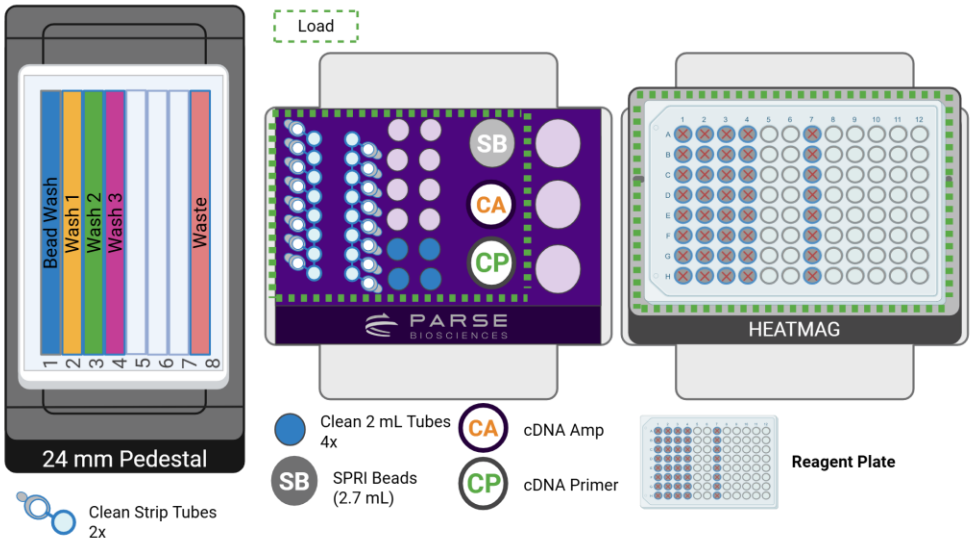
        i. Pos 1: 5 mL tube containing SPRI Beads (~2.7 mL).

        ii. Pos 2: O cDNA Amp Mix.

        iii. Pos 3: O cDNA Amp Primers.

Note: Ensure that the SPRI Beads are thoroughly mixed before placing them on the deck. Beads can settle if the program isn't started immediately after loading all reagents.

4. Unseal and place the **Reagent Plate** on the HEATMAG with 96 Well Adapter on Deck C.

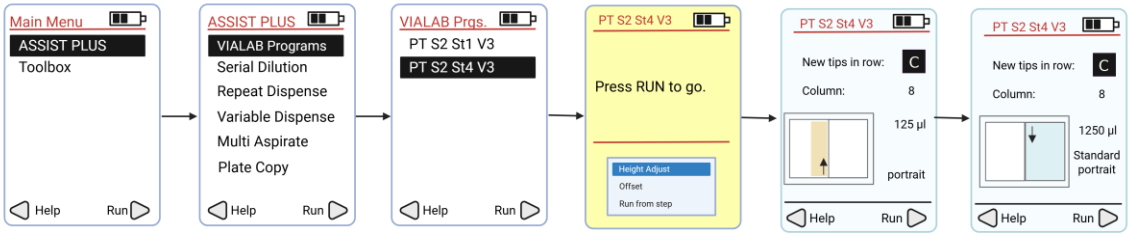


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove the VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

6. Remove reagents caps, select, and run the program **PT S2 St4 V3_6** following the diagram below.



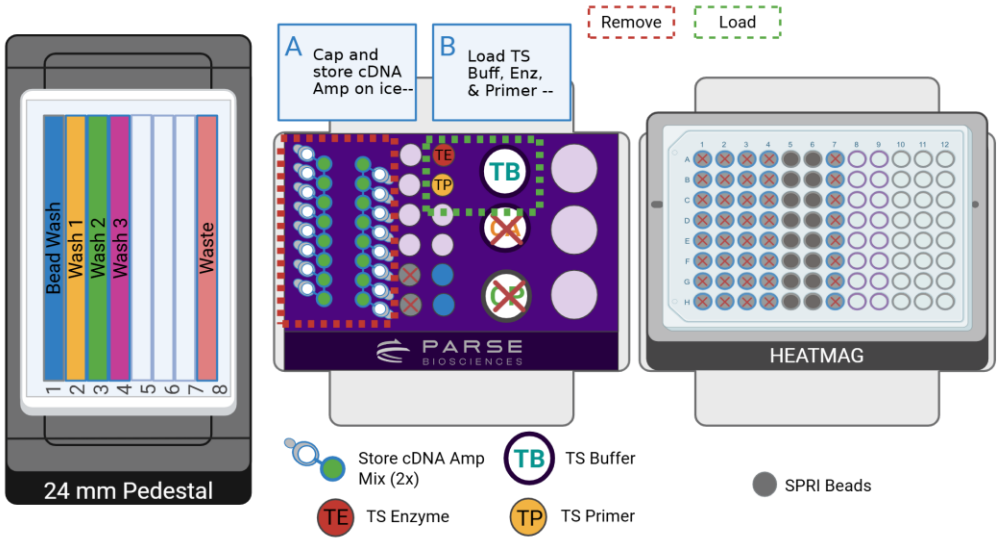
7. Press "Run" to continue the program. Follow the program prompts for manual intervention:

    a. Deck B Column 1 (both left and right position)

        i. Cap **cDNA Amp Mix** tubes on both columns left and right.

ii. Visually confirm that volumes are even across all wells of the strip tube to ensure consistent dispensing.

8. Store **cDNA Amp Mix** tubes on ice until use in a subsequent step.



9. Press "Run" to continue the program.

10. Follow the program prompts for manual intervention:

    a. Deck B Column 2:

        i. Pos 7: ● Template Switch Enzyme.

        ii. Pos 8: ● Template Switch Primer.

    b. Deck B Column 3:

        i. Pos 1: ○ Template Switch Buffer.

11. At the conclusion of the run:

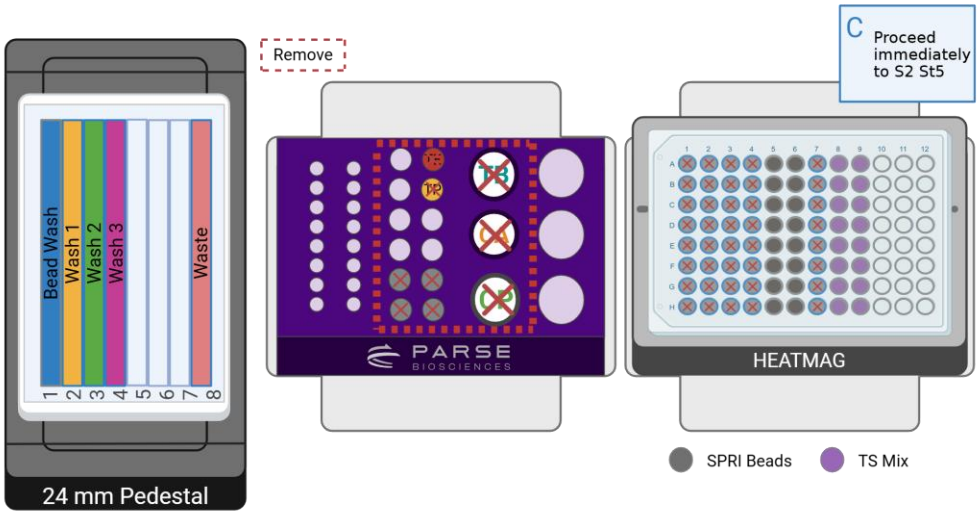
    a. Visually confirm that volumes are even across all wells to ensure consistent dispensing in the **Reagent Plate** on Deck C:

        i. Columns 5 and 6: SPRI Beads.

        ii. Columns 8 and 9: Template Switch Master Mix.

    b. Remove all labware on Deck B. Discard used reagent tubes on Deck B, columns 2 and 3.

12. Proceed immediately to Section 2.5.



2.5. Template Switch and cDNA Amplification

The Template Switch Master Mix is added to the captured cDNA and incubated for 30 minutes. The template switch reaction adds a 5′ adapter to the cDNA.

1. Gather the following items and set up the deck indicated:

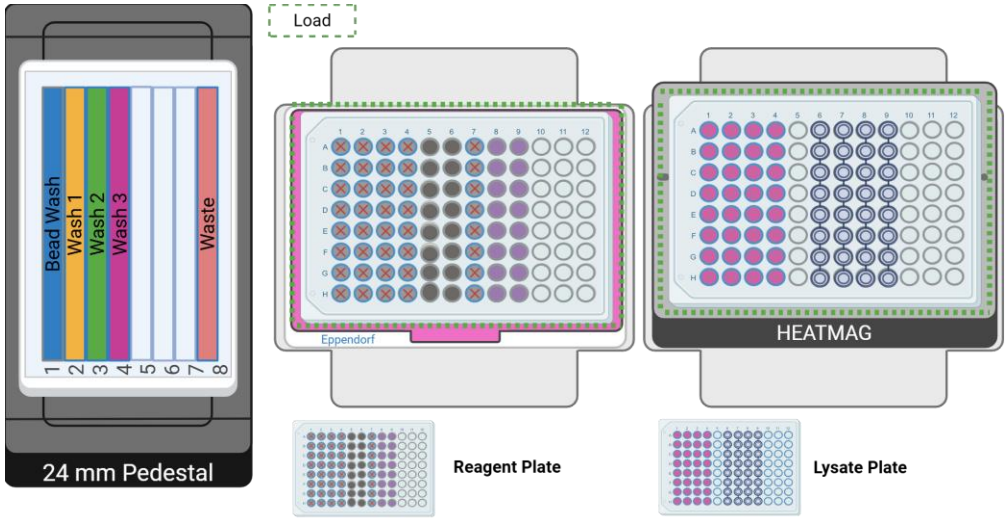
ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER 8-Ch 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
Parse Metal Cold Block	Parse	1	
Thermochromic PCR Cold Block	Parse	1	Keep at room temperature
Thermochromic PCR Cold Block Riser	Parse	1	
24 mm Labware Pedestal	INTEGRA Components	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
300 mL Reservoir Base	INTEGRA Components	1	

2. Ensure the following are loaded following the deck configuration below:
    a. Deck A with 24 mm Pedestal: unsealed **Wash Buffer Plate**.
    b. Deck B with room temperature Thermochromic PCR Block:
        i. Columns 5 and 6: **Reagent Plate** with dispensed SPRI Beads.

ii. Columns 8 and 9: Template Switch Master Mix.

c. Deck C with HEATMAG with 96 Well Adapter, load **Lysate Plate** and uncap only columns 1 - 4.

3. Deck configuration should correspond to the image below.

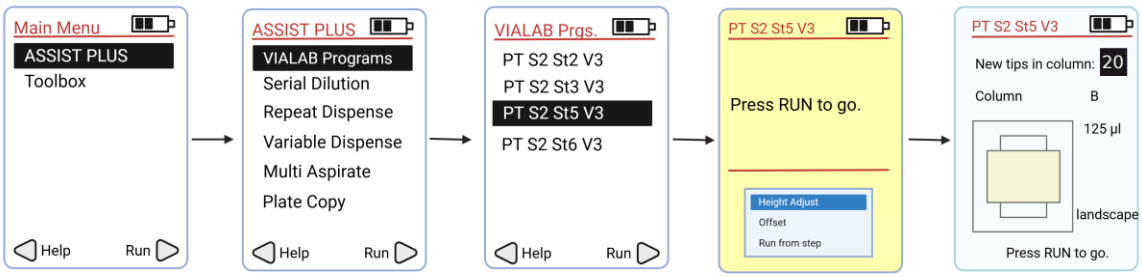


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

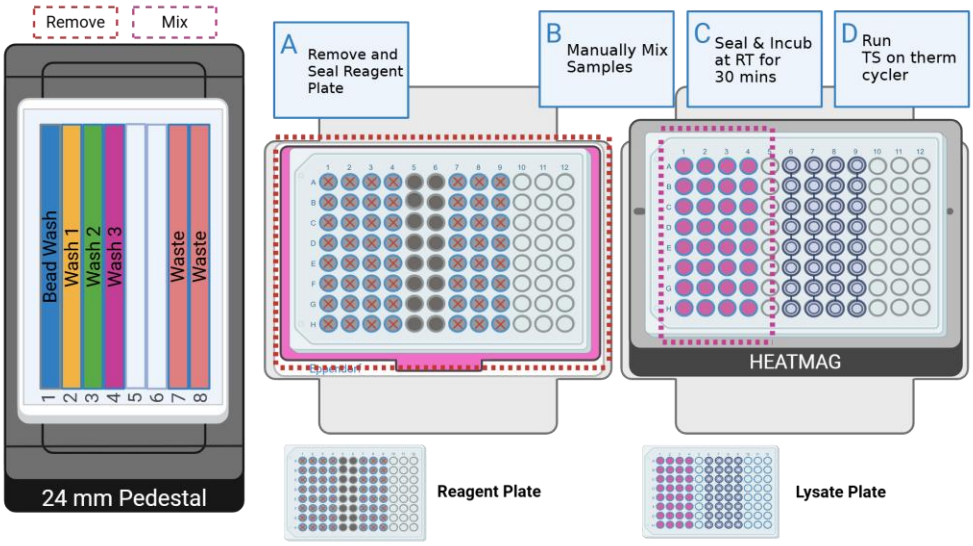
a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

b. Replace it with VOYAGER 8-Ch 5-125 µL 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.

5. On the VOYAGER Pipette select and run the program **PT S2 St5 V3_5** following the diagram below.



6. Follow the program prompts for manual intervention:



a. Remove and seal the **Reagent Plate**. Store at room temperature until use in a subsequent step.

b. Remove the **Lysate Plate** from Deck C. Set a P200 pipette to 70 µL and manually pipette up and down to ensure complete resuspension of the streptavidin pellet in the Template Switch Master Mix in columns 1-4. Avoid introducing bubbles.

Note: The Template Switch Master Mix is viscous, which can make resuspending the beads challenging. Do not directly touch the bead pellet with the pipette tip, as the beads may stick to it.

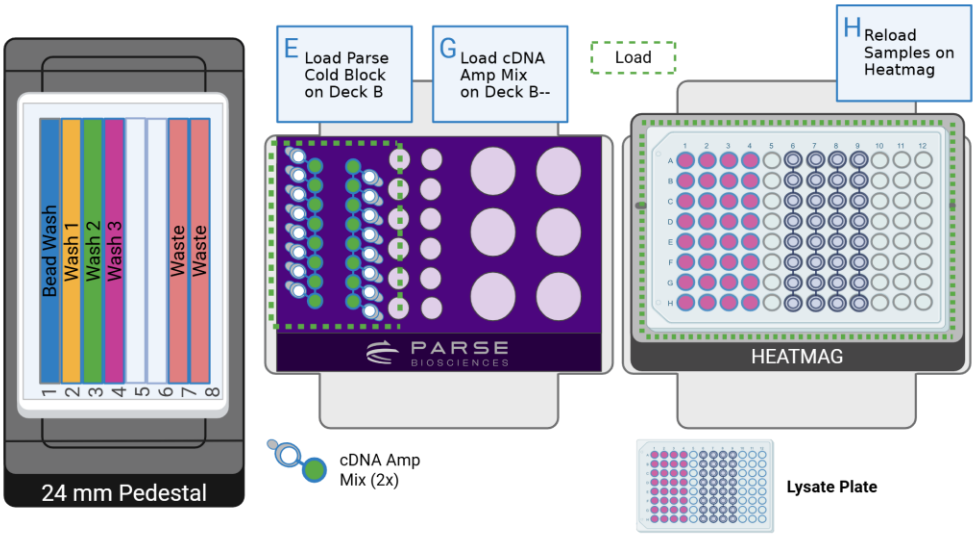
c. Cap the **Lysate Plate** (column 1-4) with domed strip caps and reload on Deck C. Incubate the **Lysate Plate** at room temperature for **30 minutes**. Add a PCR plate seal to the Wash Buffer Plate on Deck A during the 30 minute incubation.

d. After the 30 minute incubation remove the **Lysate Plate** from the Deck C and place it into a thermocycler. Run the following program.

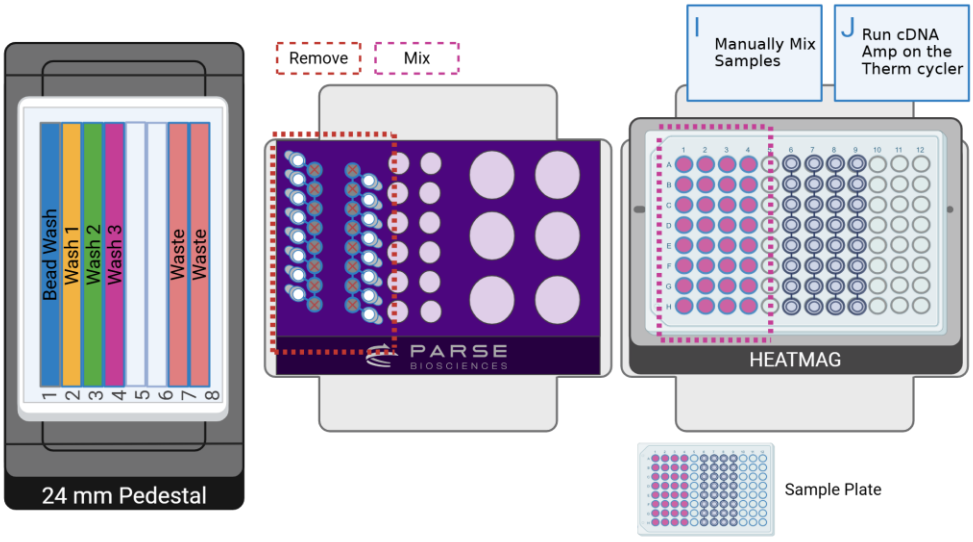
TEMPLATE SWITCH		
Total Run Time		60 minutes
Lid Temperature		70°C
Sample Volume		100 µL
Step	Time	Temperature

TEMPLATE SWITCH		
1	60 min	42°C
2	Hold	4°C

7. When the Template Switch thermocycling program has completed, Press "Run" to continue the program:



e. Replace the room temperature Thermochromic PCR Block with the Parse Cold Block on Deck B.
f. Remove the seal from the **Wash Buffer Plate** on Deck A.
g. Uncap and load the **cDNA Amp Mix tubes** from section 2.4.8 back into Deck B, column 1, right and left.
h. Uncap columns 1 - 4 of the **Lysate Plate** and reload on the HEATMAG with 96 Well Adapter on Deck C. Press "Run" to continue the program.



8. When prompted:

    i. Remove the Lysate Plate from Deck C and set a P200 pipette to 70 µL and manually pipette up and down to ensure complete resuspension of the streptavidin pellet in the cDNA Amp Mix in columns 1 - 4. Avoid introducing bubbles.

9. Determine the number of PCR cycles required for cDNA amplification based on the table below. Although these recommendations are appropriate for many cell types, the number of cycles may need to be optimized for each sample type.

NUMBER OF PCR CYCLES			
Cells/nuclei in the Sublibrary	High RNA content such as cell lines	Low RNA content such as PBMCs	Nuclei
12,500-25,000	4	6	5
25,000-62,000	3	5	4
62,500-125,000	2	4	3
125,000-156,250	2	3	2

j. When prompted, load the **Lysate Plate** into the thermocycler and run the following program.

cDNA AMPLIFICATION			
Total Run Time			40-60 minutes
Lid Temperature			105°C
Sample Volume			100 µL
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	5
3*	45 s	65°C*	
4	3 min	72°C	
5	20 s	98°C	Variable, see above
6*	20 s	67°C*	
7	3 min	72°C	
8	5 min	72°C	1
9	Hold	4°C	1

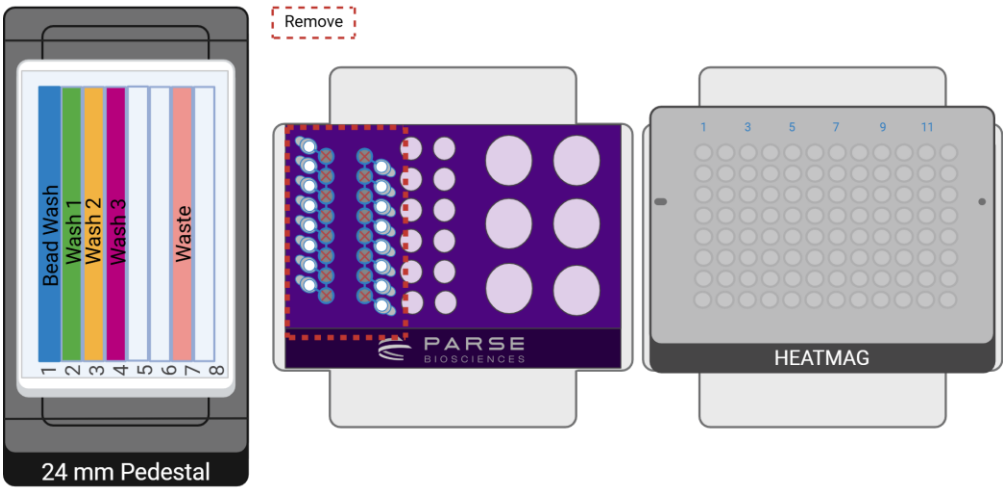
CRITICAL! If processing sublibraries with different numbers of cells/nuclei, they should be amplified in separate thermocyclers according to the recommendations above.

Note: Annealing steps 3* and 6* have different time and temperature settings. Ensure these are correct before starting the program.

10. While the thermocycler program is running:

    a. Remove and discard used strip tubes on Deck B.

    b. Seal the **Wash Buffer Plate** with a PCR Plate Seal during this time to reduce contamination.



STOP Safe stopping point: the **Lysate Plate** can be stored in the thermocycler or at 4°C for up to 18 hours.

2.6. Post-Amplification Purification

Amplified cDNA is purified with a 0.8x SPRI bead cleanup.

1. Gather the following items and set up the deck as indicated:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER 8-Ch 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
Thermochromic PCR Cold Block	Parse	1	Keep at room temperature
Thermochromic PCR Cold Block Riser	Parse	1	
24 mm Labware Pedestal	INTEGRA Components	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
300 mL Reservoir Base	INTEGRA Components	1	
Ethanol	Consumables and Reagents		
Nuclease free water	Consumables and Reagents		

2. Prepare at least **20 mL** of fresh 85% ethanol with nuclease-free water.

3. With a serological pipette, add:

    a. **20 mL** 85% Ethanol in lane **5** of the **Wash Buffer Plate** on Deck A.

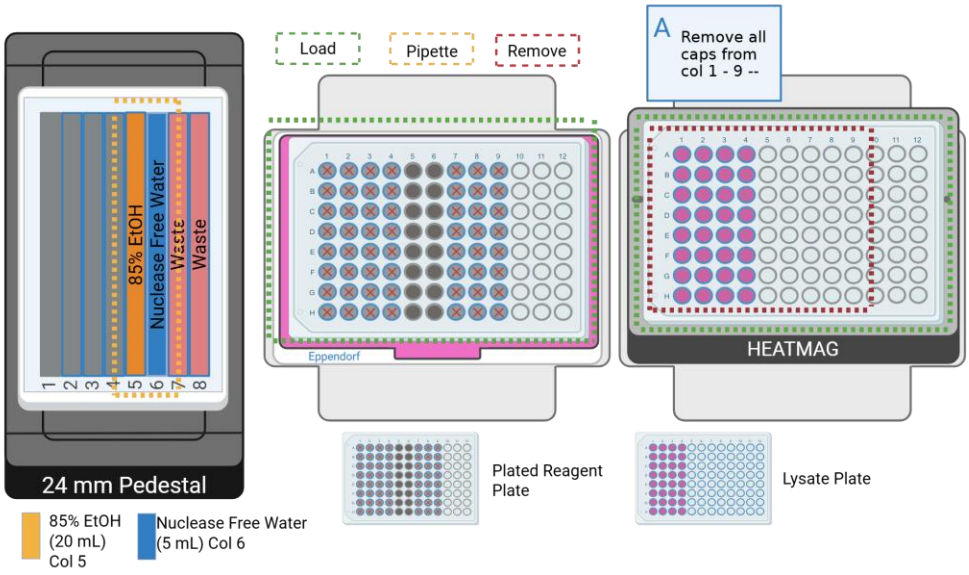
    b. **5 mL** nuclease free water in lane **6** of the **Wash Buffer Plate** on Deck A.

4. Unseal and load the Reagent Plate with SPRI beads in column 5 and 6 from Section 2.4.11 on the Room Temperature Thermochromic Cold Block on Deck B. If the beads have settled, set a P200 pipette to 70 µL and manually pipette up and down to ensure complete resuspension prior to loading them on Deck B.

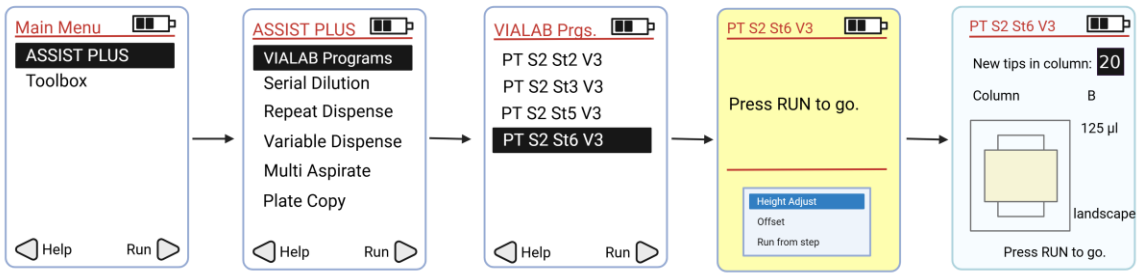
Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

5. Load the **Lysate** Plate with samples in columns 1 - 4 on the HEATMAG with 96 Well Adapter on Deck C.

    a. Remove all caps columns 1-4 and 6-9.



6. Select and run program **PT S2 St6 V3_5** following the diagram below.

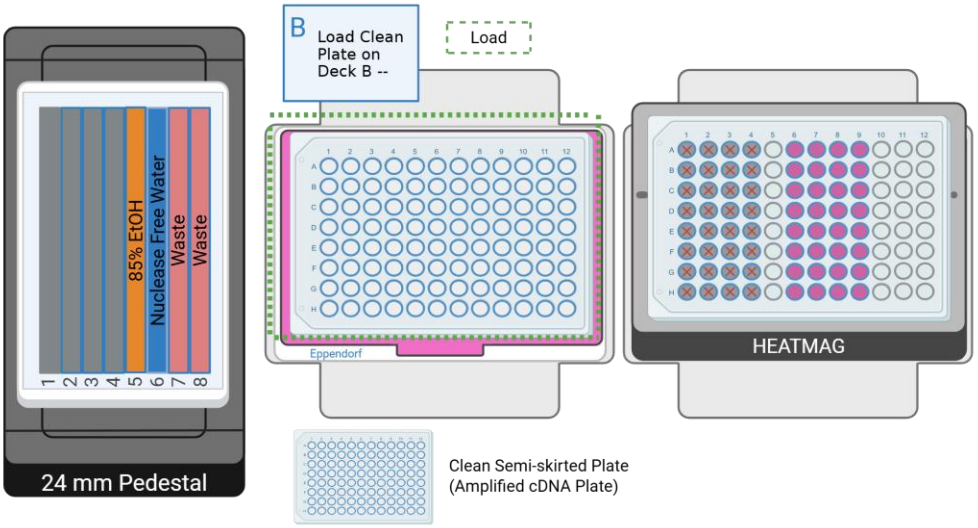


7. Press "Run" to start the program.

8. When prompted:

    a. Discard used Reagent Plate on Deck B. Load a clean 96 well semi-skirted plate on Deck B, and label it **Amplified cDNA Plate**.

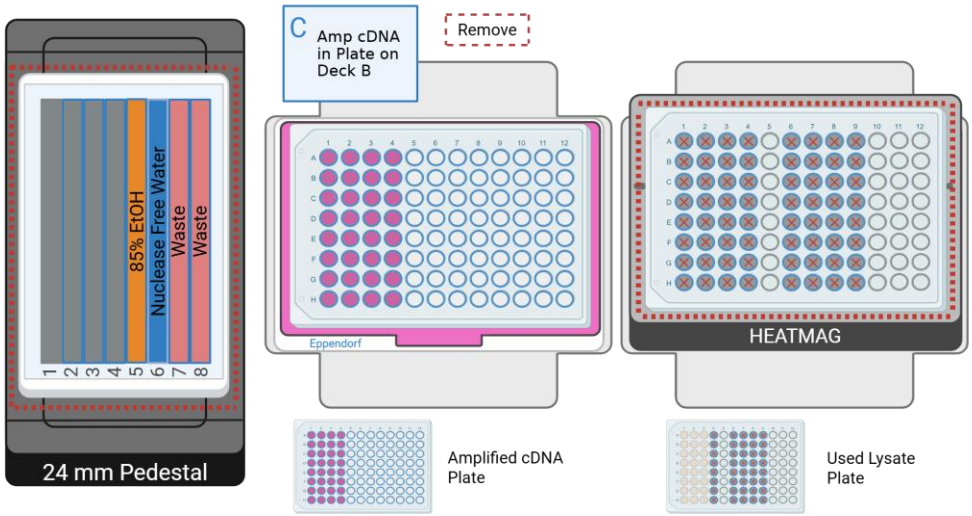
    b. Press "Run" to continue the program.



9. At the conclusion of the run:

    a. **Amplified cDNA Plate** is on Deck B. Do not discard as it will be used in the next steps.

    b. Discard the **Wash Buffer Plate** on Deck A and used **Lysate Plate** on Deck C.



Safe stopping point: **Amplified cDNA Plate** sealed with foil can be stored at 4°C for up to 48 hours or at -20°C for up to 3 months. Otherwise, proceed to Section 2.7 to quantify the cDNA before proceeding to Section 3.

CRITICAL! In section 3, you will need two Thermochromic PCR Cold Blocks with Thermochromic PCR Cold Block Risers. One block should remain cold throughout the process, while the other should be at room temperature. We recommend either leaving one block at room temperature overnight to thaw before starting, or running it under warm water to thaw quickly on the morning of Section 3.

2.7. cDNA Quantification

The concentration and size distribution of the cDNA are measured with fluorescent dyes and capillary electrophoresis. The cDNA is then stored at 4°C for up to 48 hours or at -20°C for up to 3 months.

1. Measure the concentration of each tube of purified cDNA from Section 2.6 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.

2. Assess the size distribution of each tube of purified cDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D5000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

Safe stopping point: **Amplified cDNA Plate** sealed with foil can be stored at 4°C for up to 48 hours or at -20°C for up to 3 months. Otherwise, proceed immediately to Section 3.

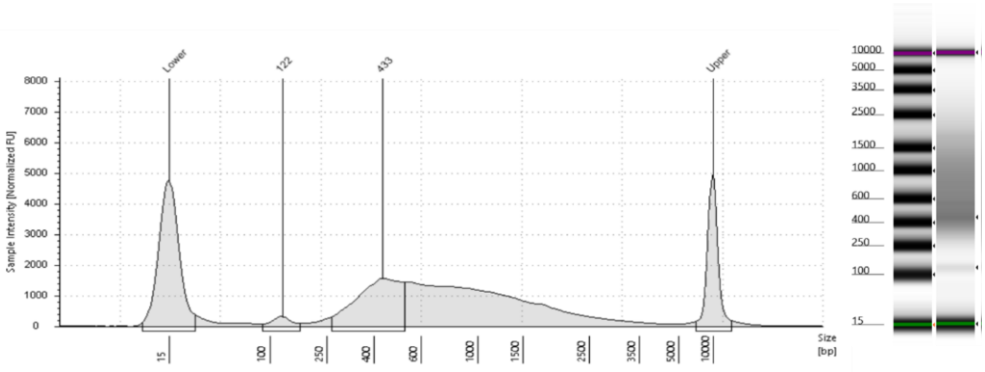


Figure 7: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution or 3-5 ng are appropriate.

Note: The traces above are representative of typical TapeStation cDNA traces. The shape and prominence of the trace is dependent on cell type, sublibrary size, and amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

Section 3: Sequencing Library Preparation

3.1. Fragmentation Mix Creation and Plating

The program makes the Fragmentation Mix and aliquots out the mix and SPRI beads such that they can be easily pipetted into sublibraries using the multichannel pipette.

Note: SPRI beads are used in several steps throughout Section 3. Ensure they are fully resuspended before use.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with risers from the -20°C freezer and leave **one** at room temperature for 10 minutes prior to use. **Allow the second one to reach room temperature**
Thermochromic PCR Cold Block Riser	Parse	2	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
Semi-Skirted 96 Well PCR Plate	Consumables	2	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
1.5 mL tube	Consumables	1	
2 mL tube	Consumables	1	
Fragm/End Prep Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice.
Fragm/End Prep Enzymes	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
SPRI beads	Room temperature	~ 2 mL	Ensure the beads are fully vortexed and suspended before use.

2. If not done already, remove the two Thermochromic PCR Cold Block with Risers from -20°C. Thaw one at room temperature for **10 minutes**, thaw the other until completely pink. Meanwhile, keep the Parse Metal Cold Block in ice.

3. If the **Amplified cDNA Plate** was stored at -20°C, start the following program to thaw the plate.

THAW AMPLIFIED cDNA PLATE		
Total Run Time		5 minutes
Lid Temperature		40°C
Sample Volume		75 µL
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

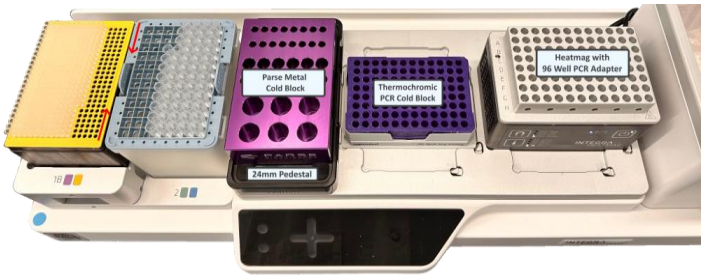
4. Vortex the **Amplified cDNA Plate** for **5 seconds**. Briefly centrifuge for **30 seconds** at 100 x g.

5. Manually transfer **35 µL** of cDNA into columns 1-4 of a new low-bind, semi-skirted PCR plate and store on ice. Label this plate **cDNA Sample Plate**. Store any remaining purified cDNA at -20°C.

6. If not already connected, connect the D-ONE Pipetting Module 1-Ch, 5-1250 µL Pipette and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

7. Place the chilled Thermochromic PCR Cold Block with Riser on Deck B.

8. Place the cooled Parse Metal Cold Block on Deck A. The deck layout should correspond to the configuration below:



9. Place the following components using the deck configuration below:

    a. Deck A, column 2:

        i. Pos 1: a clean 1.5 mL tube.

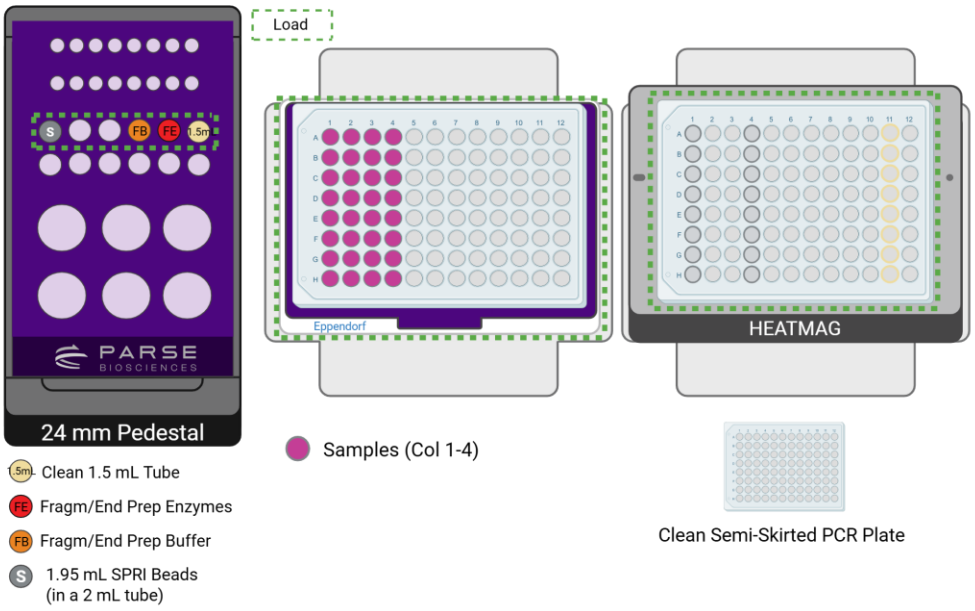
        ii. Pos 2: ● Fragm/End Prep Enzymes.

        iii. Pos 3: ● Fragm/End Prep Buffer.

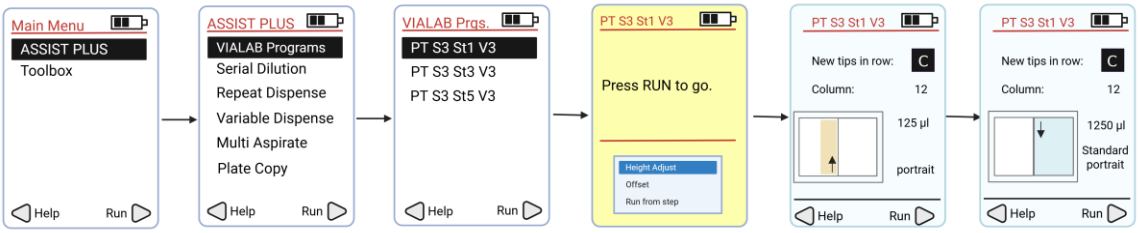
        iv. Pos 6: A 2 mL tube filled with **1,950 µL** of SPRI Beads.

    b. Load the **cDNA Sample Plate** stored on ice during Step 3.1.5 onto the Thermochromic PCR Cold Block on Deck B with A1 in the upper left corner, pressing firmly to ensure the plate is fully seated.

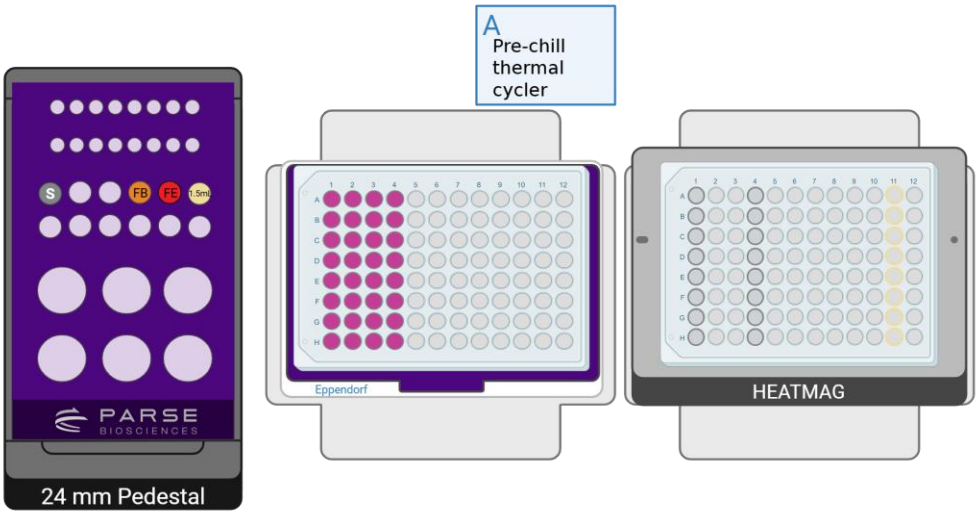
    c. Deck C: clean semi-skirted 96 well PCR plate.



10. Remove the reagent caps, then select and run the program **PT S3 St1 V3_5** following the diagram below.



11. Follow the program prompts for manual intervention:



a. When prompted, start the Fragmentation and End Prep program on the thermocycler to pre-chill, ensuring that Step 1 of the cycling program is set to 4°C, so it will be at the correct temperature when needed during Section 3.2.6 Ensure that the SPRI beads have not settled during thermocycler setup. If the beads have settled, resuspend them manually. Press "Run" on the pipette to continue.

FRAGMENTATION AND END PREP		
Total Run Time		40 minutes
Lid Temperature		70°C
Sample Volume		50 µL
Step	Time	Temperature
1	Hold	4°C
2	10 min	32°C

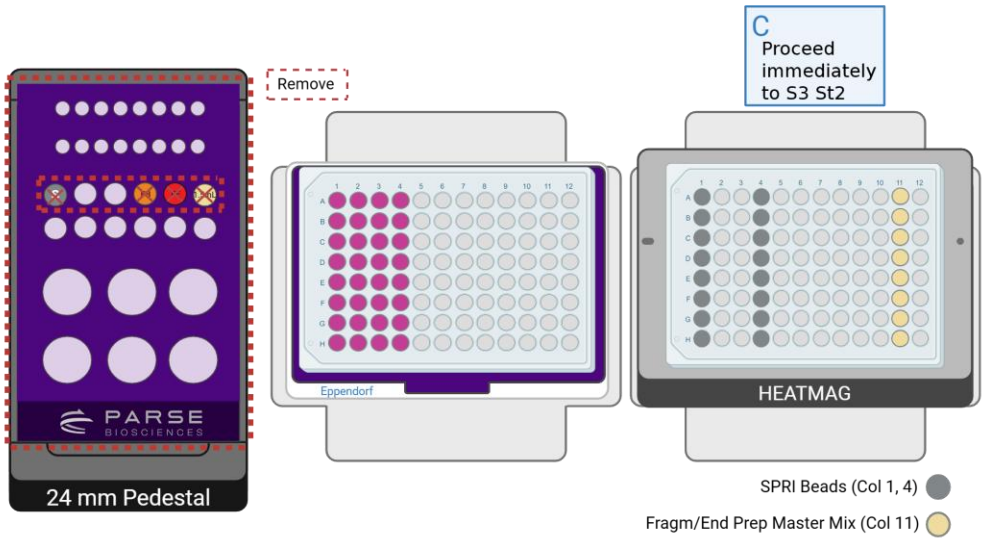
FRAGMENTATION AND END PREP		
3	30 min	65°C
4	Hold	4°C

12. At the conclusion of the run:

    a. Discard the tubes on Deck A.

    b. Keep all labware on Decks B and C as they will be used in the next step.

    c. Remove the Parse Metal Cold Block from the Deck A, place it on ice, and proceed immediately to Section 3.2.



3.2. Fragmentation Mix Addition and Post-Fragmentation SPRI Cleanup

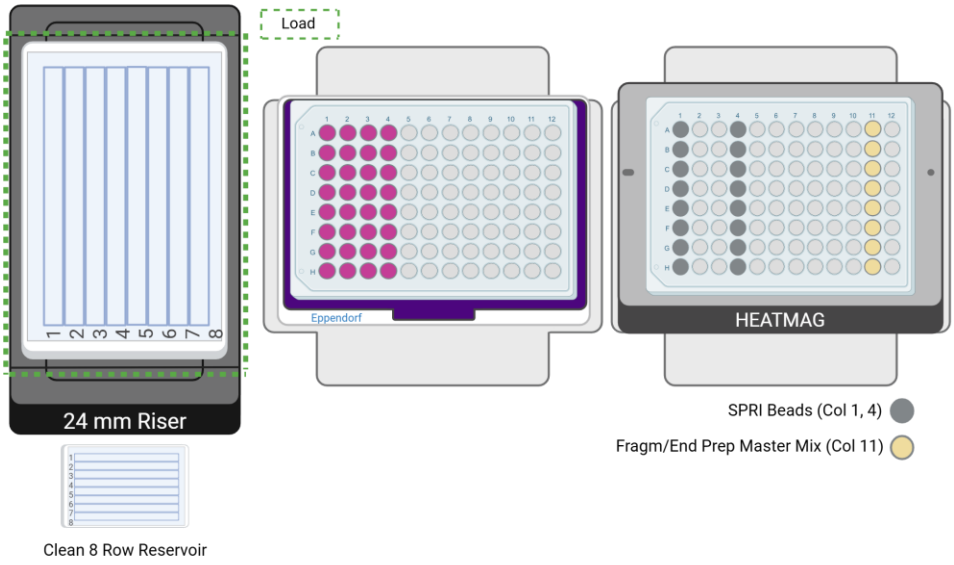
The program uses the VOYAGER multichannel pipette to add Fragmentation Mix to sublibraries. After the user runs the Fragmentation thermal cycling program, sublibraries are loaded back onto the ASSIST PLUS and a double-sided SPRI cleanup is carried out.

1. Gather the following components and reagents and set up the deck as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Module 8-Ch, 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with risers from the -20°C freezer and leave **one** at room temperature for 10 minutes prior to use. **Allow the second one to reach room temperature**
Thermochromic PCR Cold Block Riser	Parse	2	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
300 mL Reservoir Base	Consumables	1	
8 Row reservoir	INTEGRA	1	
125 µL Tip Rack	INTEGRA	1	
Ethanol	Consumables and Reagents	1	
Nuclease-free water	Consumables and Reagents	1	

2. Prepare **45 mL** of fresh 85% ethanol with nuclease-free water.

3. Place a clean 8 Row Reservoir on the 300 mL Reservoir Base on Deck A. The deck layout should correspond to the configuration below.

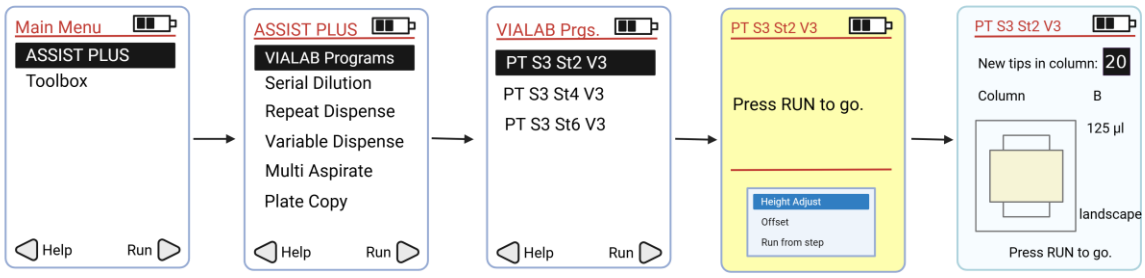


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

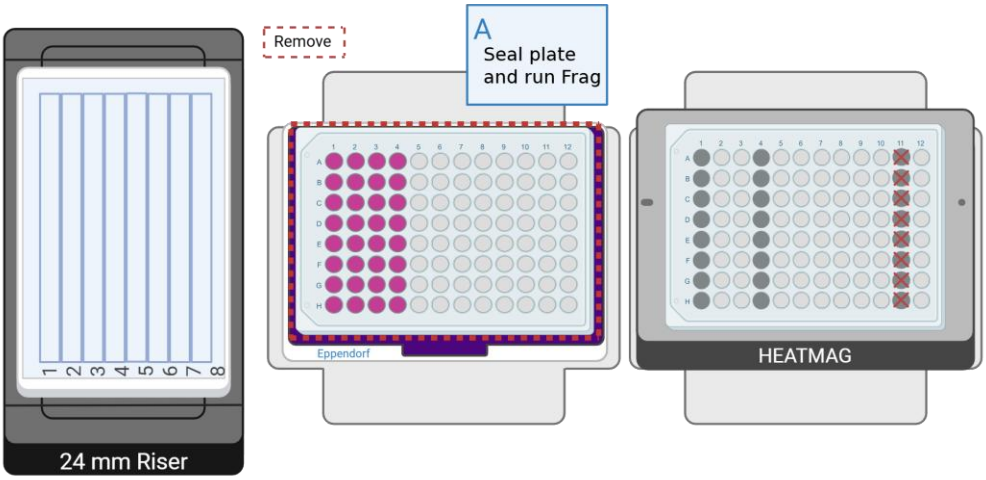
    d. Replace it with VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure the Tip Deck and tip box are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

5. Select and run the program **PT S3 St2 V3_5** following the diagram below.



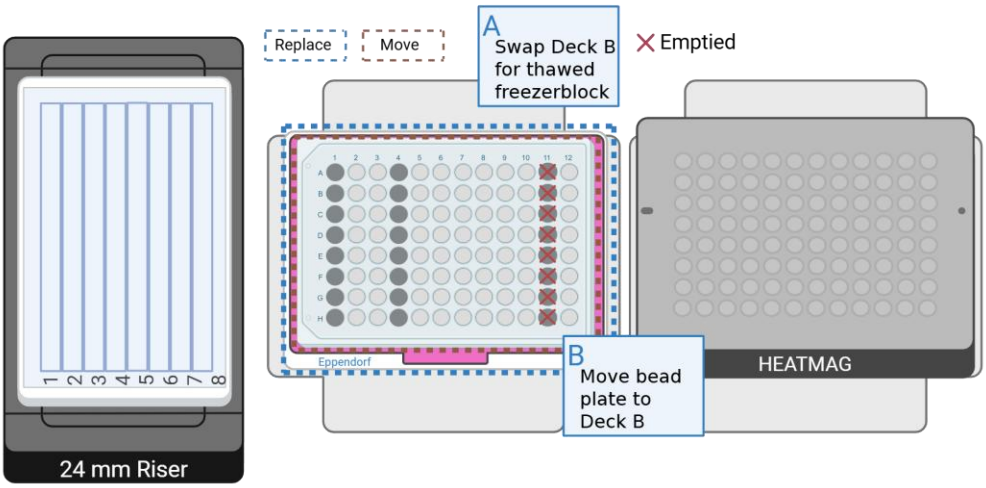
6. When prompted, follow the program prompts for manual intervention:

    a. Remove the **cDNA Sample Plate** from Deck B and seal with a PCR plate seal. Place the sealed plate into the pre-cooled thermocycler from Section 3.1.11a.



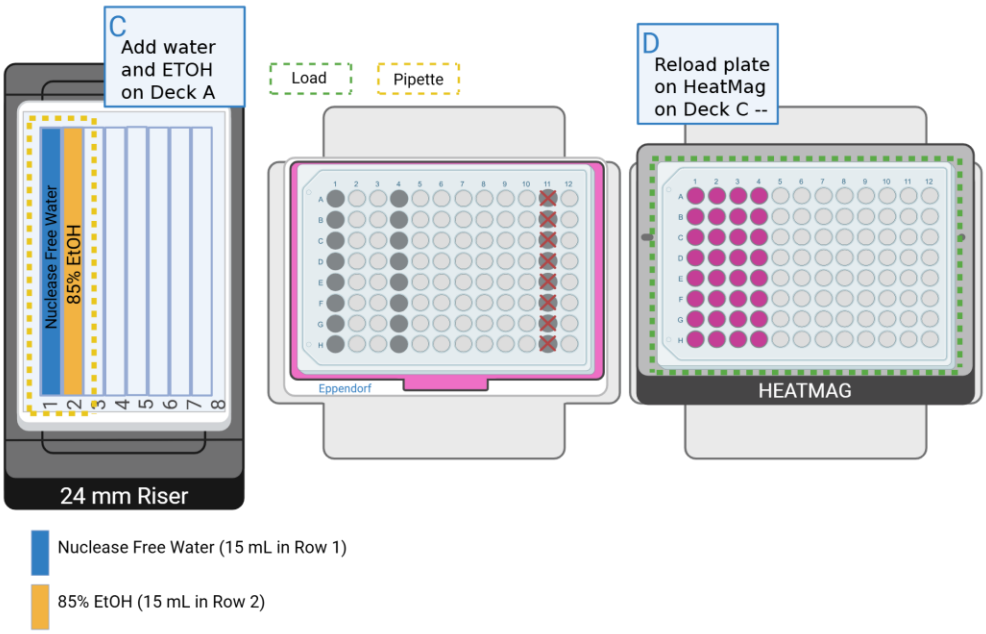
7. Ensure the thermocycler is cool prior to use and start the Fragmentation and End Prep program in the thermocycler precooled in Section 3.1.11a. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and End Prep program.

8. While the thermocycler is running, press "Run" on the pipette to continue the program. Follow the program prompts for manual intervention:



    a. Replace the frozen Thermochromic PCR Cold Block with riser on Deck B with the one that has been brought to room temperature. A fully thawed Thermochromic PCR Cold block should look pink in color. Press "Run" on the pipette to continue.

b. Move the plate on Deck C onto the fully thawed Thermochromic PCR Cold Block on Deck B. Press "Run" on the pipette to continue.



c. With a serological pipette add:

i. **15 mL** nuclease free water in lane 1 of the 8 Row Reservoir on Deck

ii. **15 mL** 85% Ethanol in lane 2 of the 8 Row Reservoir on Deck A. Press "Run" to continue.

d. When Fragmentation has completed and when prompted, load the sublibrary plate onto the HEATMAG on Deck C with A1 in the top left. Ensure the nuclease free water and the 85% Ethanol are evenly distributed within their rows in the 8-Row Reservoir. Press "Run" on the pipette to continue.

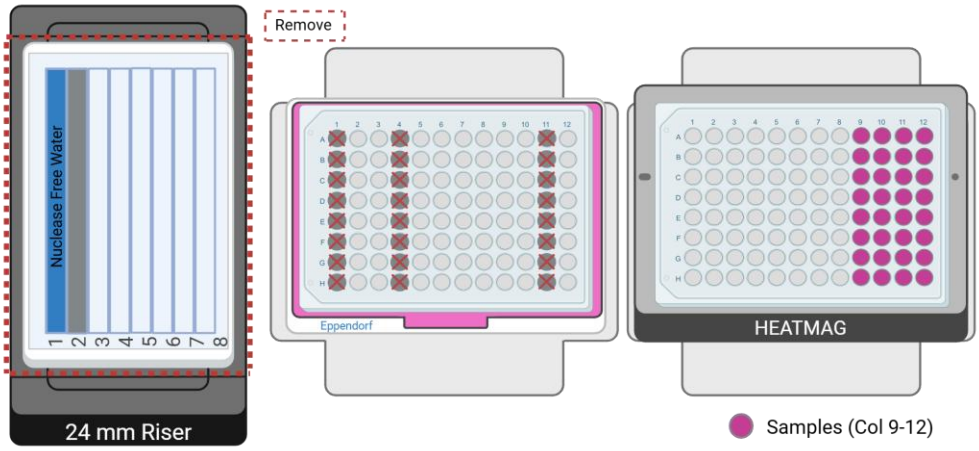
9. At the conclusion of the run:

a. Remove the 8 Row Reservoir on Deck A and seal it with a PCR plate seal. Store at room temperature for later use.

b. Keep all labware on Decks B and C. They will be used for the next step.

10. When the program is complete, continue to Section 3.3.

STOP Safe stopping point: The size-selected fragmented and end prepped DNA in columns 9-12 of the **cDNA Sample Plate** on deck C can be sealed with a foil seal and stored at 4°C for up to 18 hours or at -20°C for up to 2 weeks.


Remove
Nuclease Free Water
1 2 3 4 5 6 7 8
24 mm Riser
Eppendorf
HEATMAG
Samples (Col 9-12)

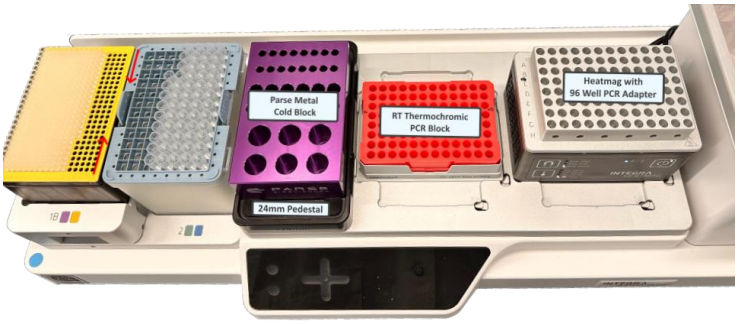
3.3. Ligation Mix Creation and Plating

The program prepares the Ligation Mix in a clean 2 mL tube, then dispenses it into column 10 of the semi-skirted 96-well plate on Deck B, allowing for easy pipetting into sublibraries using the multichannel pipette.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	1	Ensure the Thermochromic PCR Cold Block with riser is at room temperature before use.
Thermochromic PCR Cold Block Riser	Parse	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
1.5 mL tube	Consumables	3	
2 mL tube	Consumables	1	
● Ligation Adapter	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
● Adapter Ligation Buffer	-20°C Reagents	1	
● Adapter Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

2. Place the Parse Metal Cold Block on Deck A. The deck layout should correspond to the configuration below.



3. On the Parse Metal Cold Block place these components following the deck configuration below:

    a. Column 2:

        i. Pos 5: A 1.5 mL tube filled with **1,500 µL** of SPRI Beads.

        ii. Pos 6: A 1.5 mL tube filled with **1,500 µL** of SPRI Beads. Ensure the beads are fully resuspended.

    b. Column 2:

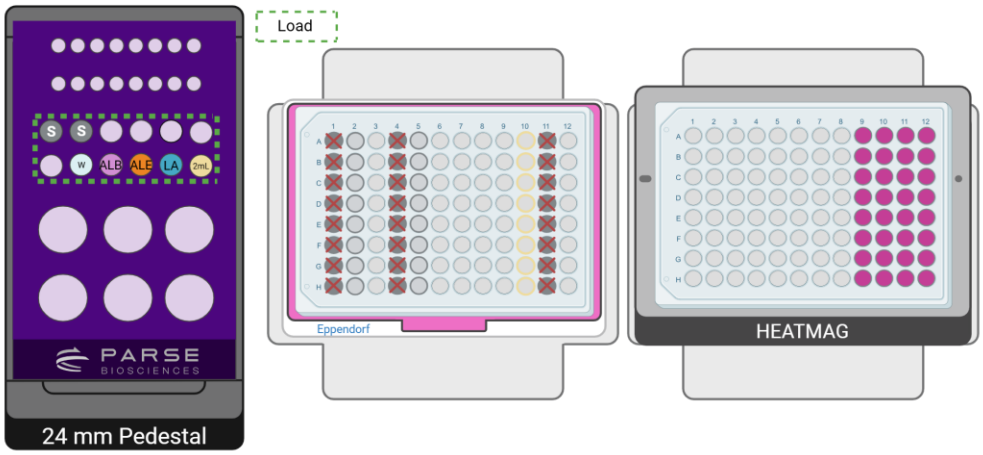
        i. Pos 7: a clean 2 mL tube.

        ii. Pos 8: ● Ligation Adapter.

        iii. Pos 9: ● Adapter Ligation Enzyme.

        iv. Pos 10: ● Adapter Ligation Buffer.

        v. Pos 11: a 1.5 mL tube filled with **800 µL** of nuclease-free water.

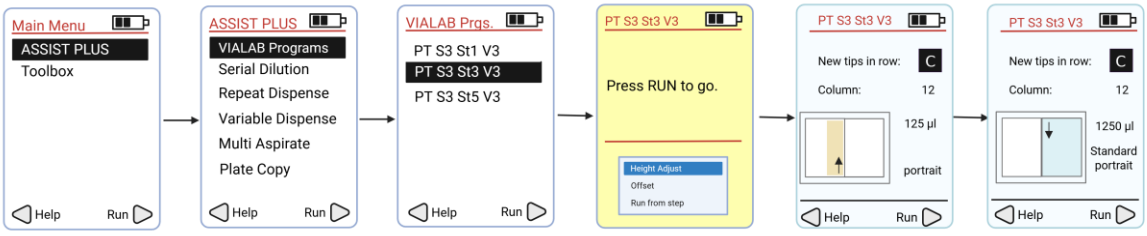


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove the VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

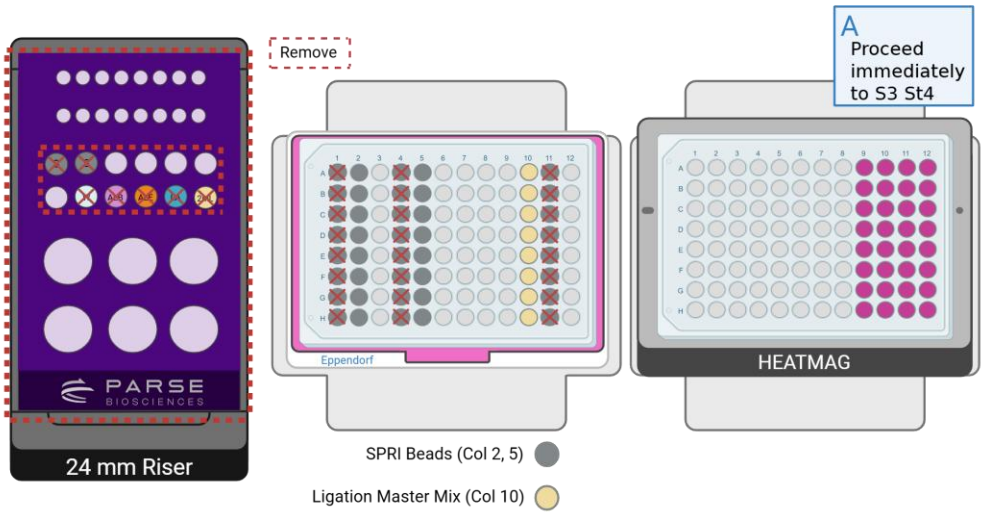
5. Remove the reagent caps, select and run the program **PT S3 St3 V3_5** following the diagram below.



6. At the conclusion of the program:

    a. Remove the Parse Metal Cold Block from Deck A. Discard all the tubes.

    b. Keep all labware on Decks B and C. Proceed immediately to section 3.4.



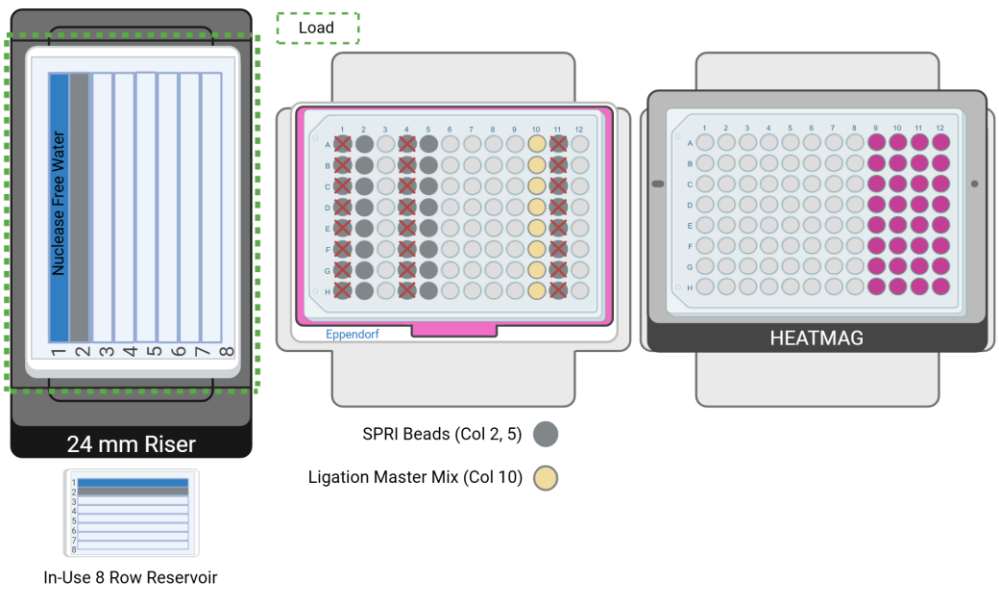
3.4. Ligation Master Mix Addition and Post-Ligation SPRI Cleanup

The program uses the VOYAGER multichannel pipette to add Ligation Mix to sublibraries. After the user runs the Ligation thermocycling program, sublibraries are loaded back onto the ASSIST PLUS and a single-sided SPRI cleanup is carried out.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch, 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	1	Ensure the Thermochromic PCR Cold Block with riser is at room temperature before use. It should look pink.
Thermochromic PCR Cold Block Riser	Parse	1	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
300 mL Reservoir Base	INTEGRA Components	1	
In-use 8 Row Reservoir	INTEGRA	1	Retrieve the reservoir that was previously stored at room temperature.
Ethanol	Consumables and Reagents		85% Ethanol was freshly made in section 3.2.2.

2. Load the 8 Row Reservoir on Deck A with the 300 mL Reservoir Base. The deck layout should correspond to the configuration below.

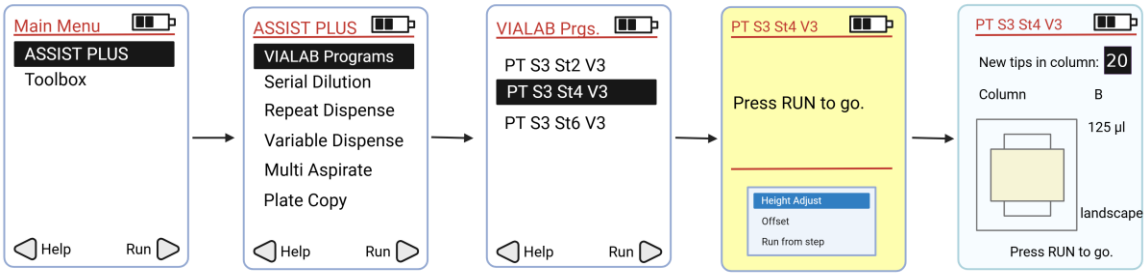


3. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

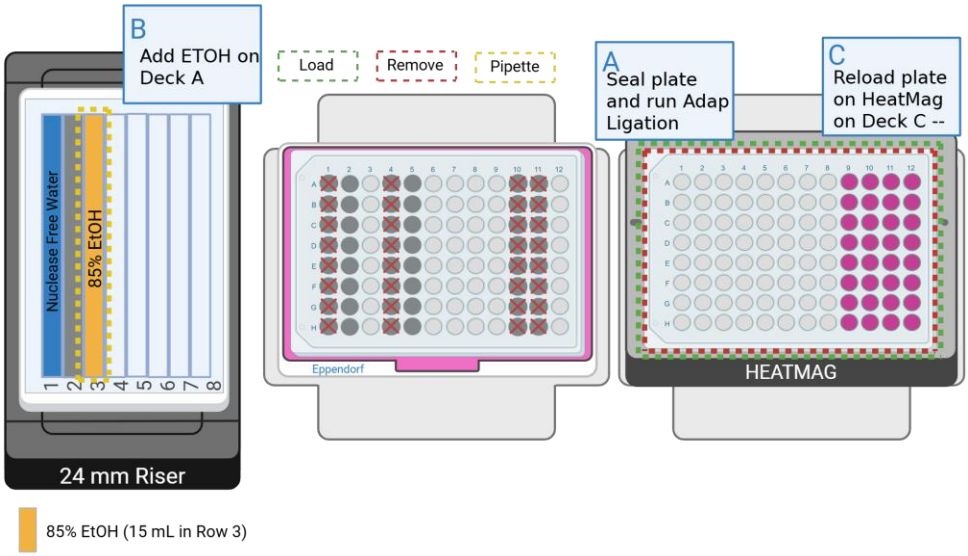
    a. Remove the D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VOYAGER 8-Ch 5-125 µL 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.

4. Navigate to the VIALAB programs on the Voyager Pipetting Module, select and run the program **PT S3 St4 V3_5** following the diagram below.



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



a. Seal the **cDNA Sample Plate** with a PCR plate seal on Deck C and place it into a thermocycler. Run the following program. Proceed to the next step while the thermocycler program is still running.

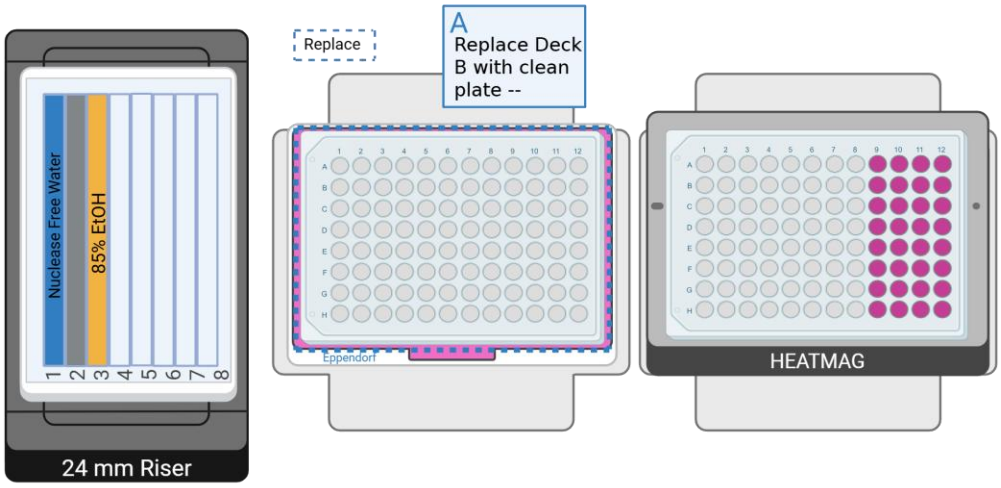
ADAPTER LIGATION		
Total Run Time		15 minutes
Lid Temperature		30°C*
Sample Volume		100 µL
Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

Note: *If the thermocycler's lid can't reach the recommended temperature of 30°C, turn the lid heating off.

b. With a serological pipette, add **15 mL** 85% Ethanol to lane 3 of the 8 Row Reservoir on Deck A.

c. Upon thermocycling completion reload the **cDNA Sample Plate** onto the HEATMAG located on Deck C and remove the seal, ensuring that A1 is in the top left. Press "Run" to continue the program.

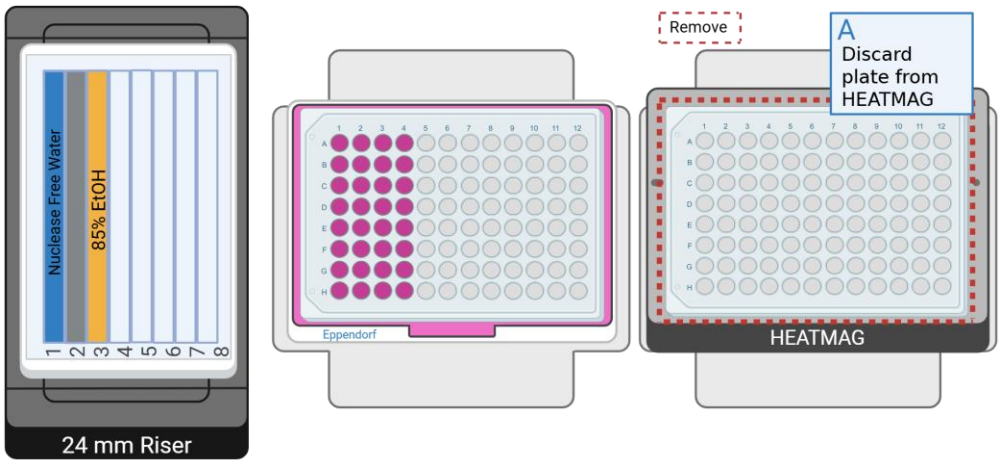
6. When prompted, follow the program prompts for manual intervention then press “Run” to continue the program:



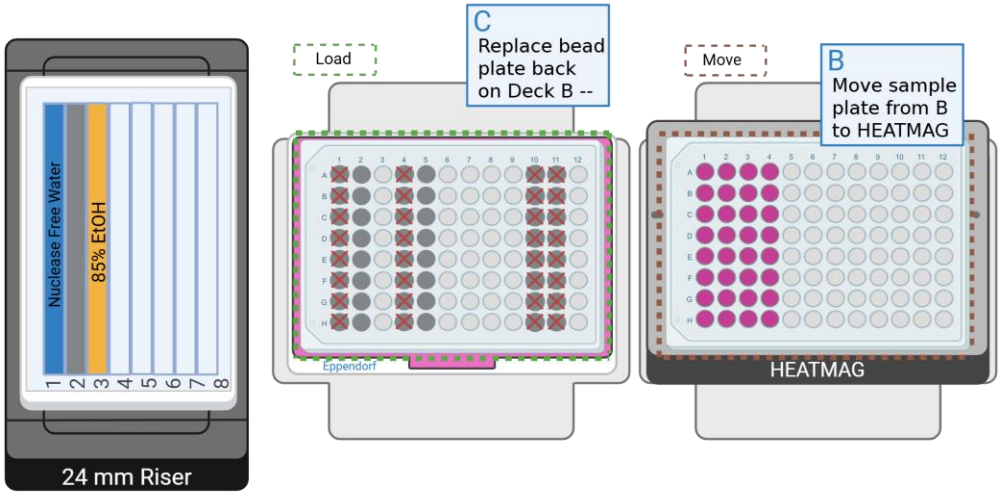
   a. Replace the plate on Deck B with a clean semi-skirted PCR plate then press “Run” to continue the program. Do not discard the plate from Deck B. Set it aside at room temperature.

CRITICAL! Do not discard plate from Deck B. It will be used again shortly.

7. When prompted, follow the program prompts for manual intervention then press “Run” to continue the program:



   a. Discard the plate from the HEATMAG on Deck C.



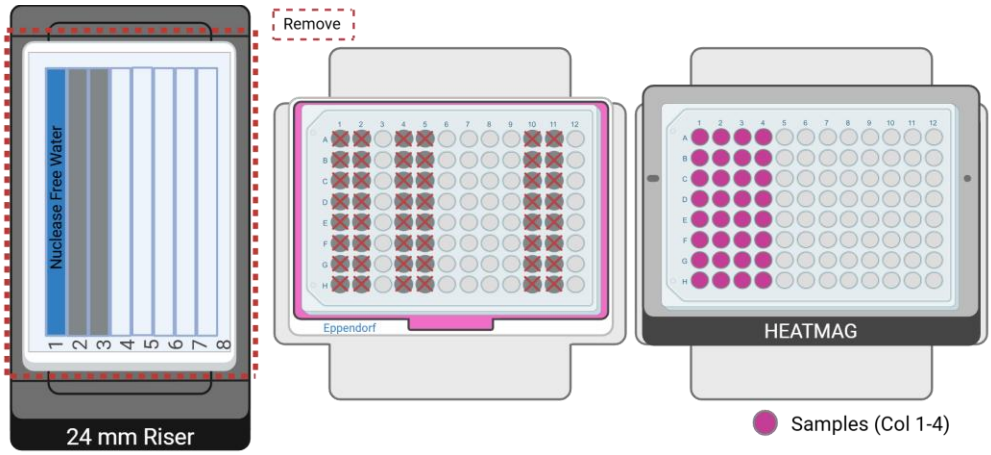
b. Label the new **cDNA Sample Plate,** then move it from Deck B to the HEATMAG on Deck C.

c. Retrieve the plate containing SPRI beads in Columns 2 and 5 that was set aside in step 3.1.7a and replace it on Deck B.

8. At the conclusion of the program:

a. Remove the 8 Row Reservoir on Deck A and seal it with a PCR plate seal. Store at room temperature for later use.

b. Keep all labware on Decks B and C. Proceed to Section 3.5.



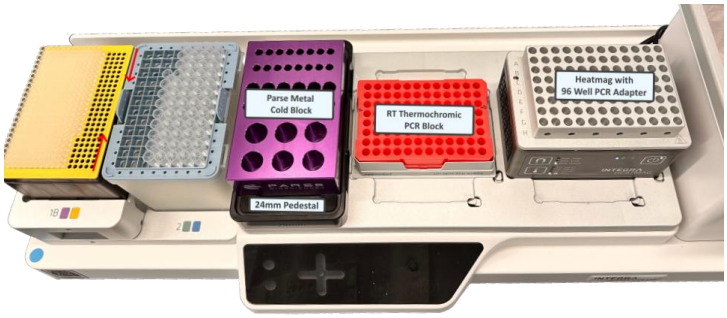
3.5. Barcoding Round 4

Program uses the pipette to aliquot out the Library Amp Mix such that it can be easily pipetted into sublibraries using the multichannel pipette. During this program, the user will manually add UDIs to sublibraries.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Components	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Parse Metal Cold Block	INTEGRA Components	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	1	Ensure the Thermochromic PCR Cold Block with riser is at room temperature before use.
Thermochromic PCR Cold Block Riser	Parse	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
2 mL tube	Consumables	1	
Strips of 8 Domed PCR Caps	Consumables	12	
● Library Amp Mix	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by inverting 3x. Briefly centrifuge before use.
UDI Plate - WT	Parse reagents	1 sealed well per sublibrary	Thaw at room temperature then place on ice.

2. Load the Parse Metal Cold Block on the 24 mm Labware Pedestal on Deck A. The deck layout should correspond to the configuration below.

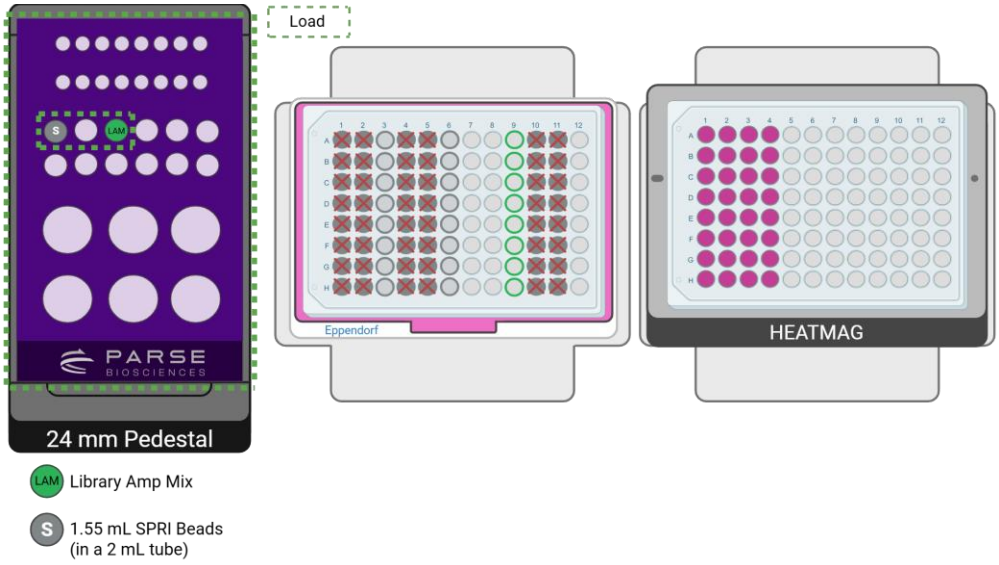


3. On the Parse Metal Cold Block place these components following the deck configuration below:

    a. Column 2:

        i. Pos 4: ● Library Amp Mix

        ii. Pos 6: A 2 mL tube filled with **1,550 µL** of resuspended SPRI Beads.



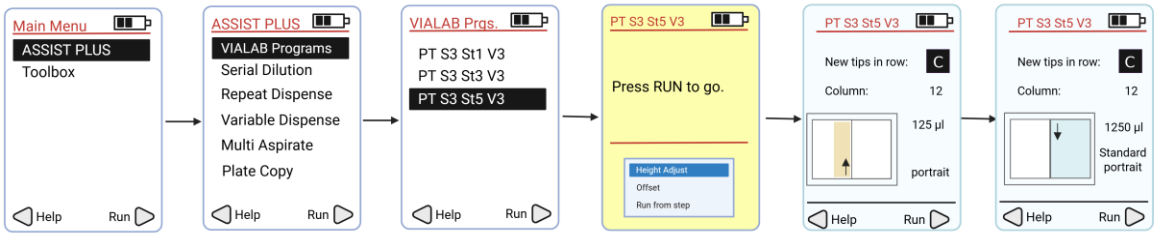
4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove the VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them

down firmly until they click into place. Remove the tip box lids prior to starting the program.

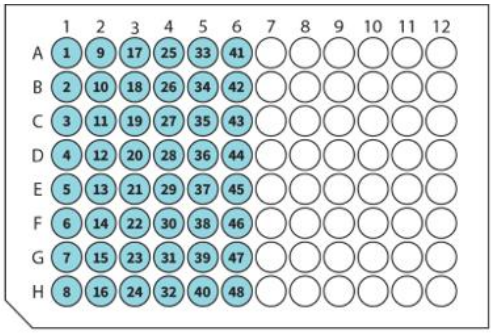
5. Remove the reagents caps, select and run the program **PT S3 St5 V3_5** following the diagram below.



6. While the program is running, centrifuge the thawed UDI Plate - WT at 100 x g for **1 minute.**

7. Wipe the surface of the UDI plate with 70% ethanol and allow it to dry.

8. Orient the UDI Plate - WT with the notch on the bottom left as pictured below. For each sublibrary being processed, choose one unused well of the UDI Plate - WT and record the well position and number for each sublibrary.



9. When prompted, with a multichannel P20, manually pierce the seal of the chosen wells of the UDI Plate - WT. Then, with a multichannel P20 and new tips, manually mix by pipetting 5x and immediately transfer **4 µL** from a chosen unused well of the UDI Plate-WT to its corresponding sample well in the **cDNA Sample Plate**, columns 1 - 4 on Deck C.

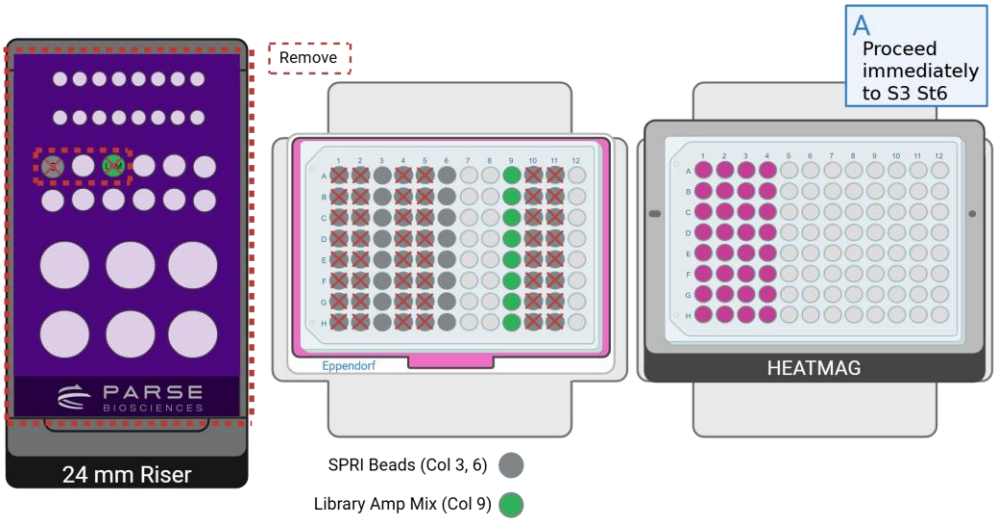
CRITICAL! Only transfer primers from 1 well of the UDI Plate - WT to 1 well of the **cDNA Sample Plate**.

10. If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse pierced wells.

11. Press "Run" to continue the program. At the conclusion of the run:

a. Remove the Parse Metal Cold Block from Deck A. Discard all the tubes.

b. Proceed immediately to Section 3.6.



3.6. Library Amp Mix Addition and Size Selection

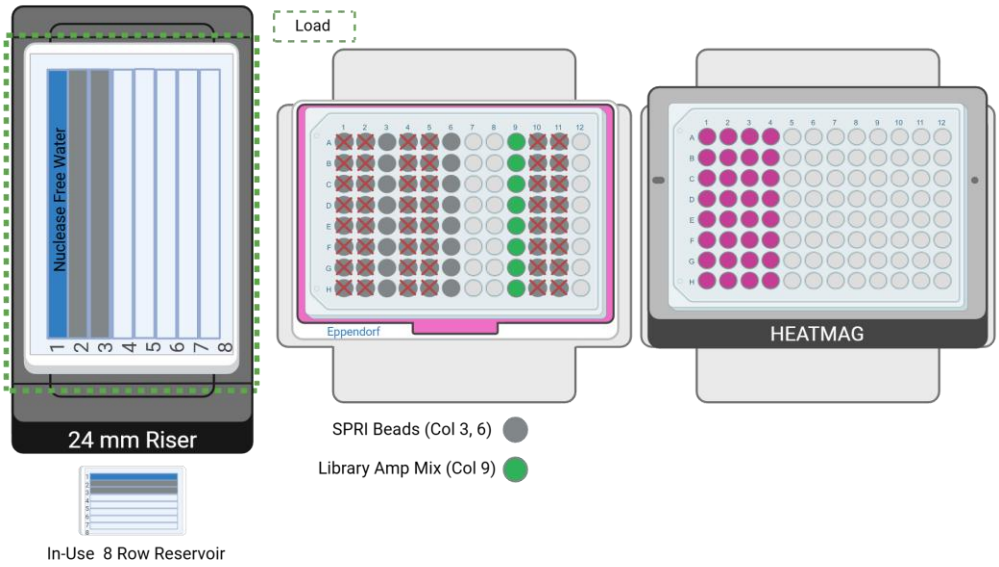
The program uses the VOYAGER multichannel pipette to add Library Amp Mix to the sublibraries. After the user runs the Indexing PCR thermocycling program, sublibraries are loaded back onto the ASSIST PLUS and a double-sided SPRI cleanup is carried out.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Components	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Components	1	
125 µL Tip Rack	INTEGRA	1	
24 mm Labware Pedestal	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse	1	Ensure the Thermochromic PCR Cold Block with riser is at room temperature before use.
Thermochromic PCR Cold Block Riser	Parse	1	
300 mL Reservoir Base	INTEGRA Components	1	
HEATMAG with 96 Well Adapter	INTEGRA Components	1	
In-use 8 Row Reservoir	INTEGRA	1	Retrieve the reservoir that was previously stored at room temperature.
Ethanol	Consumables and Reagents		85% Ethanol was freshly made in section 3.2.2.

2. Gather the 85% ethanol prepared in Section 3.2.2, set aside at room temperature for later use.

3. Load the 8 Row Reservoir stored at room temperature onto Deck A. The deck layout should correspond to the configuration below.

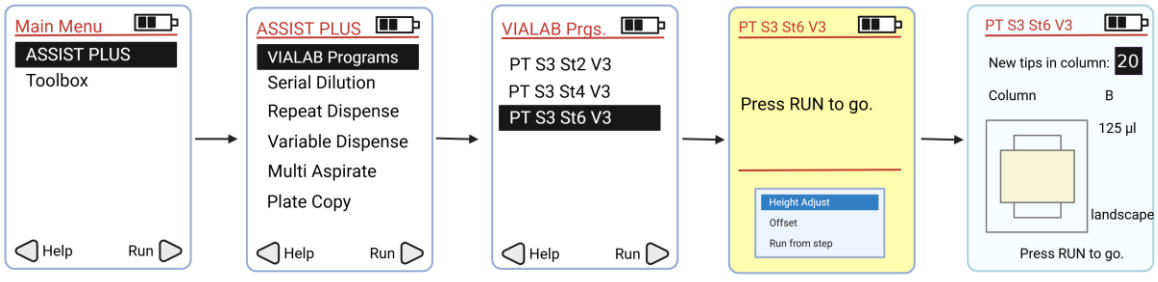


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    b. Replace it with VOYAGER 8-Ch 5-125 µL 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.

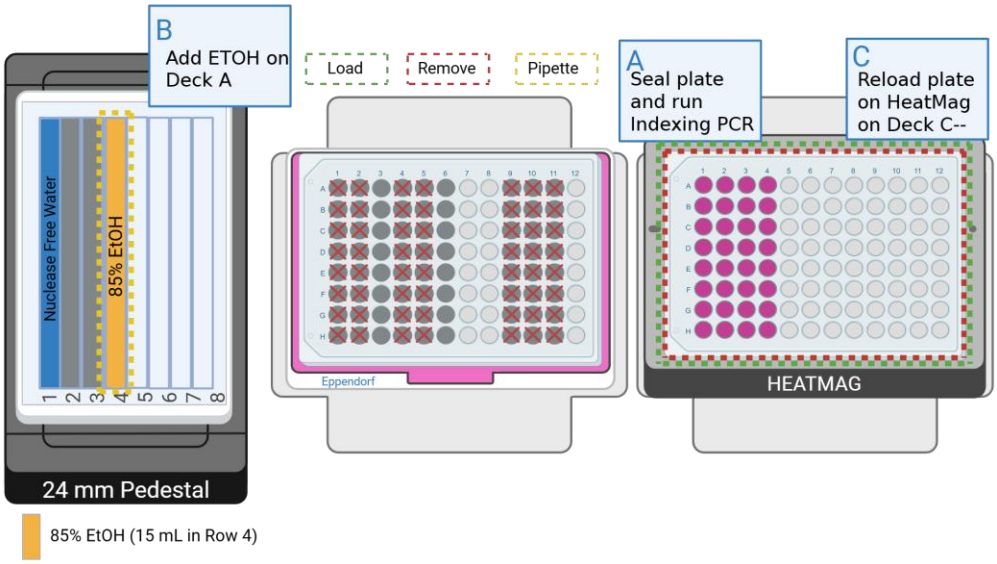
5. Select and run the program **PT S3 St6 V3_5** following the diagram below.



6. While the program is running, determine the number of PCR cycles required for the Indexing PCR based on the **average** amount of cDNA inputted into the fragmentation and end prep reaction as recorded in Section 2.7.

NUMBER OF PCR CYCLES		
cDNA concentration (End of Section 2.7)	cDNA Input (ng)	PCR Cycles
0.35 - 0.7 ng/µL	10-24	13
0.71 - 1.4 ng/µL	25-49	12
1.41 - 2.84 ng/µL	50-99	11
2.85 - 8.56 ng/µL	100-299	10
8.57- 28.4 ng/µL	300-999	8
28.5+ ng/µL	1,000 or more	7

7. When prompted, follow the program prompts for manual intervention:



8. Remove the **cDNA sample plate** from Deck C and seal the plate with a PCR plate seal. Place it into a thermocycler and run the following program.

INDEXING PCR			
Total Run Time			~30 minutes
Lid Temperature			105°C
Sample Volume			50 µL
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 s	98°C	Varies, see table above
3	20 s	67°C	
4	1 min	72°C	
6	5 min	72°C	1
6	Hold	4°C	1

9. When the Indexing PCR thermocycling program is complete, unseal the 8 Row Reservoir on Deck A. With a serological pipette, add **15 mL** 85% Ethanol to row 4 of the reservoir. Press "Run" to continue the program.

10. Reload the **cDNA sample plate** onto the HEATMAG with 96 Well Adapter on Deck C. Press "Run" to continue the program.

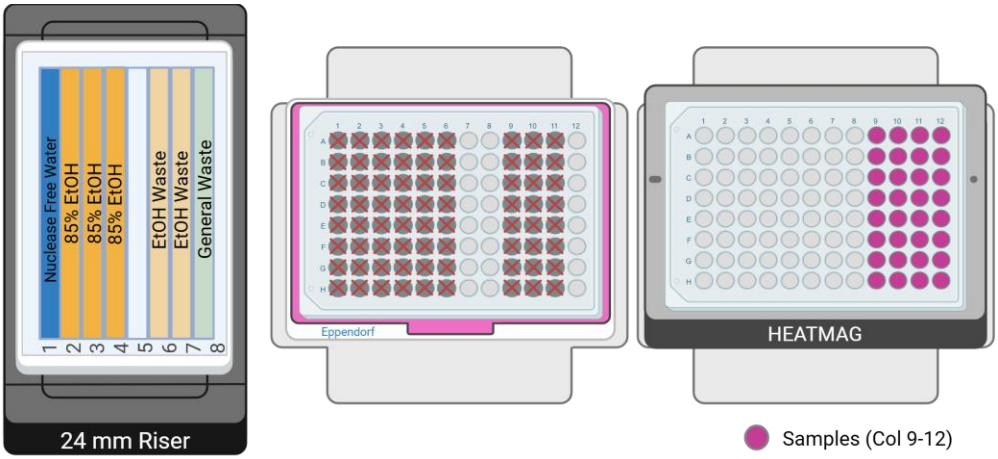
11. At the conclusion of the run:

    a. The sequencing libraries will be in columns 9 - 12 on Deck C. Sequencing libraries can be stored at -20°C for up to 3 months.

    b. Discard the used 8 Row Reservoir on Deck A and their contents.

    c. Discard the used semi-skirted plate on Deck B.

STOP Safe stopping point: Sequencing libraries can be either sealed with a foil seal or transferred into PCR strip tubes, and stored at -20°C for up to 3 months.


Nuclease Free Water
85% EtOH
85% EtOH
85% EtOH
EtOH Waste
EtOH Waste
General Waste
1 2 3 4 5 6 7 8
24 mm Riser
Eppendorf
HEATMAG
Samples (Col 9-12)

3.7. Sequencing Library Quantification

The concentration and size distribution of the sequencing libraries are measured with fluorescent dyes and capillary electrophoresis. Sequencing libraries can be stored at -20°C for up to 3 months.

1. Measure the concentration of each purified sequencing library from Section 3.7 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

2. Assess the size distribution of each purified sequencing library with a High Sensitivity DNA Kit on the Agilent Bioanalyzer System or High Sensitivity D1000 ScreenTape and Reagents on the Agilent TapeStation System according to the manufacturer's instructions.

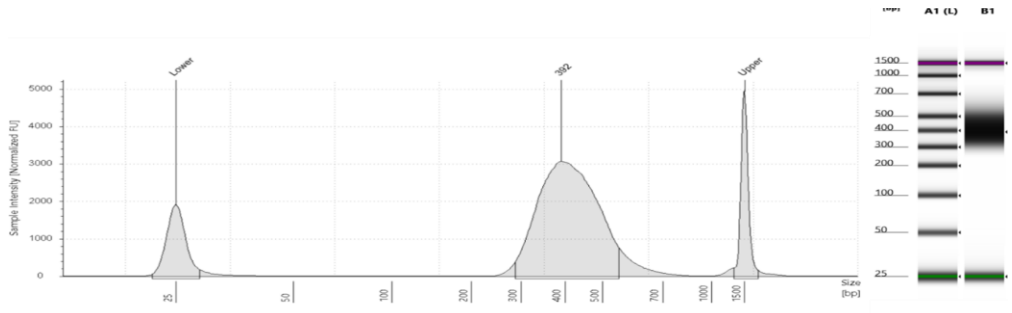


Figure 8: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

Note: Samples may need to be diluted to be within the manufacturer's recommended concentration range. Typically, between a 1:3 to 1:10 dilution or 3-5 ng are appropriate.

Note: The traces above are representative of typical TapeStation of DNA from indexed sublibraries. There should be a peak between 400-500 bp. The prominence of the trace is dependent on the amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

Note: If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 400-500 bp). Do not use this additional peak when estimating amplicon size.

Appendices

Appendix A: Sequencing Information

We recommend a minimum sequencing depth of 20,000 reads per cell for whole transcriptome libraries. However, ideal sequencing depth is dependent on the sample type and experimental goals. For example, if trying to capture rare cell types in a heterogenous population, deeper sequencing is required. Alternatively, analyzing a homogenous population, more shallow sequencing may be appropriate.

Whole transcriptome sequencing libraries should be diluted and denatured according to the manufacturer's instructions for the relevant sequencing instrument. If using the Illumina platform, we strongly recommend adding 5% PhiX for optimal sequencing quality.

Details of the final whole transcriptome sequencing library structure are below.

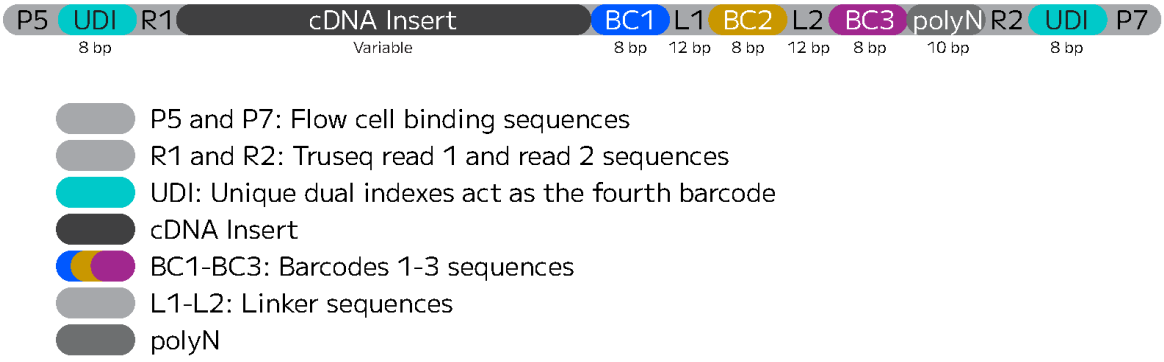


Figure 9: Whole transcriptome sequencing library structure.

Libraries should be sequenced with paired reads using the read structure in the table below. Read lengths longer than those recommended below are acceptable, but additional bases in read 2 are trimmed by the Parse Analysis Pipeline.

READ	FUNCTION	INSERT
Read 1	cDNA Insert	64
i7 Index (Index 1)	Barcode 4 / UDI	8
i5 Index (Index 2)	Barcode 4 / UDI	8
Read 2	Barcodes 1-3	58

The fourth barcode that tags each sublibrary act as a standard Illumina i7 and i5 indexes. Please refer to the following table to demultiplex the whole transcriptome libraries.

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_1	A1	CAGATCAC	ATGTGAAG	CTTCACAT
UDI_Plate_WT_2	B1	ACTGATAG	GTCCAACC	GGTTGGAC
UDI_Plate_WT_3	C1	GATCAGTC	AGAGTCAA	TTGACTCT
UDI_Plate_WT_4	D1	CTTGTAAT	AGTTGGCT	AGCCAACT
UDI_Plate_WT_5	E1	AGTCAAGA	ATAAGGCG	CGCCTTAT
UDI_Plate_WT_6	F1	CCGTCCTA	CCGTACAG	CTGTACGG
UDI_Plate_WT_7	G1	GTAGAGTA	CATTCATG	CATGAATG
UDI_Plate_WT_8	H1	GTCCGCCT	AGATACGG	CCGTATCT
UDI_Plate_WT_9	A2	GTGAAACT	TACAGACT	AGTCTGTA
UDI_Plate_WT_10	B2	TCATTCCT	AATGCCTG	CAGGCATT
UDI_Plate_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
UDI_Plate_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
UDI_Plate_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT

UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
UDI_Plate_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG
UDI_Plate_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
UDI_Plate_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
UDI_Plate_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix B: Pipetting Programs

Section 1.1. Sample Normalization

PT S1 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Dilute Cells	40 min
3	“Thaw R1 Plate” message	

Section 1.2. Loading and Pooling Round 1 Plate

PT S1 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Transfer Diluted Fixed Sample to Round 1 Barcoding Plate (35 µL/well)	7 min
3	“Remove R1 plate on Deck C” message	
4	“Remove labware on Deck B” message	
5	“Move hardware on Deck B” message	
6	“Load R1 Plate on Deck B - -” message	
7	Volume Change	
8	Pool Cells into Row A (42 µL/well)	1 min
9	Pool Cells into Row A (15 µL/well)	1 min
10	Pool Cells into Row E (42 µL/well)	1 min
11	Pool Cells into Row E (15 µL/well)	1 min

Section 1.3. Round 2 Ligation Preparation

PT S1 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Pool row A into 10 mL Tube with 1000 µL tips (117 µL/well)	4 min
3	Pool row E into 10 mL Tube with 1000 µL tips (117 µL/well)	4 min
4	Pool row into 10 mL Tube with 125 µL tips (10 µL/well)	2 min
5	Add Spin Additive	30 sec
6	"Remove 10mL Tube from Deck C" message	
7	"Thaw R2 Plate" message	
8	"Load 10mL Tube on Deck C –" message	
9-16	Remove Supernatant (3.9 mL)	2 min
17	"Checkpoint: Supernatant Removed - - " message	
18-19	Resuspend Cell Pellet with Resuspension Buffer (2 mL)	1 min
20	Add R2 Ligation Enzyme to Buffer (20 µL)	1 min
21	Mix Ligation Mix	1 min
22	Volume Change	
23	Transfer Cells to Ligation Mix (2.3 mL)	30 sec
24	Mix Cells in Ligation Mix	30 sec
25-28	Transfer Ligation Mix to Basin (~4.2 mL)	1.5 min

STEPS	ACTION	DURATION
29-31	Mix Sample in Basin	30 sec

Section 1.4. Round 2 Ligation

PT S1 St4 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-4	Mix samples in basins	1.5 min
5-12	Load Sample into Round 2 Plate (40 µL/well)	8 min
13	“Remove R2 Plate for incubation” message	
14	"Replace the left basin liner" message	
15	"Load R2 plate on Deck B" message	
16	"Pipette R2 Stop in Deck A" message	
17	Volume Change	
18	Add R2 Stop (10 µL/well)	5 min
19	"Remove R2 Plate for incubation” message	
20	"Replace right basin liner" message	
21	"Load R2 Plate on Deck B" message	
22-23	Pool R2 Plate to basin (65 µL/well)	4 min

Section 1.5. Round 3 Ligation Preparation

PT S1 St5 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	"Move basin to slanted holder" message	
3	"Load cell strainer on 10 mL tube" message	
4	“Thaw R3 Plate - -” message	
5	Wash basin mix	30 sec
6	Volume change	
7-11	Strain cells/nuclei (6 mL)	2 min
12	"Move Basin Holder to Deck A" message	
13	"Remove cell strainer" message	
14	Labware change	
15	Add R3 Ligation Enzyme (20 µL)	1 min
16	Mix Ligation Enzyme with sample	1.5 min
17	Volume change	
18-22	Transfer cells/nuclei to right basin (~6.04 mL)	2 min

Section 1.6. Round 3 Ligation

PT S1 St6 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-4	Mix sample in basin	1.5 min
5-12	Add sample to Round 3 Plate (50 µL/well)	8 min
13	"Remove R3 Plate for incubation" message	

STEPS	ACTION	DURATION
14	"Replace both basin liners" message	
15	"Reload R3 Plate on Deck B" message	
16	"Pipette R3 Stop in Deck A --" message	
17	Volume change	
18	Add R3 Stop to plate (20 µL/well)	4.5 min
19	Pool R3 Plate (105 µL/well)	4.5 min

Section 1.7. Pre-Lysis

PT S1 St7 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	“Move Basin Holder to Deck B” message	
3	"Load cell strainer on 10 mL tube" message	
4	Wash basin	30 sec
5	Volume change	
6-12	Strain cells (~7.7 mL)	3.5 min
13	“Move Basin Holder to Deck A” message	
14	"Remove cell strainer - -" message	
15	Add Spin Additive (70 µL)	30 sec
16	“Remove 10mL tube from Deck C” message	
17	"Load 10 mL tube on Deck C --" message	
18	Volume change	
19-23	Remove supernatant (~8 mL)	4 min

STEPS	ACTION	DURATION
24	"Checkpoint: Supernatant Removed--" message	
25-26	Resuspend Pre-Lyse (4 mL)	2 min
27	“Remove 10mL tube from Deck C” message	
28	"Load 10 mL tube on Deck C --" message	
29	Volume change	
30-34	Remove supernatant (~4.4 mL)	2.5 min
35	“Checkpoint: Supernatant Removed - -” message	
36	"Count cells/nuclei" message	

Section 1.8. Lysis and Sublibrary Generation

PT S1 St8 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	Aliquot 25 µL of sample (25 µL/tube)	4 min
6	Lysis Mastermix	1.5 min
7	Mix Lysis Mastermix	1 min
8	Add Lysis Mastermix to sample (30 µL)	10 min
9	“Vortex and centrifuge samples” message	

Section 2.1. Reagent Plating

PT S2 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	

STEPS	ACTION	DURATION
2-3	Dispense Streptavidin Beads (120 µL/well)	1.5 min
4	Dispense Binding Buffer (120 µL/well)	1.5 min
5	Dispense Enhancer (20 µL/well)	1 min
6	"Load Bead Wash (2x) –" - message	
7-9	Dispense Bead Wash (entire volume)	2.5 min
10	"Load Wash 1 (3x) –" - message	
11-14	Dispense Wash Buffer 1 (entire volume)	3 min
15	"Load Wash 2 (3x) –" - message	
16-19	Dispense Wash Buffer 2 (entire volume)	3 min
20	"Load Wash 3 (3x) –" - message	
21-24	Dispense Wash Buffer 3 (entire volume)	3 min
25	"Proceed immediately to S2 St2–" message	

Section 2.2. cDNA Capture

PT S2 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	"Thaw Lysate After Prog Start –" message	
3-6	Remove Streptavidin Bead Supernatant (entire volume)	2 min
7-12	1st Bead Wash (122 µL/well)	7 min
13-14	Add 2nd Bead Wash (122 µL/well)	3 min
15	"Load Lysates Plate on Deck B –" message	

STEPS	ACTION	DURATION
16	Add Enhancer (2.5 µL/well)	2 min
17-20	2nd Bead Wash Removal (122 µL/well)	6 min
21-26	3rd Bead Wash (122 µL/well)	7 min
27-31	Add Binding Buffer (110 µL/well)	6 min
32	“Check strep beads for full resusp” message	
33	“Additional mixing might be req –” message	
34-35	Add Bounded Beads to Sample (50 µL/well)	6.5 min
36	“Vortex at 800-1k rpm for 30 mins –” message	
37	“Cover 8 Row Res & Plated Reagents” message	

Section 2.3. Streptavidin Beads Wash

PT S2 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-6	Supernatant Removal (entire volume)	3.5 min
7-18	1st Wash 1 (120 µL/well)	14 min
19-30	2nd Wash 1 (120 µL/well)	14 min
31-41	Wash 2 (120 µL/well)	14 min
42-45	Wash 3 (120 µL/well)	4 min
35	“Proceed immediately to S2 St4” message	

Section 2.4. Master Mixes Preparation

PT S2 St4 V3_6

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Dispense SPRI Beads (160 µL/well)	2 min
2-9	cDNA Amp Mix Prep: Enzyme: 1050 µL (2 tubes) Primer: 1050 µL (2 tubes)	5 min
10-11	Dispense cDNA Amp Mix to Strip Tubes (230 µL/well)	3 min
12	"Cap and store cDNA Amp on ice–" message	
13	"Load TS Buff, Enz, & Primer –" message	
14-22	Template Switch Mix Prep: Buffer: 1710 µL (total) (2 tubes) Enzyme: 92.4 µL (2 tubes) Primer: 42.6 µL (2 tubes)	9 min
23-24	Dispense Template Switch to Reagent Plate Column 8 & 9 (220 µL/well)	2.5 min
25	"Proceed immediately to S2 St5" message	

Section 2.5. Template Switch and cDNA Amplification

PT S2 St5 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	Remove Wash 3 Supernatant Column 1 & 3 (entire volume)	4 min

STEPS	ACTION	DURATION
6-7	Adds Template Switch Mix to Samples Column 1 & 3 (100 µL/well)	4.5 min
8-9	Remove Wash 3 Supernatant Column 2 & 4 (entire volume)	4 min
10-12	Adds Template Switch Mix to Samples Column 2 & 4 (100 µL/well)	4.5 min
13	"Remove and Seal Reagent Plate" message	
14	"Manually Mix Samples" message	
15	"Seal and incubate at RT for 30 mins" message	
16	"Run TS on therm cycler" message	
17	"Reload Samples on Heatmag" message	
18-19	"Load Parse Cold Block on Deck B" message	
20	"Load cDNA Amp Mix on Deck B–" message	
21-25	Remove Template Switch Supernatant Column 1 & 3 (entire volume)	3.5 min
26-27	Wash 3 Column 1 & 3(120 µL/well)	1 min
28-29	Remove Template Switch Supernatant Column 2 & 4 (entire volume)	3.5 min
30-31	Wash 3 Column 2 & 4(120 µL/well)	1 min
32-34	Remove Wash 3 Column 1 & 3 (entire volume)	1.5 min
35-36	Add cDNA Amp Mix to Sample Column 1 & 3 (100 µL/well)	1 min
37-38	Remove Wash 3 Column 2 & 4 (entire volume)	1.5 min
39-41	Add cDNA Amp Mix to Sample Column 2 & 4 (100 µL/well)	1 min
42	"Manually Mix Samples" message	
43	"Run cDNA Amp on the Thermocycler" message	

Section 2.6. Post-Amplification Purification

PT S2 St6 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	“Remove caps from col 6-9 -” message	
2-3	Mix Samples	1 min
4	Activate Magnet	
5-6	Mix SPRI Beads	2 min
6-10	Transfer Sample (Col 1 -4) to (Col 6-9) on Heatmag (90 µL/well)	2.5 min
11-14	Add SPRI Beads to Samples (72 µL/well)	12 min
15-19	Remove Supernatant Column 6 & 7 (entire volume)	8.5 min
20-21	1st EtOH Wash Column 6 & 7 (180 µL/well total)	1 min
22-23	Remove Supernatant Column 8 & 9 (entire volume)	3 min
24-25	1st EtOH Wash Column 8 & 9 (180 µL/well total)	1 min
26-28	Remove Supernatant Column 6 & 7 (entire volume)	8.5 min
29-30	2nd EtOH Wash Column 6 & 7 (180 µL/well total)	1 min
31-32	Remove Supernatant Column 8 & 9 (entire volume)	3 min
33-34	2nd EtOH Wash Column 8 & 9 (180 µL/well total)	1 min
35-39	Remove Supernatant Column 6 -9 (entire volume)	5 min
40-46	Add Elution Column 6 - 9 (75 µL/well)	16.5 min
47	“Load Clean Plate on Deck B-” message	
48-51	Transfer Elution to New Plate Column 1 -4 (entire volume)	3 min
52	“Samples in Plate on Deck B” message	

Section 3.1. Fragmentation Mix Creation and Plating

PT S3 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Pre-chill thermal cycler message	
3	Plate out SPRI beads (110 µL/well)	3 min
4	Create Fragmentation Mix	4 min
5	Plate out Fragmentation Mix into column 11 (66 µL/well)	2 min
6	Proceed to S3 St2 message	

Section 3.2. Fragmentation Mix Addition and Post-Fragmentation SPRI Cleanup

PT S3 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	Stamp Fragmentation Mix into samples (15 µL/well)	3 min
6-10	Deck loading messages	
11	Volume change	
12	Ensure magnet is deactivated	
13	Mix SPRI beads	4 min
14-17	Add SPRI beads to samples (30 µL/well)	4 min
18	5 minute bead incubation	5 min
19	Activate magnet	
20	2 minute bead immobilization	2 min
21-24	Transfer supernatant (75 µL/well)	2 min

STEPS	ACTION	DURATION
25	Deactivate magnet	
26-29	Add SPRI beads to samples (20 µL/well)	4 min
30	3 minute bead incubation	3 min
31	Activate magnet	
32	3 minute bead immobilization	3 min
33	Volume change	
34-37	Discard supernatant	3 min
38-45	Ethanol addition 1 (180 µL/well total)	4 min
46-71	Discard ethanol and ethanol addition 2 (180 µL/well total)	17 min
66,69,72	Add water to resuspend	1.5 min
73	Air dry beads	30 sec
74	Deactivate magnet	
75	Add water to resuspend	30 sec
76-87	Resuspend beads in water (50 µL/well)	4 min
88	3 min bead incubation	3 min
89	Activate Magnet	
90	2 minute bead immobilization	2 min
91-94	Transfer eluate (50 µL/well)	2 min
95	Deactivate magnet	

Section 3.3. Ligation Mix Creation and Plating

PT S3 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	

STEPS	ACTION	DURATION
2-3	Plate out SPRI beads (175 µL/well)	5 min
4	Create Ligation Mix	7 min
5	Slow mix to reduce volume stuck in tip	18 sec
6	Plate out Ligation Mix into column 10 (220 µL/well)	4 min
7	Proceed to S3 St4 message	

Section 3.4. Ligation Mix Addition and Post-Ligation SPRI Cleanup

PT S3 St4 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-4	Stamp Ligation Mix into samples (50 µL/well)	4 min
6-8	Deck loading messages	
9	Ensure magnet is deactivated	
10-11	Mix SPRI beads	3 min
12-15	Add SPRI beads to samples (80 µL/well)	6 min
16	3 minute bead incubation	3 min
17	Activate magnet	
14	8 minute bead immobilization	8 min
19	Volume change	
20-23	Discard supernatant	5 min
28-35	Ethanol addition 1 (180 µL/well total)	4 min
36-61	Discard ethanol and ethanol addition 2 (180µL/well total)	17 min
56,59,62	Add water to resuspend	1.5 min
63	Air dry beads	30 sec

STEPS	ACTION	DURATION
64	Add water to resuspend	30 sec
65	Deactivate magnet	
66-69	Resuspend beads in water (23 µL/well)	3 min
70	3 minute bead incubation	3 min
71	Activate magnet	
72	2 minute bead immobilization	2 min
73	Prompt to add clean plate	
74	Volume change	
75-78	Transfer eluate (21 µL/well)	2 min
79-81	Deck loading messages	
82	Deactivate magnet	

Section 3.5. Barcoding Round 4

PT S3 St5 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Plate out SPRI beads (90 µL/well)	3 min
3	Plate out Amplification Mix into column 9 (120 µL/well)	1 min
4	Add UDIs message	
5	Proceed to S3 St 6 message	

Section 3.6. Library Amp Mix Addition and Size Selection

PT S3 St6 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	Stamp Amplification Mix into samples (25 µL/well)	3 min
6-8	Deck loading messages	
9	Ensure magnet is deactivated	
10-11	Mix SPRI beads	4 min
12-15	Add SPRI beads to samples (30 µL/well)	4 min
16	3 minute bead incubation	3 min
17	Activate magnet	
18	3 minute bead immobilization	3 min
19-22	Transfer supernatant (75 µL/well)	2 min
23	Deactivate magnet	
24-27	Add SPRI beads to samples (10 µL/well)	4 min
28	3 minute bead incubation	3 min
29	Activate magnet	
30	3 minute bead immobilization	3 min
31	Volume change	
32-35	Discard supernatant	3 min
36-43	Ethanol addition 1 (180 µL/well total)	4 min
44-69	Discard ethanol and ethanol addition 2 (180 µL/well total)	17 min
64,67,70	Add water to resuspend	1.5 min
71	Air dry beads	30 sec

STEPS	ACTION	DURATION
72	Deactivate magnet	
73	Add water to resuspend	30 sec
74-85	Resuspend beads in water (20 µL/well)	30 sec
86	3 min bead incubation	4 min
87	Activate magnet	
88	2 minute bead immobilization	2 min
89-92	Transfer eluate (20 µL/well)	2 min
93	Deactivate magnet	

Appendix C: 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 D: Revision History

Version	Description	Date
1.0	Initial Release	September 2025
1.1	Section 2.4: updated scripts to increase TS Primer Volume	February 2026

Appendix E: Acknowledgements

All graphics were created with BioRender.com.

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