User Manual

Version 1.2 – UMWT3530INT

Evercode™ WT Mega 384 v3 with INTEGRA ASSIST PLUS

For use with

ECWT3530

INTEGRA ASSIST PLUS

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Table of Contents

Overview 5

Workflow 5

Important Guidelines 10

Parse Reagents 17

Parse-Provided Equipment 22

INTEGRA Components 23

Consumables and Reagents 24

Equipment 26

Reading and Understanding Visuals and Diagrams 27

Section 1: Automation Setup & In Situ Barcoding 31

1.1. Set Up for Standard Fixation Samples 31

1.2. Loading and Pooling the Round 1 Plate 38

1.3. Round 2 Ligation Preparation 48

1.4. Round 2 Ligation 57

1.5. Round 3 Ligation Preparation 63

1.6. Round 3 Ligation 69

1.7. Pre-Lysis 73

1.8. Lysis and Sublibrary Generation 81

Section 2: cDNA Capture and Amplification 85

2.1. Reagents Plating 85

2.2. cDNA Capture 91

2.3. Streptavidin Beads Wash 93

2.4. Master Mixes Preparation 95

2.5. Template Switch and cDNA Amplification 99

2.6. Post-Amplification Purification 107

2.7. cDNA Quantification 110

Section 3: Sequencing Library Preparation 111

3.0. cDNA Normalization (Optional) 111

3.1. SPRI Bead Plating 117

3.2. Fragmentation Mix Creation and Plating 120

3.3. Fragmentation Mix Addition and Post-Fragmentation SPRI Cleanup 124

3.4. Ligation Mix Creation and Plating 130

3.5. Ligation Master Mix Addition and Post-Ligation SPRI Cleanup 134

3.6. Barcoding Round 4 138

3.7. Library Amp Mix Addition and Size Selection 142

3.8. Sequencing Library Quantification 147

Appendices 148

Appendix A: Sequencing Information 148

Appendix B: Pipetting Programs 151

Appendix C: Troubleshooting 168

Appendix D: Revision History 169

Appendix E: Acknowledgements 169

Overview

Workflow

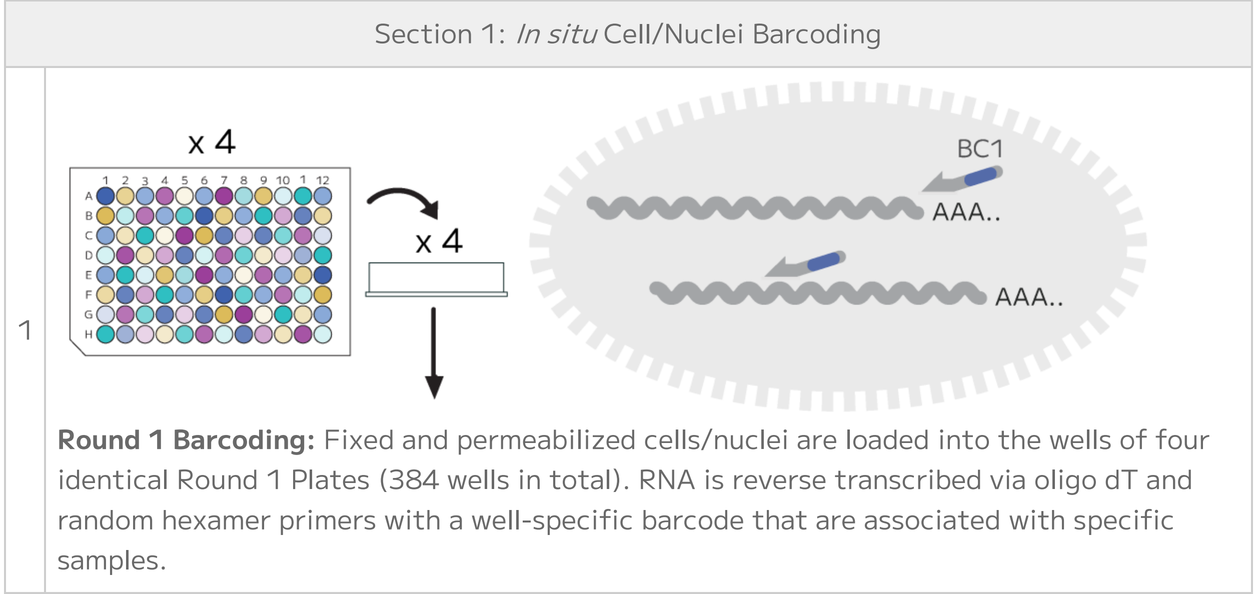
The Evercode combinatorial barcoding workflow is compatible with the INTEGRA ASSIST PLUS to enable large-scale single cell RNA-Seq experiments through a robust, semi-automated process.

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 Mega 384 v3 with INTEGRA ASSIST PLUS kit can profile up to 1,000,000 cells/nuclei across 384 different biological samples or experimental conditions.

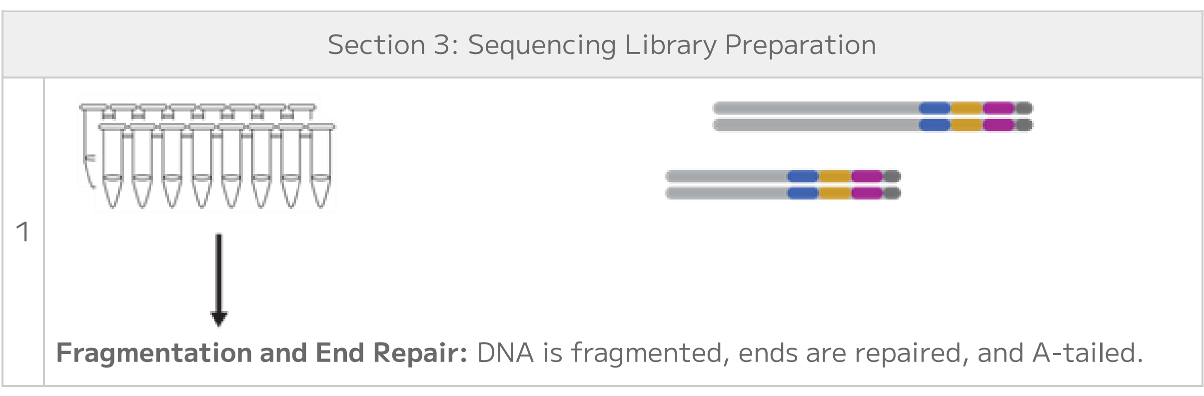
Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding can yield a very large number of possible barcode combinations which is more than sufficient to uniquely label up to 1,000,000 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.

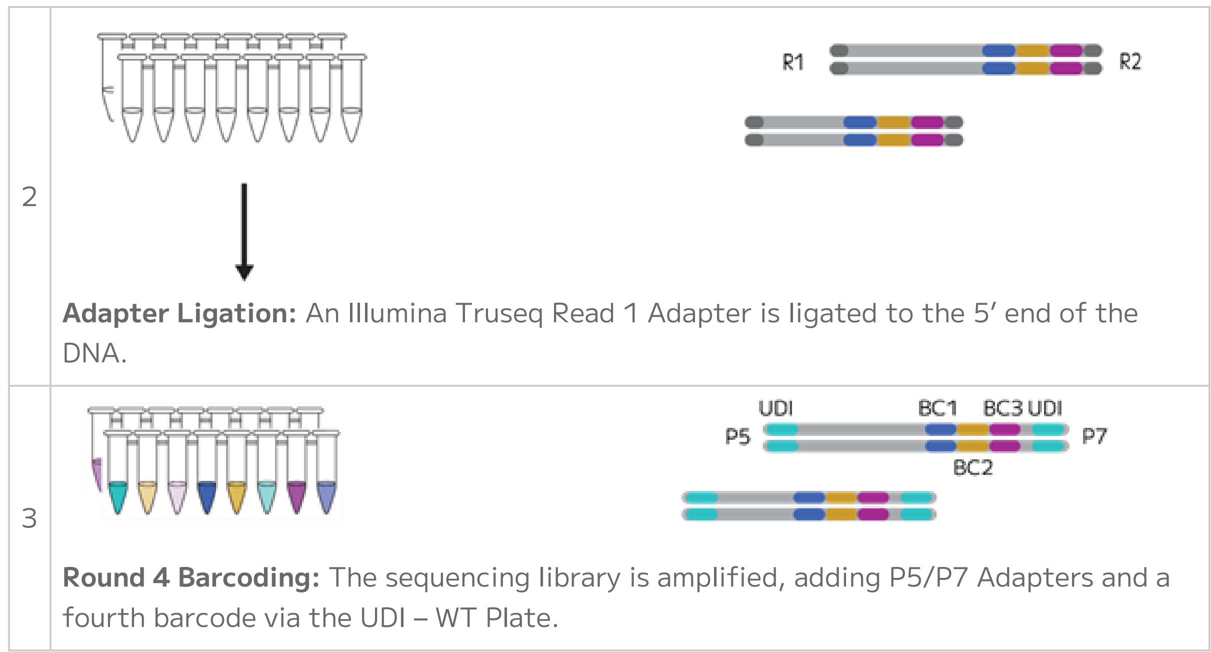
The tables below provide a high-level overview of the whole transcriptome workflow.

Section 1: *In situ* Cell/Nuclei Barcoding	
1	 **Round 1 Barcoding:** Fixed and permeabilized cells/nuclei are loaded into the wells of four identical Round 1 Plates (384 wells in total). 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 the Round 2 Plate. 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 the Round 3 Plate. A third barcode is ligated to the cDNA, which also contains an Illumina Read 2 sequence, and biotin.
4	**Lysis and Sublibrary Generation:** Cells/nuclei are split across 16 sublibraries and lysed.

Section 2: cDNA Capture and Amplification	
1	Streptavidin magnetic bead AAA... AAA... **cDNA Capture:** Biotinylated cDNA is captured via streptavidin beads.
2	TS AAA... AAA... **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 Read 1 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.

Figure 1 shows the Mega 384 workflow. This kit can barcode up to 1 million cells/nuclei across 384 samples in a single reaction. The Mega 384 utilizes four identical Round 1 Plates, each holding a unique set of 96 samples, totaling 384 samples. These samples are pooled from the four Round 1 Plates into a single Round 2 Plate. The barcodes from Round 1 and Round 2 serve as sample identifiers.

Samples from Round 2 are pooled and distributed into the Round 3 plate. Cells/nuclei are then pooled and subsequently split to generate 16 sublibraries (Figure 1). Sample plates are easily identified by labeling color-coded stickers (Figure 2).

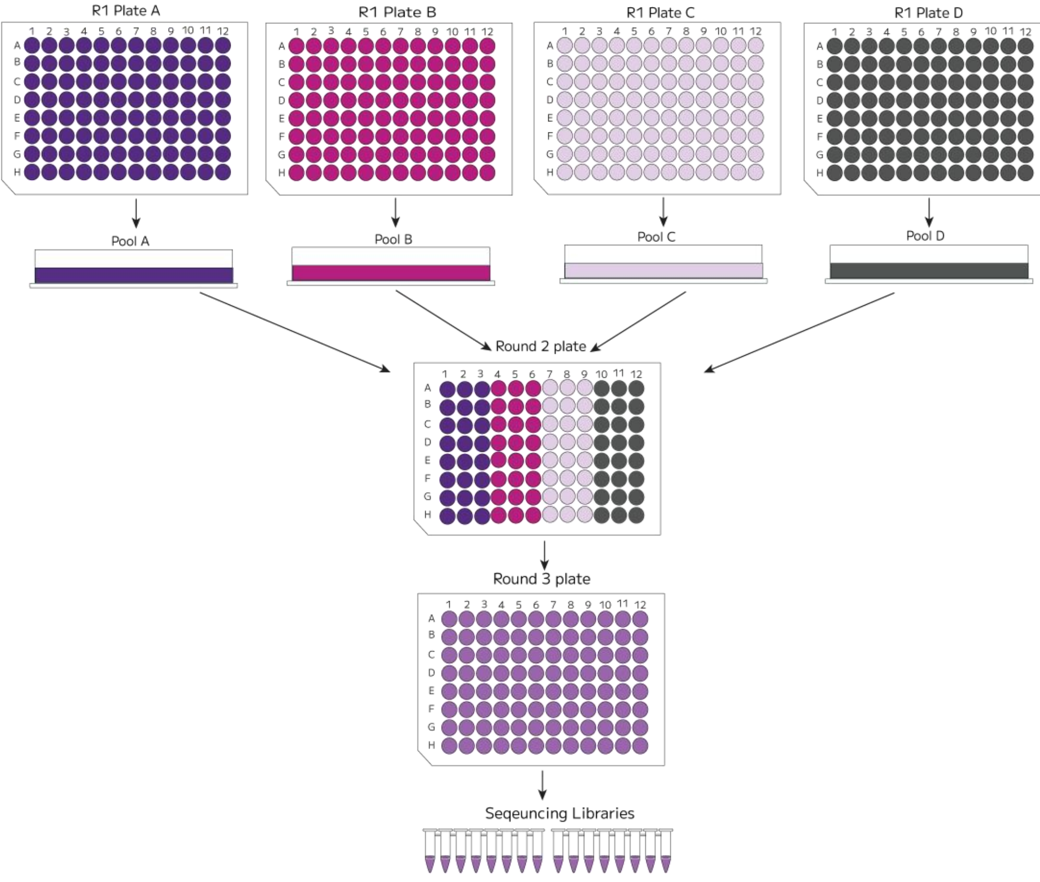


Figure 1: Evercode WT Mega 384 workflow pools 384 samples from four Round 1 Barcoding plates into one Round 2 and Round 3 Barcoding Plates respectively, generating 16 Sequencing Libraries

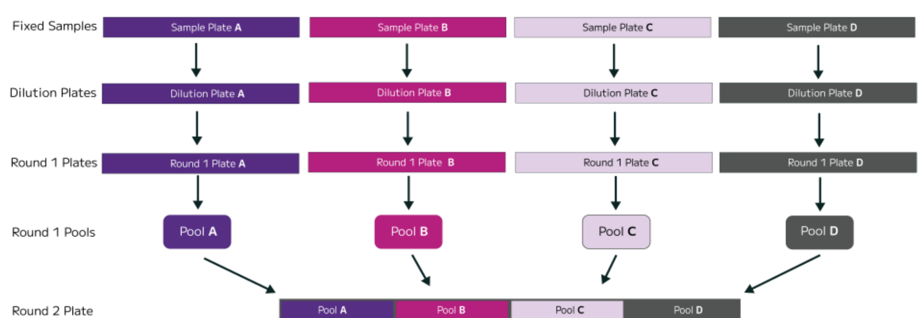


Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

This protocol is intended to be followed when performing the Evercode Mega 384 assay on the INTEGRA ASSIST PLUS liquid handler.

Important Guidelines

The following guidelines provide additional information to obtain optimal performance with the Evercode WT Mega 384 v3 with INTEGRA ASSIST PLUS barcoding workflow.

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

Sample Input

- This protocol begins with cells or nuclei previously fixed with an Evercode Cell Fixation v3 or Evercode Nuclei Fixation v3 kit.

- Samples fixed with the Standard Fixation and the Low Input Fixation kits are fully compatible with the INTEGRA automated barcoding workflow. Samples fixed with the Low Input Fixation Workflow can be uploaded after cell/nuclei capture.

- Even if samples were counted before freezing, we strongly recommend counting cells/nuclei again after thawing to account for any changes in cell/nuclei concentration during storage and freeze/thaw. Typically, a 5-15% decrease in concentration after thawing should be expected. These counts are used to determine how cells/nuclei are loaded in the Round 1 Plate, and counting accuracy at this stage is critical to recover the desired number of cells/nuclei following barcoding.

- When planning on processing many fixed samples, we recommend setting aside a small "counting aliquot" of each sample at the end of fixation. These counting aliquots can be counted the day prior to starting Section 1 of any Evercode assay kit. The Evercode Fixation User Guides outline recommendations for generating aliquots. Because aliquots have undergone a similar storage time and a freeze/thaw, cell/nuclei counts from these aliquots will be more representative than using counts from immediately after fixation.

- Aliquots should be thawed in a water bath set to 37°C in sets of 2-4 and counted with a hemocytometer or alternative counting device. Cell/nuclei counts should be recorded in the Sample Loading Table, and any remaining cell/nuclei material in the thawed counting aliquot should be discarded.

- Once fixed samples have been thawed, they should not be refrozen.

- Samples need to be diluted into a dilution plate prior to Round 1 Barcoding. The Sample Loading Table will recommend the appropriate volume of dilution buffer needed to achieve the correct cell concentration in the required volume. If the required "Number of Sample Dilution Tubes” is greater than 1, three additional dilution buffer tubes for highly concentrated samples can be purchased as a Sample Dilution Accessory Kit (PN

ECAC3901). This kit is included in the initial INTEGRA bundle, but will need to be separately purchased for subsequent orders.

Cell/Nuclei Counting and Quality Assessment

- We recommend a hemocytometer for counting, but alternative counting devices can also be used. If possible, validate counts from alternative devices against a hemocytometer when using Evercode kits for the first time.
- We suggest saving images of cells/nuclei at each counting step. A 20x and 40x magnification provide the most useful information.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- Examples of 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.

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 Mega 384 INTEGRA Sample Loading Table being used is the most up-to-date version. The Sample Loading Table can be downloaded from the Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.
- The Parse Biosciences Evercode WT Mega 384 INTEGRA Sample Loading Table v2.1.xlsm (Excel spreadsheet) 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 Mega 384 v3 kit requires 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.

- 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. Do not use SureFlo basins, as their microwell design—intended to minimize dead volume—can unintentionally trap cells or nuclei, resulting in poor retention. Validated basins are listed in the Consumables and Reagents section.

Sample Concentrations

- For an Evercode WT Mega 384 kit, samples can be pre-diluted with the Sample Dilution Buffer. The input sample concentration should NOT be lower than 651 cells/nuclei per µL. If the required Sample Dilution volumes are too high, sample may need to be serially diluted.

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

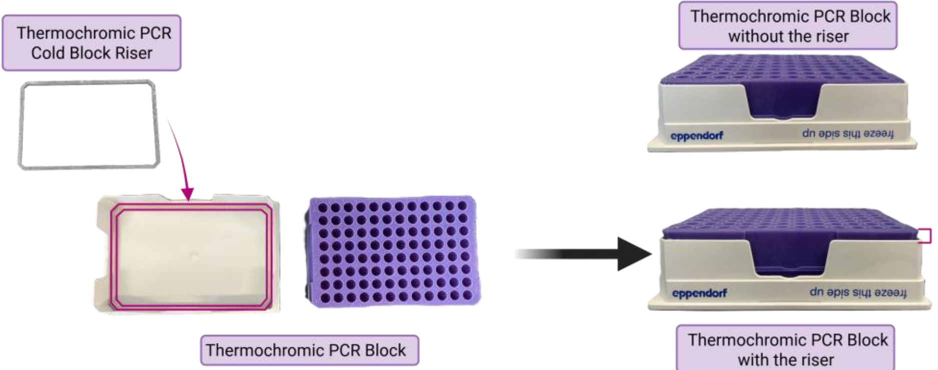
- For each sample we recommend preparing a 20 µL post-fixation counting aliquot and two 30-50 µL post-fixation sample aliquots. Prior to aliquoting, the samples should be thoroughly resuspended. The counting aliquot may be thawed in advance of the barcoding day to evaluate post-thaw cell recovery. The cell/nuclei loss observed in this aliquot is expected to closely reflect that of the sample aliquots. Concentrations measured from the counting aliquot can then be used to update the Sample Loading Table accordingly.

- If the sample stock concentrations are too high the Sample Loading Table may indicate that more than 1 tube of Sample Dilution Buffer is needed. Additional Sample Dilution

Buffer can be purchased in the Dilution Accessory Box (Parse PN ECAC3901) before starting the barcoding workflow.

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.

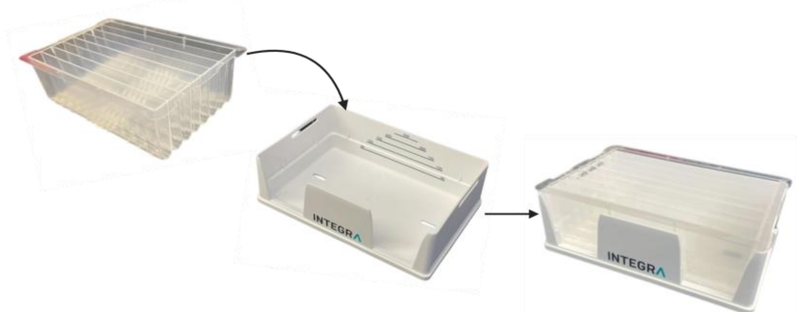


Note: Uneven freezing of the liquid inside the Thermochromic PCR Block can cause slanting of the cooling position. The Thermochromic PCR Cold Block Riser reduces the variability caused by the freezing process. If the PCR Cold Block riser is not used, there will be extra liquid left in the wells of the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

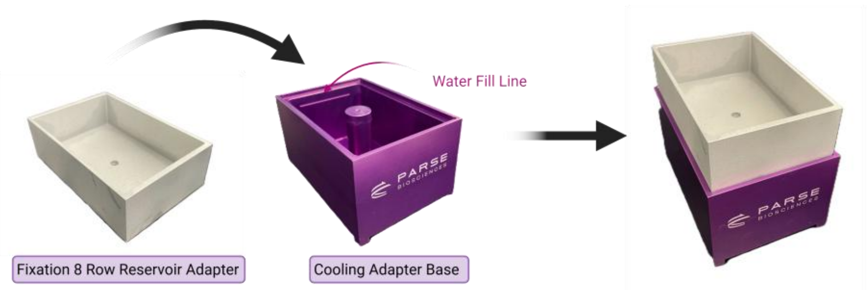
Bases and Reservoirs

- 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.

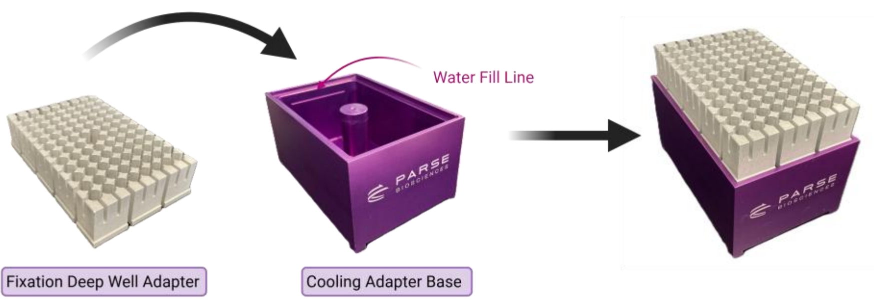


Cooling Adapter Bases

- 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 Biosciences Evercode WT Mega 384 INTEGRA Sample Loading Table v2.1.xlsm ("Mega384DilutionWorksheetPlate**AB**_YYYYMMDD.csv and Mega384DilutionWorksheetPlate**CD**_YYYYMMDD.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 Mega 384 v3 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store at -20°C, MGT100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	MG101	Green semi-skirted 96 well plate	4
	Round 2 Plate	MG102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
Resuspen Buf	Resuspension Buffer	MG131	5 mL tube	1
Sample Dil	Sample Dilution Buffer	MG132	5 mL tube	4
R2 Lig Buf	Round 2 Ligation Buffer	MG133	5 mL tube	1
R2 Lig Enzy	Round 2 Ligation Enzyme	MG107	1.5 mL tube	1
R2 Stop	Round 2 Stop Buffer	MG108	2 mL tube	1
R3 Stop	Round 3 Stop Buffer	MG109	5 mL tube	1
Pre-Lysis Wash	Pre-Lysis Wash Buffer	MG110	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
R3 Lig Enzy	Round 3 Ligation Enzyme	MG111	1.5 mL tube	1
Pre-Lysis Dil	Pre-Lysis Dilution Buffer	MG112	2 mL tube	1
Lysis Enzy	Lysis Enzyme	MG113	1.5 mL tube	1
Bead Wash	Bead Wash Buffer	MG114	5 mL tube	1
Wash 1	Wash Buffer 1	MG115	5 mL tube	1
Wash 2	Wash Buffer 2	MG116	5 mL tube	1
Enhancer	Capture Enhancer	MG117	1.5 mL tube	1
Binding Buf	Binding Buffer	MG118	1.5 mL tube	1
Wash 3	Wash Buffer 3	MG119	5 mL tube	1
TS Buffer	Template Switch Buffer	MG120	2 mL tube	1
TS Enzyme	Template Switch Enzyme	MG121	1.5 mL tube	1
TS Primer	Template Switch Primer	MG122	1.5 mL tube	1
cDNA Amp Mix	cDNA Amp Mix	MG123	1.5 mL tube	1
cDNA Amp Primers	cDNA Amp Primers	MG124	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
Fragm/End Prep Buf	Fragm/End Prep Buffer	MG125	1.5.mL tube	1
Frag/End Prep Enzy	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1
Lig Adapter	Ligation Adapter	MG127	1.5 mL tube	1
Adapt Lig Buffer	Adapter Ligation Buffer	MG128	1.5 mL tube	1
Adapt Lig Enzy	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
Library Amp Mix	Library Amp Mix	MG130	1.5 mL tube	1

4°C Reagents and Stickers. Store 4°C MGT200

LABEL	ITEM	PN	FORMAT	QTY
Spin Add	Spin Additive	MG204	1.5 mL tube	1
Lysis Buffer	Lysis Buffer	MG202	1.5 mL tube	1
Strep Beads	Streptavidin Beads	MG203	1.5 mL tube	1

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

LABEL	ITEM	PN	FORMAT	QTY
Sample Dil	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.

The Evercode WT Mega 384 v3 kit requires the following box. Store at -20°C. UDI Plate - WT box is included in the INTEGRA bundles, but will need to be separately purchased if necessary for subsequent orders.

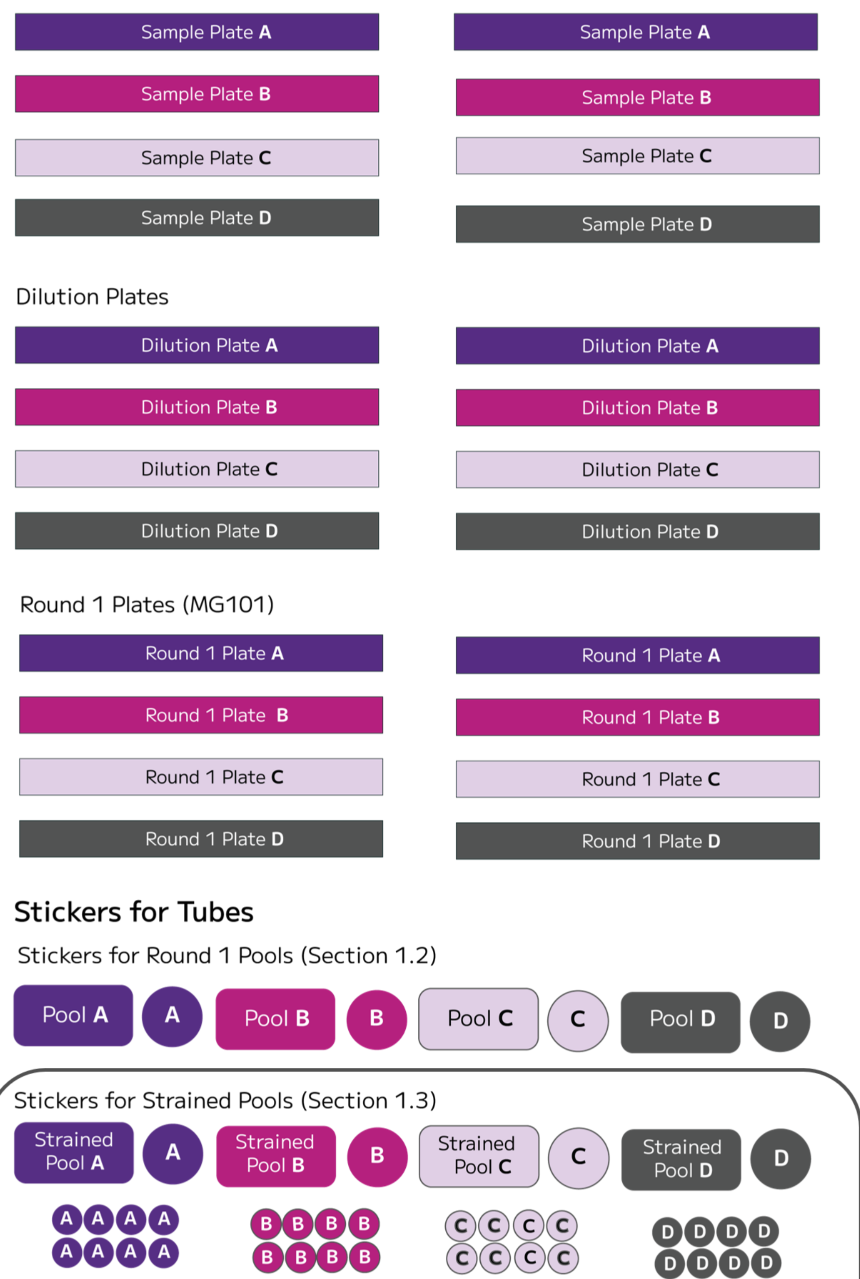
ITEM	SUPPLIER	PN	NOTES
UDI Plate - WT	Parse Biosciences	UDI1001	Each 96 well plate contains 48 unique single-use reactions sufficient for multiple Evercode kits (48 sublibraries). UDI Plate - WT can be purchased separately or bundled with Evercode kits.

4°C Reagents and Stickers. Store at room temperature, MGT200

LABEL	ITEM	PN	FORMAT	QTY
N/A	Sticker Labels	N/A	Sticker Sheet	1

To avoid confusion while handling the plates, specifically labeled color-coded stickers are provided with the kit. When working with four fixed sample plates, we recommend labeling each as Fixation Plate A, Fixation Plate B, Fixation Plate C, and Fixation Plate D respectively.

For subsequent plates, use the corresponding stickers (e.g., Sample Plate A, Dilution Plate A, Round 1 Plate A, Pool A). Below is a visual representation of the stickers.



Note: The stickers for strained pools in section 1.3 (boxed portion of the figure) will not be used in the automated workflow.

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	6
Thermochromic PCR Cold Block Riser	NTAC1103	6
Parse Metal Cold Block	NTAC1107	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
Dual Reservoir Adapter (ANSI/SLAS footprint)	Adapter	4547	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
25 mL Basin Reservoir Liner	4316	7
8 Row Polypropylene Reservoir	6374*	2
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 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	5
10 mL Transport Tube	GlobeScientific™	6102S	2
1.5 mL Microtube	Genesee Scientific	21-257	7
PCR Strip Tubes	USA Scientific®	1402-4700	8
2 mL Microtubes	Genesee Scientific	21-255	4
Semi-skirted 96 well plates	Eppendorf®	E951020362	8
5mL Screw cap Tubes	Genesee Scientific	21-398	8
SealPlate®	Excel Scientific	100-SEAL-PLT	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.
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 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.
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.

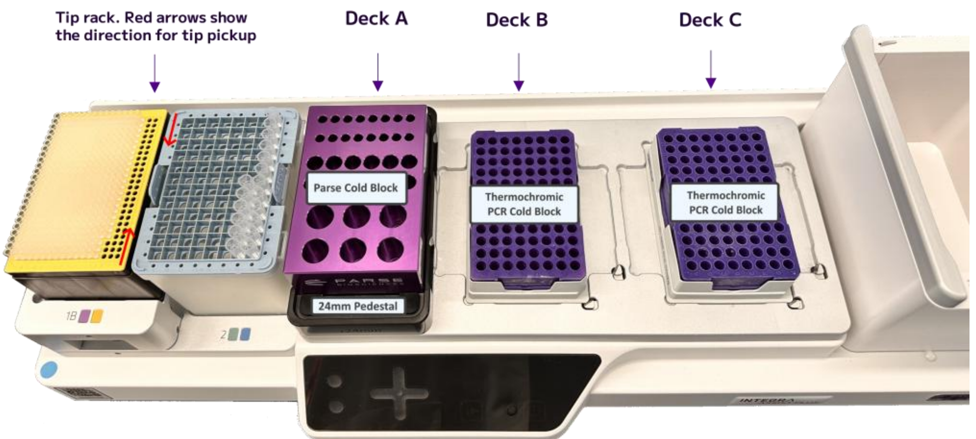
There are three types of figures in this manual.

- 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

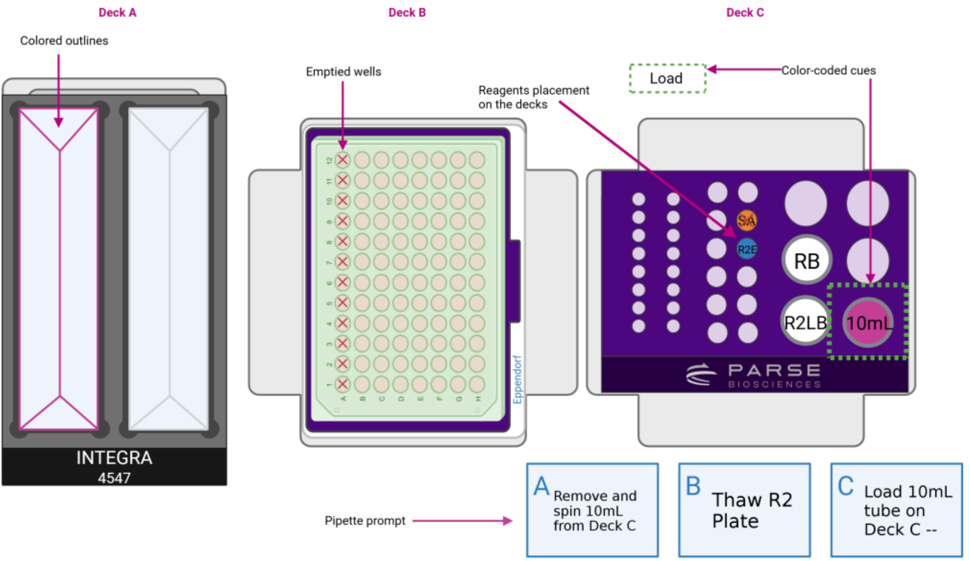
Each subsection includes multiple deck configurations that illustrate the steps executed by the program. These configurations display the exact placement of reagents and samples on the

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.

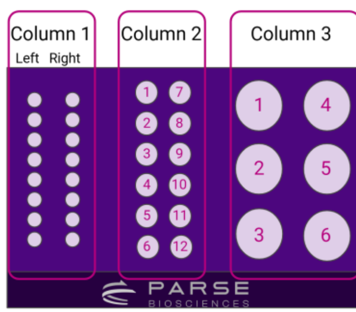
Letter-coded pipette prompts indicate the specific action the robot is about to perform when initiated. A double dash ("--") at the end of a prompt signifies that all prompts have been entered, and the robot will begin executing the programmed movements immediately after.

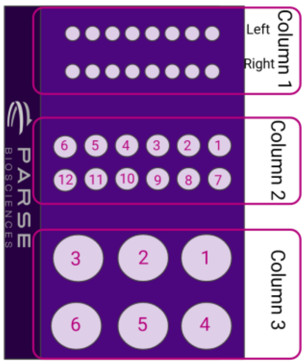


The Parse Metal Cold Block

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.

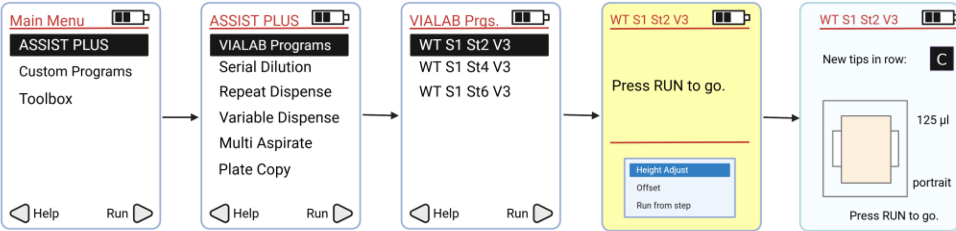
- **Load**: Indicates labware and/or reagents that need to be placed or reloaded onto the deck.
- **Remove**: Identifies labware that should be taken off the deck, either for incubation, disposal, or because it is not needed in the upcoming step.
- **Replace**: Highlights labware that has been used and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

Prior to barcoding, users are expected to count cells or nuclei using a counting plate in order to ascertain the concentration of all 384 samples. This will allow for using variable Sample Dilution Buffer volumes to dilute with a constant volume of fixed samples. This will streamline the loading process into the Round 1 plate and minimize dedicated processing time. In the following protocol, we will designate the four plates of fixed samples as A, B, C and D. Appropriate dilutions, loading concentrations, and loading positions are determined by the Sample Loading Table.

The dilution plates can be made in advance, sealed with the aluminum plate seal that can withstand storage at -20°C, and frozen at -20°C prior to barcoding.

Prepare prior to set up

1. Prepare a hemocytometer, flow cytometer, or other cell counting device.

2. Using a counting plate aliquot of fixated samples, count the number of cells/nuclei in the sample with an automated cell counter or alternative cell counting device. Record the cell/nuclei count.

Note: Do NOT thaw samples until prompted in Section 1.2.

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

<table>
<tr><th>ITEM</th><th>SOURCE</th><th>QTY</th><th>HANDLING AND STORAGE</th></tr>
<tr><td>D-ONE Pipetting Module 1-Ch, 5-1250 µL</td><td>INTEGRA Component</td><td>1</td><td></td></tr>
<tr><td>Tip Deck for D-ONE Pipetting Module</td><td>INTEGRA Component</td><td>1</td><td></td></tr>
<tr><td>125 µL Tip Rack</td><td>INTEGRA</td><td>1</td><td></td></tr>
<tr><td>1250 µL Tip Rack</td><td>INTEGRA</td><td>1</td><td></td></tr>
<tr><td>Thermochromic PCR Cold Block</td><td>Parse</td><td>2</td><td rowspan="2">Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.</td></tr>
<tr><td>Thermochromic PCR Cold Block Riser</td><td>Parse</td><td>2</td></tr>
</table>

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate Sample Dilution Buffer	Consumables	4	
○ Sample Dilution Buffer	-20°C Reagents	4	Thaw at room temperature then store on ice. Mix by inverting 3x.
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

4. Label four (user-provided) PCR Semi-skirted plates uniquely with stickers "Dilution Plate - [A-D]" provided in the 4°C Reagents and Stickers box (figure 3).

Figure 3: Image of sticker placement on a Semi-Skirted 96 Well PCR Plate. Place one sticker on the left side of the plate, adjacent to column 1, and the other sticker along the bottom edge, next to row H. This reduces contact with the plate seal.

5. Fill out the "Parse Biosciences Evercode WT Mega 384 INTEGRA Sample Loading Table v2.1.xlsm" (Excel spreadsheet) with cell counts and sample names.

6. Navigate to the “INTEGRA Loading Table” tab and check that the Minimum Sample Diluent Needed (µL) does not exceed 20,000 µL. (figure 4).

Min Diluent Needed (uL)	Required Number of Sample Dilution Tubes	Sample Dilution Tube Locations
8955.5	2	

Sample Loading Table | Integra Loading Table | Diluent Volumes | R1 Plates Config

Figure 4: INTEGRA Loading Table tab noting Minimum Diluent Needed and Sample Locations.

Note: If the required number of sample dilution tubes exceeds 4 or surpasses the available supply, the samples must be further diluted manually using serial dilutions to minimize the total volume of Sample Dilution Buffer.

7. 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 "Mega384DilutionWorksheet_”) for later use (Figure 5). There will be two worklists.

    a. The first worklist CSV file (Mega384DilutionWorksheetPlate**AB**_YYYYMMDD) will create the variable dilution plate for Sample plates A and B.

    b. The second worklist CSV file (Mega384DilutionWorksheetPlate**CD**_YYYYMMDD) will create the variable dilution plate for Sample plates C and D.

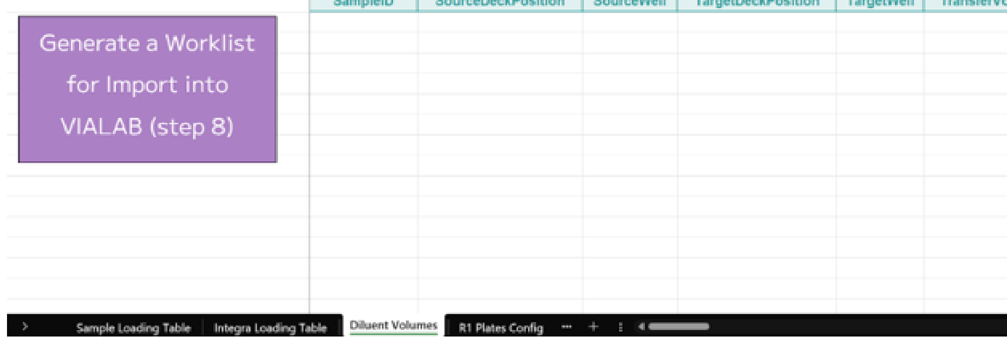


Figure 5: Diluent Volumes tab for generating a VIALAB worklist.

CRITICAL! These two CSV files are NOT interchangeable. Ensure that the nomenclature matches with the dilution plates being created.

8. Open the VIALAB program **MG4 S1 St1 V3_5 DONE** and navigate to the "Method" section.

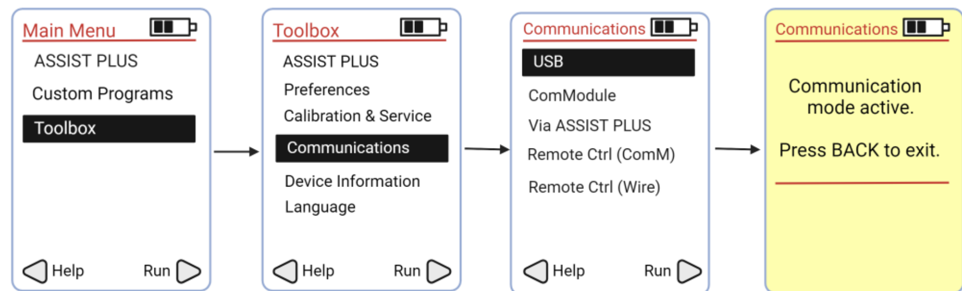
9. In the "02 Worklist", under the "Worklist and Volumes" tab, upload the "Mega384DilutionWorksheetPlate**AB**_YYYYMMDD.CSV" worklist file generated in Step 8 using the "Import" button (Figure 6).

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

10. Save and transfer your program to your D-ONE Pipetting Module (1-Ch, 5-1250 µL) as follows:

    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:

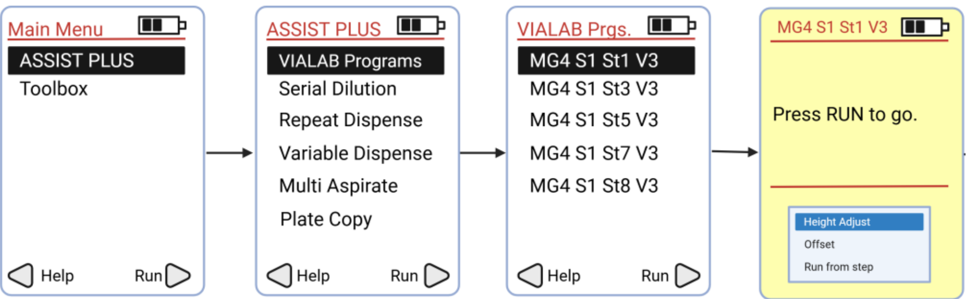


    c. In the VIALAB on your computer, select "Transfer".

    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 **MG4 S1 St1 V3_5 DONE** 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 **MG4 S1 St1 V3_5 DONE** will be found on your pipette as shown in the diagram below.



11. Remove two Thermochromic PCR Cold Blocks with Thermochromic PCR Cold Block Risers from -20°C and thaw at room temperature for 10 minutes. Ensure that the colored Thermochromic PCR Cold Block has the gray Thermochromic PCR Cold Block Riser.

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

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

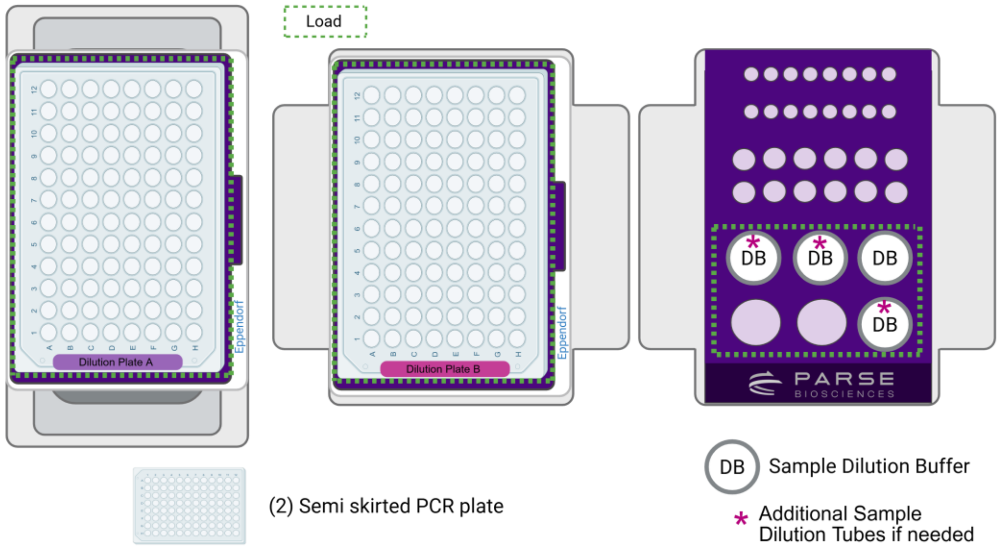
    a. Deck A: a clean semi-skirted plate on Deck A with A1 corner in the bottom left labeled “Dilution Plate A”

    b. Deck B: a clean semi-skirted plate on Deck B with A1 corner in the bottom left labeled “Dilution Plate B”

    c. Deck C, column 3

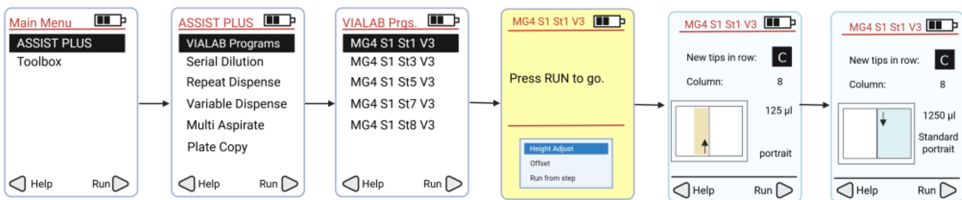
i. Pos 1 - 4: ○ Sample Dilution Buffer.

Note: Use the "Integra Loading Table" tab to determine the location of the dilution buffer tubes on the Parse Metal Cold Block especially if there is more than one Sample Dilution Tube required (figure 4).



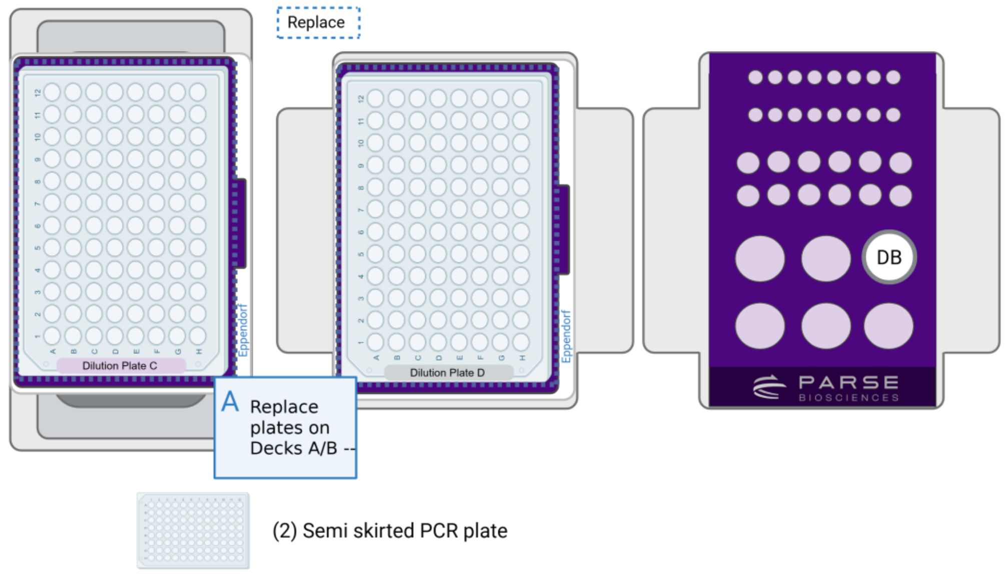
14. Attach D-ONE Pipetting Module 1-Ch, 5-1250 µL and the corresponding Tip Deck.

   a. Remove the reagent caps, then select and run the program **MG4 S1 St1 V3_5 DONE**. Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.



15. When prompted, seal and move the dilution plates from Decks A and B on ice. Replace them with the new semi skirted plates for Dilution plates C and D.

Note: Dilution plates can be made in advance, sealed with the aluminum plate seal that can withstand storage at -20°C, and frozen at -20°C prior to barcoding.



16. Repeat steps 8-14 with the second worklist file created in step 7 (Mega384DilutionWorksheetPlateCD_YYYYMMDD) for Plates C & D.

Note: The dilution buffer tubes should NOT be moved.

17. At the conclusion of the run:
    a. Seal Dilution Plates C & D and store on ice.
    b. Remove the Thermochromic PCR Cold Blocks located on Decks A and B and store upside down in the freezer.
    c. Discard the used Sample Dilution Tubes on Deck C. Remove the Parse Metal Cold Block and store on ice for future use.
    d. Remove three Thermochromic PCR Cold Blocks with Thermochromic PCR Cold Block Risers from -20°C and thaw at room temperature for 10 minutes. Proceed to the next step during the thawing process.

Safe Stopping Point: Dilution plates can be made in advance, sealed with the aluminum plate seal that can withstand storage at -20°C, and frozen at -20°C prior to barcoding.

1.2. Loading and Pooling the Round 1 Plate

This program aliquots a fixed sample amount into the respective dilution plate to normalize the samples. Then the normalized samples are loaded into the Round 1 plate. This process is done for Round 1 Plates A, B, C, and D. Once the Round 1 Plate D finishes incubating, all the Round 1 Plates are pooled in different columns of an 8 Row Reservoir. To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D. If four thermocyclers are available, they can be operated simultaneously.

1. Cool a centrifuge with swinging bucket rotors to 4°C.

2. Fill a bucket with ice.

3. If available, have four thermocyclers on standby.

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

<table>
<tr><th>ITEM</th><th>SOURCE</th><th>QTY</th><th>HANDLING AND STORAGE</th></tr>
<tr><td>VIAFLO Pipette 12-Ch, 5-125 µL</td><td></td><td>1</td><td></td></tr>
<tr><td>Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL</td><td></td><td>1</td><td></td></tr>
<tr><td>5-125 µL Tip Rack</td><td></td><td>1</td><td></td></tr>
<tr><td>8 Row Reservoir</td><td></td><td>1</td><td></td></tr>
<tr><td>Thermochromic PCR Cold Block</td><td></td><td>3</td><td rowspan="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.</td></tr>
<tr><td>Thermochromic PCR Cold Block Riser</td><td></td><td>3</td></tr>
<tr><td>Round 1 Plate</td><td>-20°C Reagents</td><td>4</td><td>Place directly on ice.</td></tr>
<tr><td>Sticker Sheet</td><td>4°C Reagents and Stickers</td><td>1</td><td>Remove sheet and follow instructions for plate and tube labeling.</td></tr>
</table>

5. Remove three Thermochromic PCR Cold Blocks with Thermochromic PCR Cold Block Risers from -20°C and thaw at room temperature for **10 minutes**. Ensure that the colored Thermochromic PCR Cold Block has the gray Thermochromic PCR Cold Block Riser.

6. If the Dilution Plates are stored at -20°C, thaw the four pre-prepared Dilution Plates in the thermocyclers according to the following protocol. Then gently remove the Dilution Plates from the thermocyclers, place in PCR plate racks, and centrifuge for **1 minute** at 100 x g at 4°C.

THAW DILUTION PLATES (4)		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	100 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

7. Place the Dilution Plates on ice, and label with corresponding adhesive labels, if not done previously.

8. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

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

9. Gently remove the Round 1 Plates from the thermocyclers, place in PCR plate racks, and centrifuge for **1 minute** at 100 x g at 4°C.

10. Remove the Round 1 Plates from the centrifuge and store on ice.

11. Label Round 1 Plates with a sticker “Round 1 - Plate _” sticker provided, taking care that each plate is associated with a unique letter from A-D.

Note: Each Round 1 Plate is identical until samples are loaded. Ensure that traceability is ensured using the provided adhesive labels.

12. From the -80°C storage remove the sample plate that is associated with Plate A loading in the Sample Loading Table and thaw it according to the following protocol.

THAW CELLS/NUCLEI		
Run Time	Lid Temperature	Sample Volume
3 min	40°C	26 µL
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

13. Gently remove the sample plate from the thermocycler and place on ice. Label sample plate with “Sample - Plate A” from the provided stickers. If the sample plate has more than 50 µL of volume, manually mix all the wells using new pipette tips for each well to ensure proper mixing.

Note: At this point, there will be 9 plates on ice: four Dilution Plates, four Round 1 Plates, and the Sample Plate A. Be sure to have each plate labeled for traceability using the provided sticker sheet. To avoid confusion, when not in use, Dilution Plates and Round 1 Plates can be stored in the fridge.

14. Place three Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser that was thawed during the previous step on all decks following the configuration below.

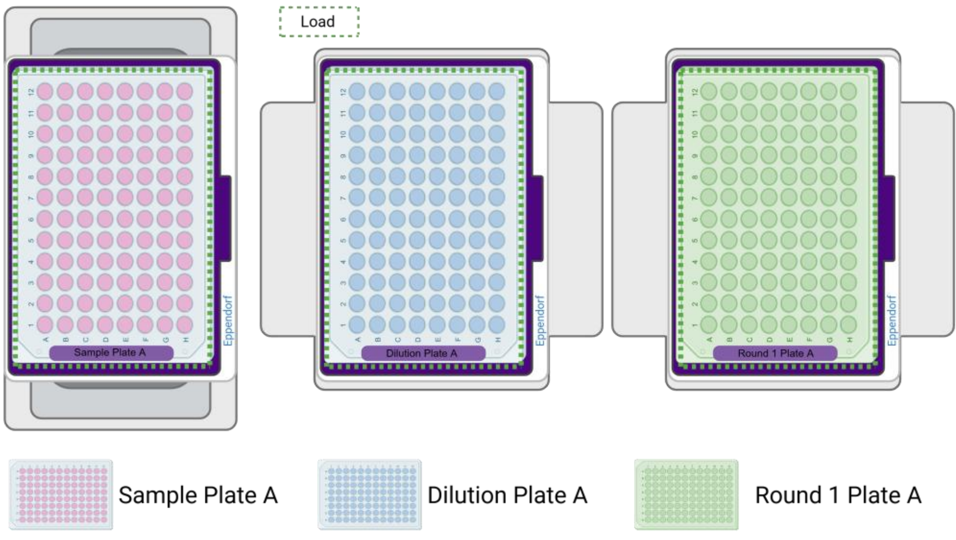
15. While secured in a PCR plate rack on a flat surface, remove the plate seal from the Sample plate, Dilution plate, and Round 1 plate.

16. Load all of the items associated with Plate A on ASSIST PLUS:

    a. Deck A: Sample Plate A

    b. Deck B: Dilution Plate A

    c. Deck C: Round 1 Plate A



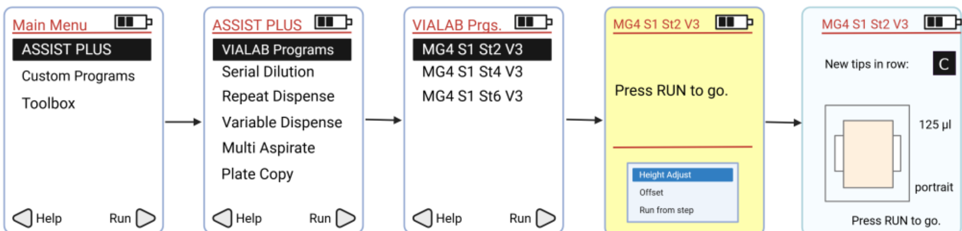
17. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    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.

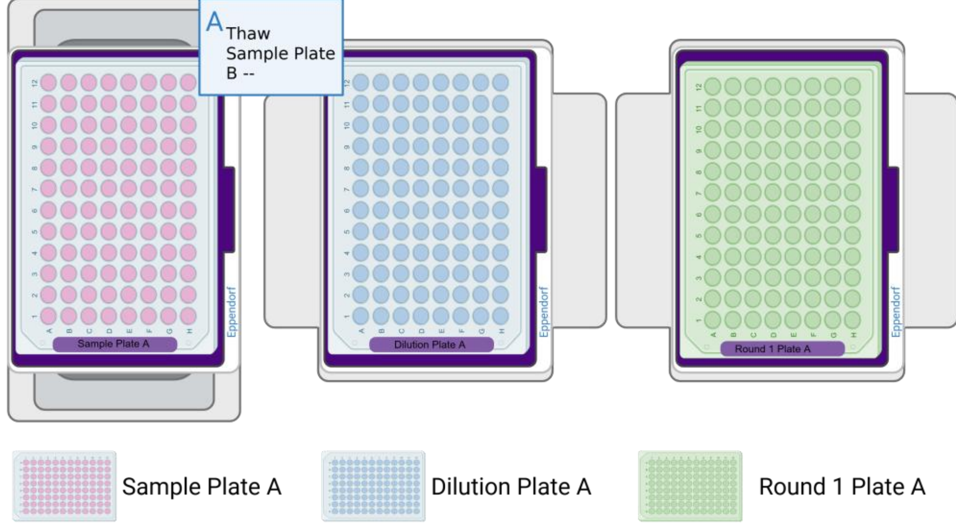
    b. Replace it with VIAFLO 12-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure the Tip Deck and tip box are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

Note: 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.

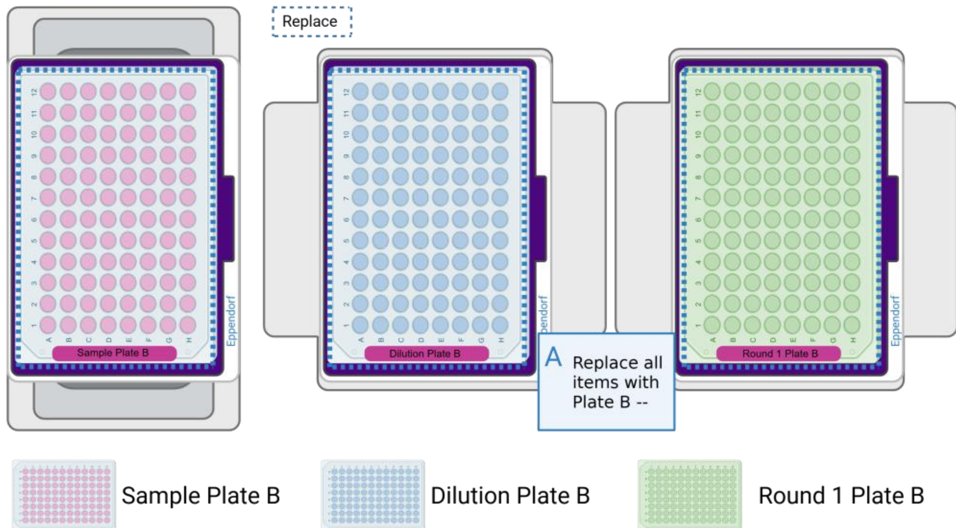
18. On the VIAFLO Pipette 12-Ch, select and run the program **MG4 S1 St2 V3_5** following the diagram below.



19. While the program is running, thaw and label Sample plate B. Press "Run" to continue the program.



20. Follow the prompt instructions and do the following:



a. Replace Sample Plate A with Sample Plate B on Deck A. Sample Plate A can be discarded.

b. Replace Dilution Plate A with Dilution Plate B on Deck B. Dilution plate A can be discarded.

c. Replace Round 1 Plate A with Round 1 Plate B on Deck C. Run the Barcoding Round 1 thermocycler program for Round 1 Plate A.

BARCODING ROUND 1			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 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	Hold

21. After placing Round 1 plate A in the thermocycler, press "Run" to continue the program.

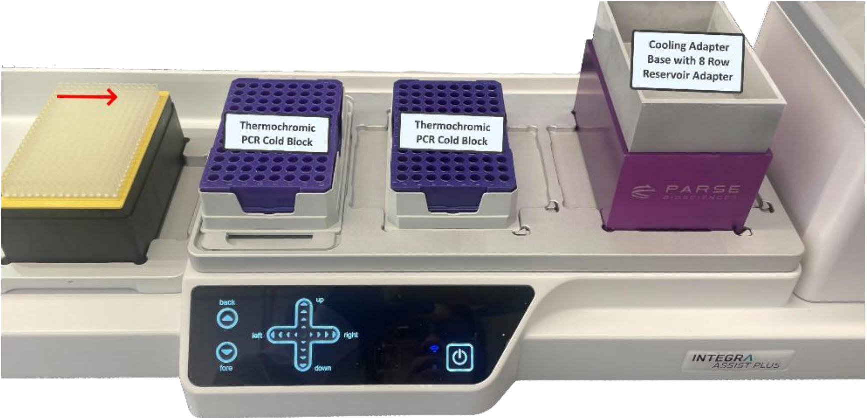
22. Repeat steps 19-21 to thaw the sample plate, replace the plates on Deck, remove the seals, and run the Barcoding Round 1 thermocycling program for sample plate sets B, C, and D respectively.

23. After placing Round 1 Plate D into the thermocycler and when prompted for a labware change:

    a. Remove the Thermochromic PCR Cold Block from Deck C.

    b. Fill the Cooling Adapter Base with pebble ice and place one 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.

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



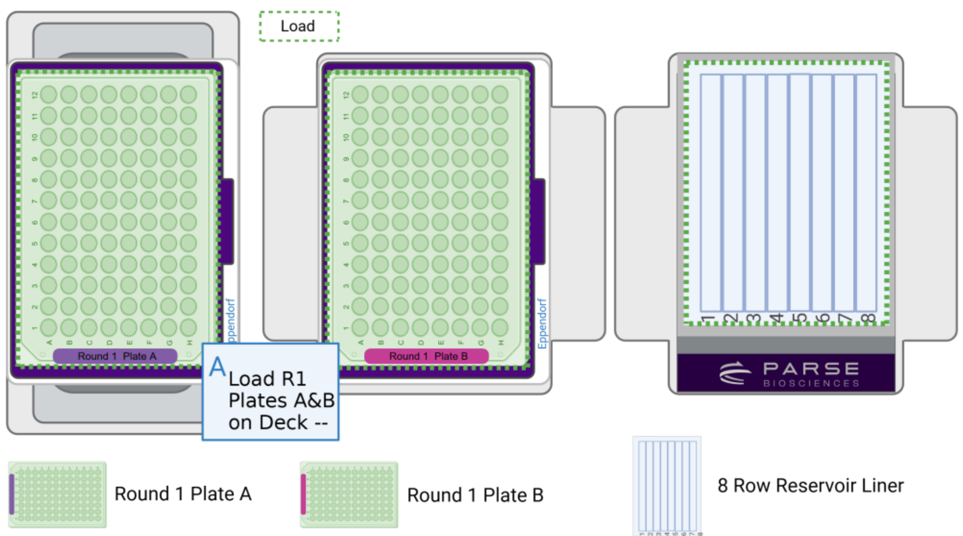
24. At the conclusion of the Barcoding Round 1 Thermocycling program for **Plate D**, load on deck:

a. Deck A: Round 1 Plate A on the thermochromic PCR cold block with A1 at the bottom left.

b. Deck B: Round 1 Plate B on the thermochromic PCR cold block with A1 at the bottom left.

c. Deck C: Place a clean 8 Row Reservoir on the Fixation 8 Row Reservoir Adapter on Deck C with row 1 facing the left side.

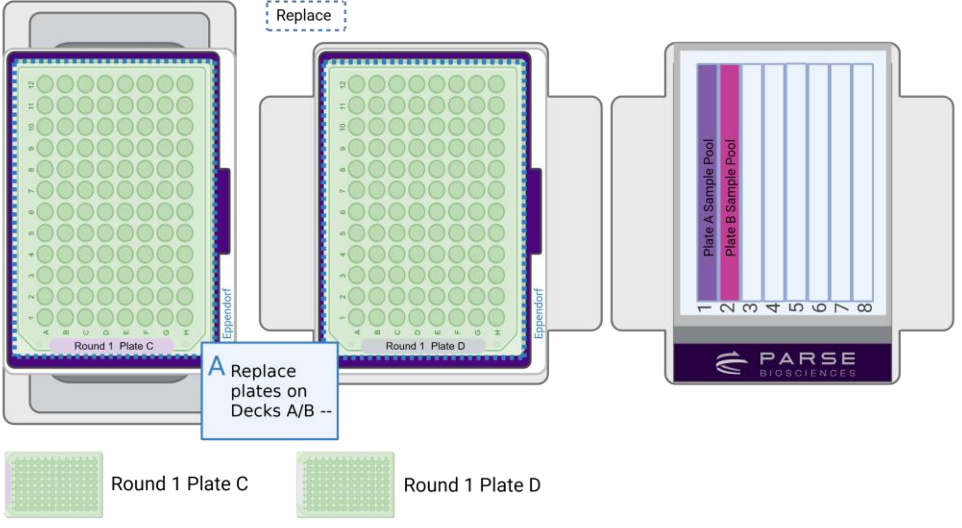
d. Press "Run" to continue the program.



25. When prompted:

a. Replace Round 1 Plate **A** with Round 1 Plate **C** on the thermochromic PCR cold block on Deck A with A1 at the bottom left

b. Replace Round 1 Plate **B** with Round 1 Plate **D** on the thermochromic PCR cold block on Deck B with A1 at the bottom left.



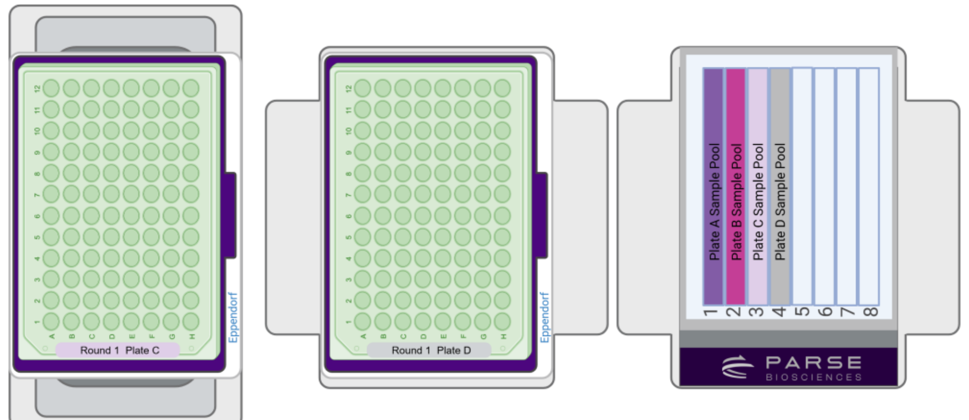
26. At the conclusion of the run:

    a. Discard the empty Round 1 plates on Decks A and B.

    b. Remove the Thermochromic PCR Cold Blocks with the Thermochromic PCR Cold Block Risers from the deck and store them in the freezer

    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 **as it contains the samples**.

CRITICAL! The 8 row reservoir will be used for the next step but needs to stay cold until the next step is started.



Round 1 Plate C
Eppendorf
Round 1 Plate D
Eppendorf
Plate A Sample Pool
Plate B Sample Pool
Plate C Sample Pool
Plate D Sample Pool
1
2
3
4
5
6
7
8
PARSE
BIOSCIENCES

1.3. Round 2 Ligation Preparation

This program transfers the pooled samples from Section 1.2 into 5 mL tubes to be centrifuged. While the samples are being centrifuged, the Round 2 Ligation Master Mix is created in a column 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 6 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	
Thermochromic PCR Cold Block	Parse-Provided	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-Provided	1	
ASSIST PLUS Slanted Plate Holder	INTEGRA Component	1	
Parse Metal Cold Block	Parse-Provided	1	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate	Consumables	1	
5 mL screwcap v bottom tubes	Consumables	8	
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	-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	
300 mL Reservoir Base	INTEGRA		

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.

        i. 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. Label four 5 mL screw cap transport tubes with the provided stickers “Round 1 Pool - Plate [A-D]” (figure 7).

Figure 7: Ensure the stickers on the side of the tube are as close to the top of the tube as possible to ensure the 5 mL tube fits correctly in the Parse Metal Cold Block.

    c. Place the labeled 5 mL transport tubes in its respective location in the Parse Metal Cold Block. The tube layout should correspond to the image below.

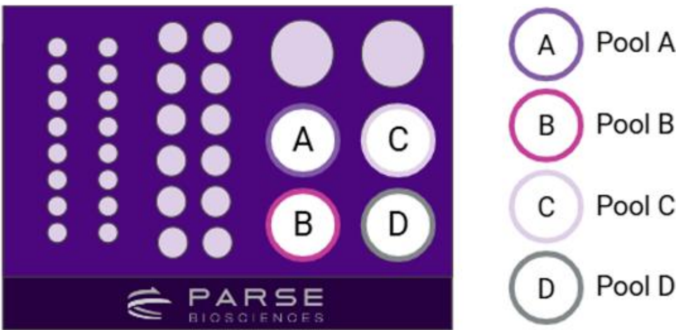
        i. Column 3:

            1. Pos 2: Pool A

            2. Pos 3: Pool B

            3. Pos 5: Pool C

            4. Pos 6: Pool D



d. Load the reagents on the Parse Metal Cold Block

  i. Column 2:

    1. Pos 8: Spin Additive

    2. Pos 9: Round 2 Ligation Enzyme

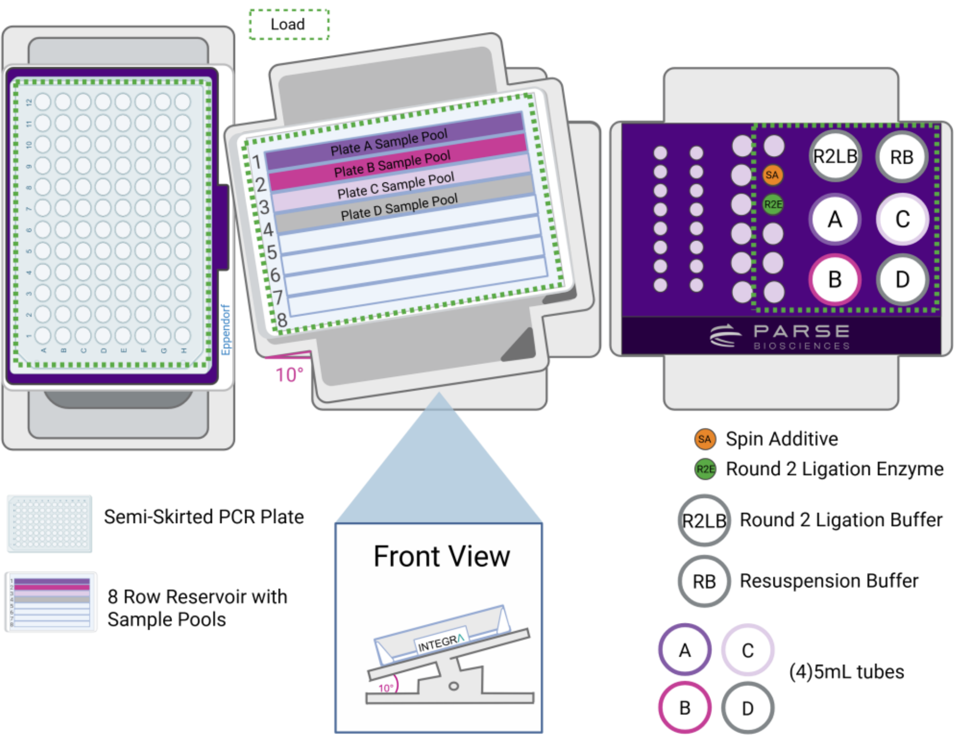
  ii. Column 3:

    1. Pos 1: Round 2 Ligation Buffer.

    2. Pos 4: Resuspension Buffer

e. Carefully Load the 8 Row Reservoir filled with the Round 1 plate pools from Section 1.2 step 32 onto the Integra 8 Row Reservoir Plastic Base with row 1 on the back left corner.

Note: When moving the dual reservoir adapter onto the slanted plate holder on Deck B, take extra care when moving the sample to avoid spills.

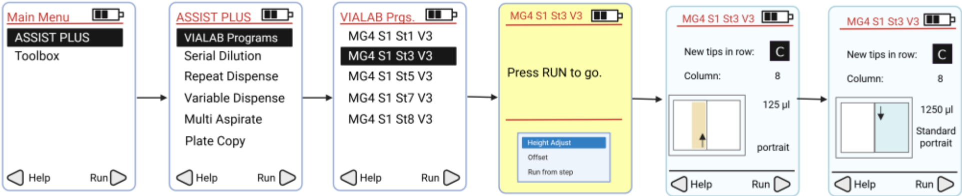


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    a. Remove the VIAFLO 12-Ch 5-125 µL Pipette and corresponding Tip Deck A. Ensure it is disconnected by pressing the back button until the main menu is displayed.

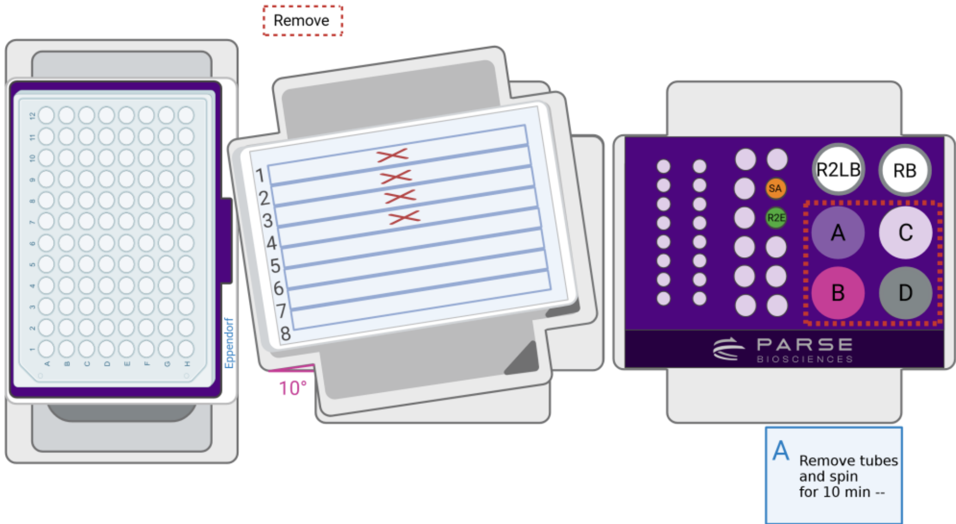
    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

5. Remove the reagent caps, select and run the program **MG4 S1 St3 V3_5** following the diagram below.



6. When prompted, cap, invert once, and centrifuge the four separate pools in 5 mL tubes using a swinging bucket rotor for **5-10 minutes** at 200-500 x g at 4°C. Immediately move to the next step after centrifugation.

CRITICAL! Ideal centrifugation speed and duration should be determined empirically for each sample type to optimize retention and resuspension efficiencies. Overly aggressive centrifugation can damage cell integrity and decrease data quality. If the centrifugation speeds used during fixation gave satisfactory retention, they should be used throughout this protocol.



7. Press "Run" to continue while the samples are centrifuging. The Round 2 Ligation Master Mix will be created in column 8 of the 8 Row Reservoir.

   a. While the Round 2 Ligation Master Mix is being made, thaw the Round 2 Plate using the program below for later use. While the thermocycling program is running, remove a new Thermochromic PCR Cold Block with Riser from -20°C freezer and thaw at room temperature and continue to the next step.

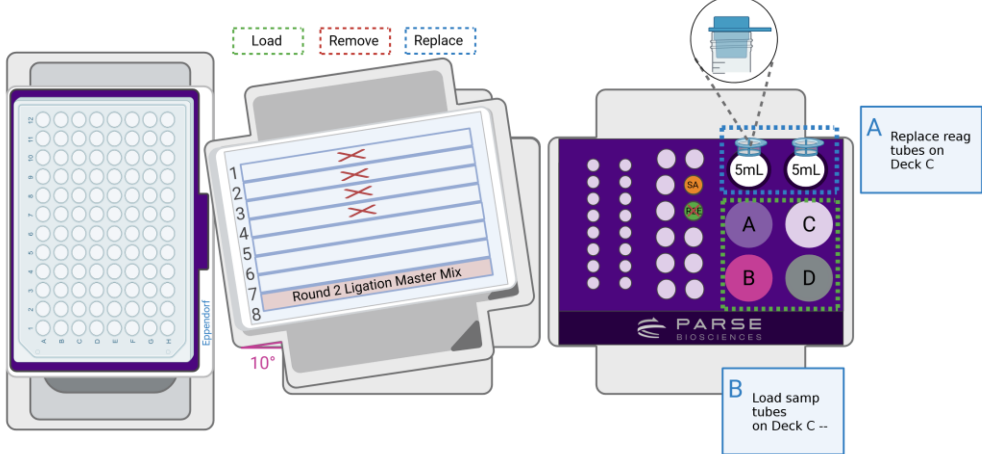
THAW ROUND 2 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	10 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

8. Follow the prompt instructions and do the following:

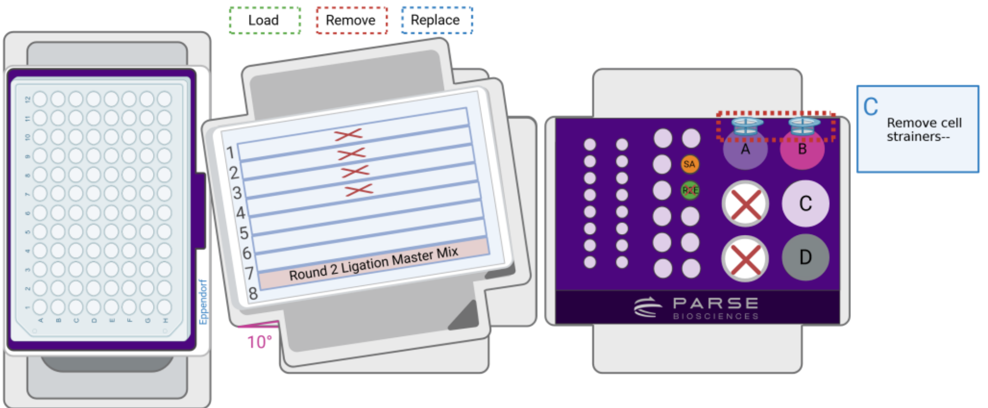
   a. Replace the used Resuspension Buffer and Round 2 Ligation Buffer tubes from Deck C with new 5 mL tubes. Place a strainer on each of these 5 mL tubes.

   b. Immediately after the centrifugation is complete, load the sample tubes on Deck C, remove the caps, and press "Run" to continue the program. Ensure that the sample pools are in the correct positions. Sample specific tube location is important for Round 2 sample loading.

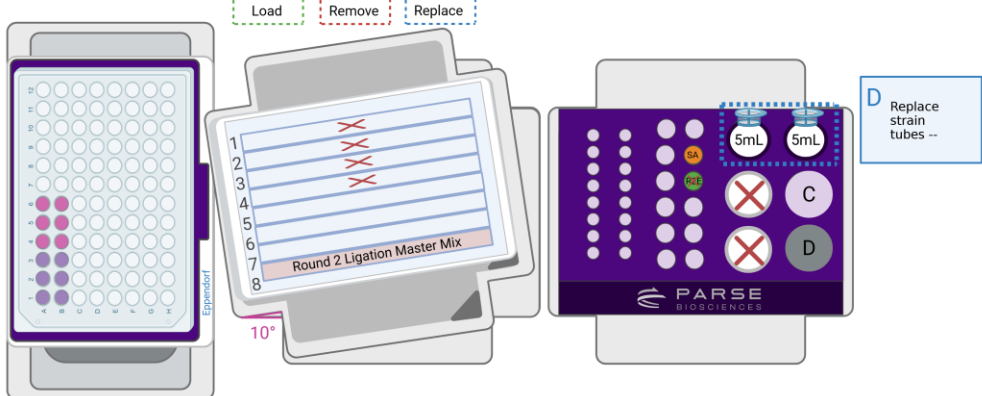
CRITICAL! Move quickly and handle the sample tubes gently to avoid dislodging the pellet, which will have a significant impact on data quality.



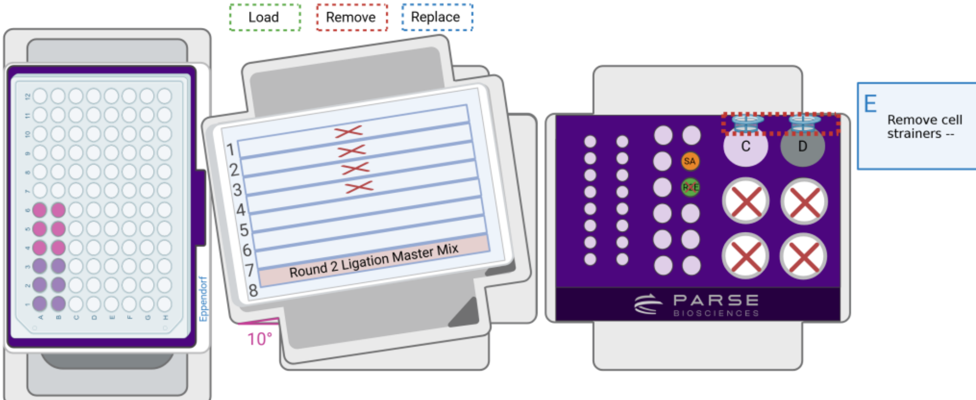
   c. When prompted, after the Pools A and B are strained, remove the strainers. The samples will be transferred to the semi-skirted plate on Deck A. Press "Run" to continue the program.



d. When prompted, replace the used strain tubes with new 5 mL tubes. Place new strainers on the new 5 mL tubes. Press “Run” to continue the program.



e. When prompted and after the Pools C and D are strained, remove the strainers. The samples will be transferred to the semi-skirted plate on Deck A. Press “Run” to continue the program.



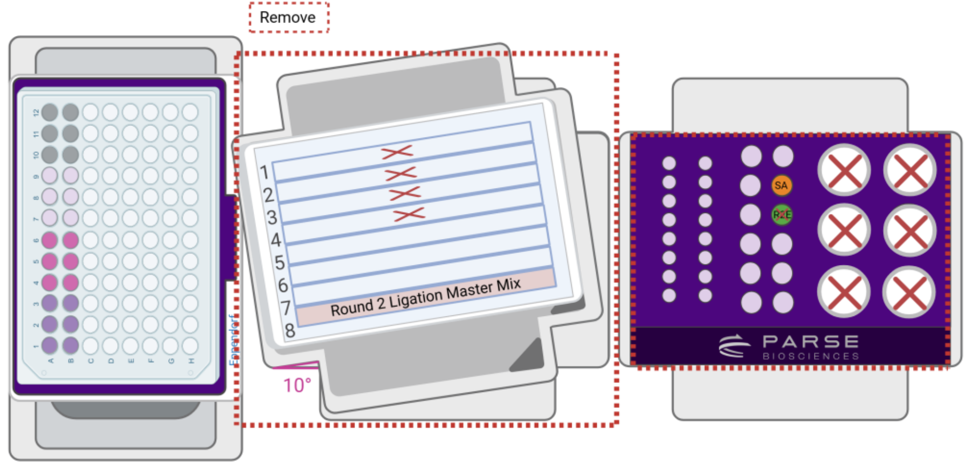
9. At the conclusion of the run:

    a. Store the spin additive reagent at room temperature for later use.

    b. Remove all labware on Deck B. The 8 Row Reservoir can be discarded.

    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.


Remove
1
2
3
4
5
6
7
8
Round 2 Ligation Master Mix
10°
SA
R2E
PARSE
BIOSCIENCES

1.4. Round 2 Ligation

This program loads the Round 2 Plate on Deck B with the sample pools on Deck A. After Round 2 incubation, Round 2 Stop Mix is mixed into the Round 2 Plate from the reservoir on Deck C. Once Round 2 Stop incubation is completed, the sample is then pooled into the left 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	
INTEGRA Dual 25mL Basin Reservoir Adapter	INTEGRA Components	1	
25 mL Basin Reservoir Liners	INTEGRA Components	1	
Thermochromic PCR Cold Block	Parse-Provided	2	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-Provided	2	
Round 2 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

2. Remove the thawed Round 2 Plate 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 Plate and store on ice.

4. Prepare and load the hardware:

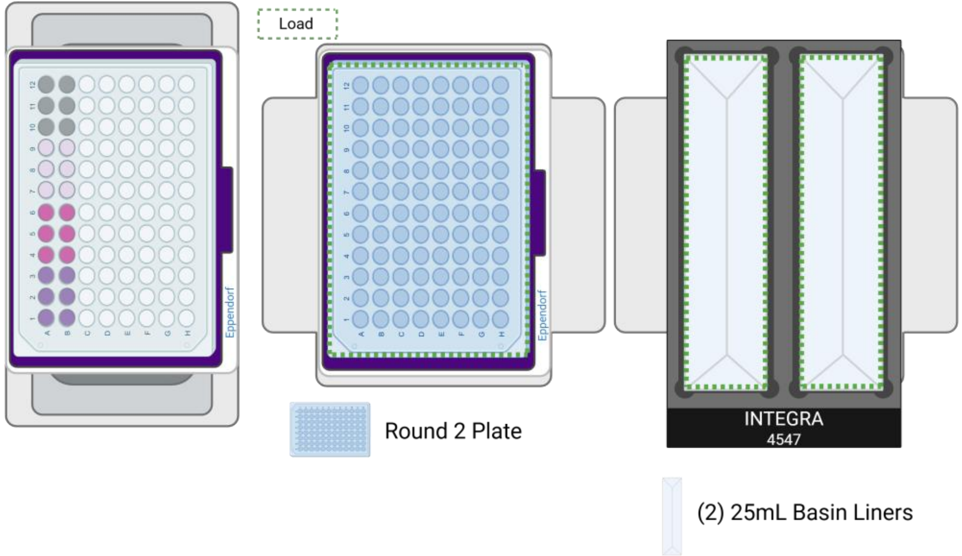
    a. Place a Thermochromic PCR Cold Block on Deck B with A1 orientated towards the bottom left.

    b. Load an INTEGRA Dual 25 mL Basin Reservoir Adapter with the INTEGRA logo facing the front on Deck C.

5. Load the consumables and reagents on Deck:

    a. Load the Round 2 plate on Deck B.

    b. Place (2) new 25 mL Basin Liners in both adapter slots on Deck C.



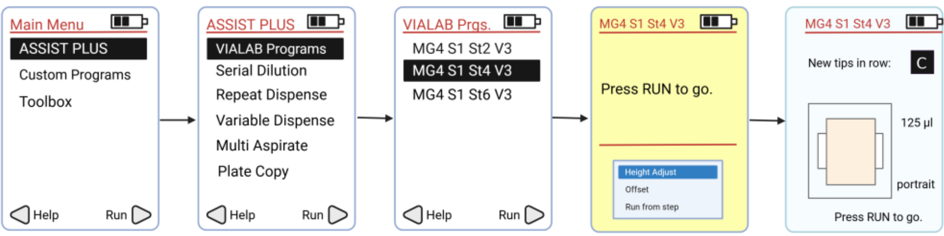
6. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    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.

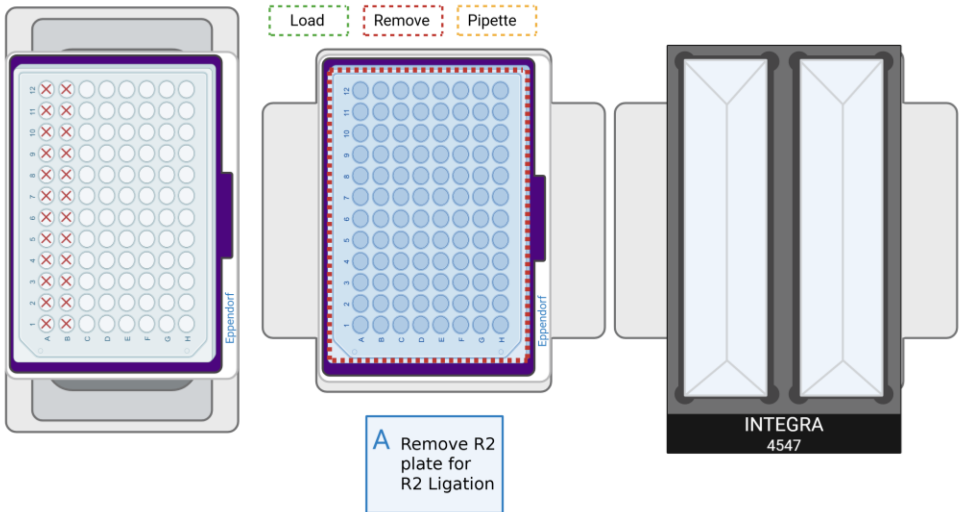
7. Run the program **MG4 S1 St4 V3_5** following the diagram below.



8. When prompted:

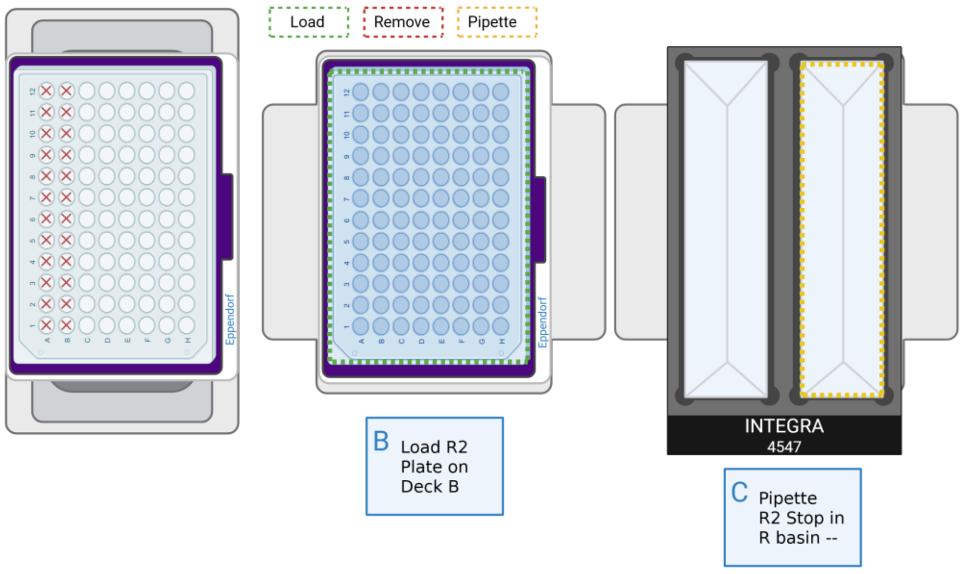
    a. Remove the Round 2 Plate from Deck B. Reseal the Round 2 Plate with an adhesive seal, place it into a thermocycler, and run the following program.

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



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

c. Briefly vortex (2-3 seconds) and centrifuge the ● Round 2 Stop Buffer. Pipette the total volume (~1.4 mL) to the right basin 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.

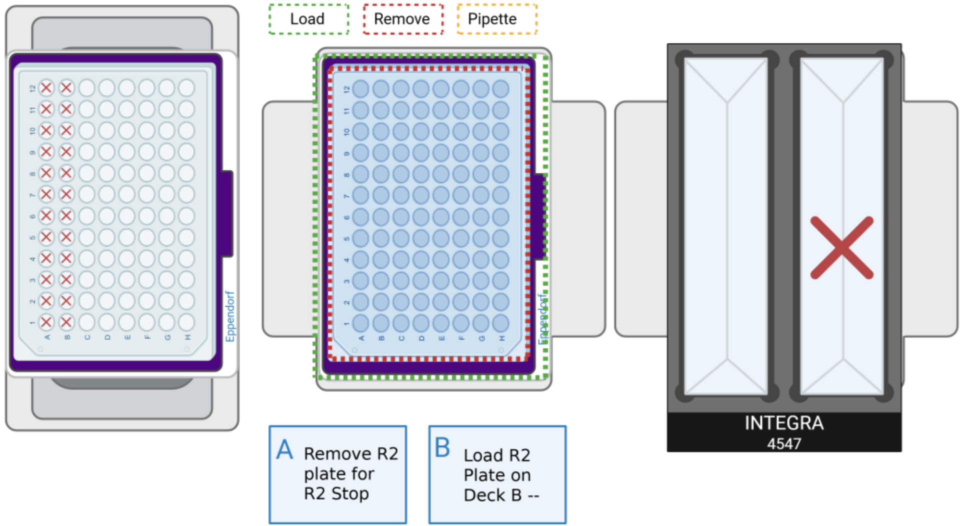


9. When prompted:

a. Remove the Round 2 Plate from Deck B. Reseal the Round 2 Plate with an adhesive seal, and incubate the Round 2 Plate in a thermocycler using the following protocol.

ROUND 2 STOP		
Run Time	Lid Temperature	Sample Volume
5 min	50°C	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 on the Thermochromic PCR Cold Block on Deck B with A1 oriented towards the lower left corner. Remove the plate seal before continuing the program.



10. At the conclusion of the run:

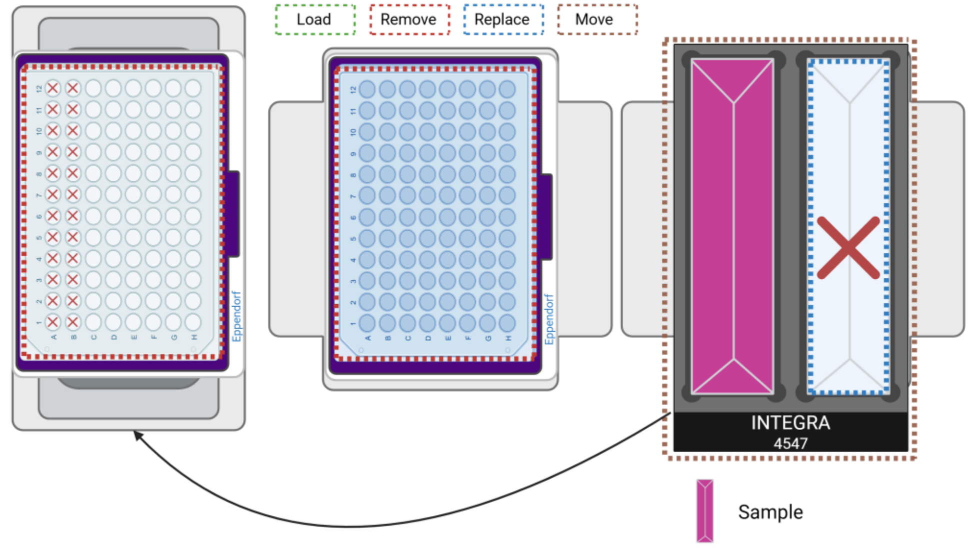
    a. Remove all labware on Deck A. The used semi-skirted can be discarded.

    b. Remove all labware on Deck B. Discard the empty Round 2 plate.

    c. KEEP the left reservoir liner in the dual reservoir adapter on Deck C. This contains the cell suspension and will be used in the next step.

    d. Replace the used right reservoir liner on Deck C with a new 25 mL reservoir liner.

    e. Move the Dual Reservoir Adapter with the cell suspension to Deck A.


Load
Remove
Replace
Move
INTEGRA
4547
Sample

1.5. Round 3 Ligation Preparation

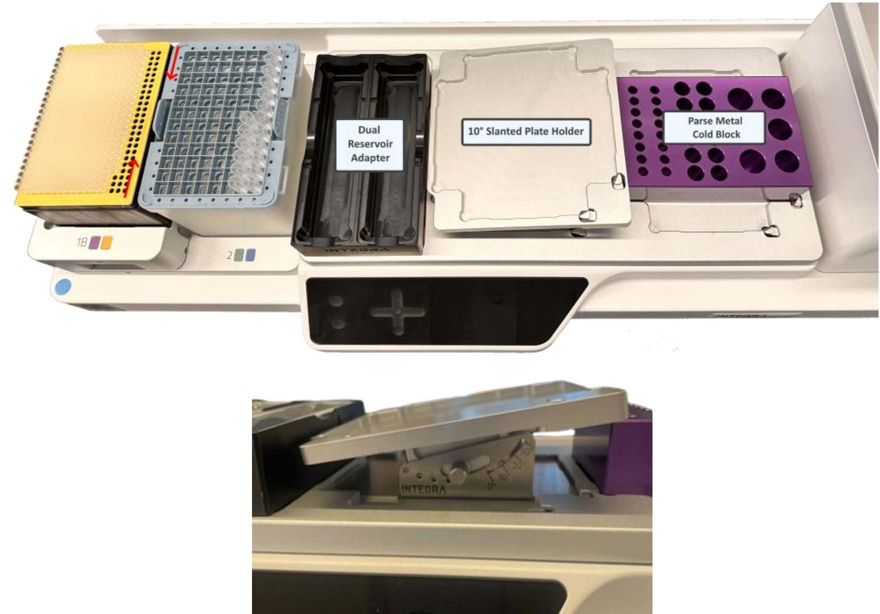
The pooled cell suspension in the left reservoir on Deck A is strained into the 10 mL transport tube on Deck C. The program adds Round 3 Ligation Enzyme into the cells and mixes. It then transfers the cell/nuclei suspension mix to the top reservoir within the slanted plate holder on Deck B.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse-Provided	1	Keep on ice when not in use.
Dual Reservoir Adapter (ANSI/SLAS footprint)	INTEGRA Component	1	
ASSIST PLUS Slanted Plate Holder	INTEGRA Component	1	
10 mL transport tube	Consumables	1	
25 mL Basin Reservoir Liners	INTEGRA	2	
25 µm, 40 µm, or 70 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Round 3 Plate	-20°C Reagents	1	Place directly on ice.
● Round 3 Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

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



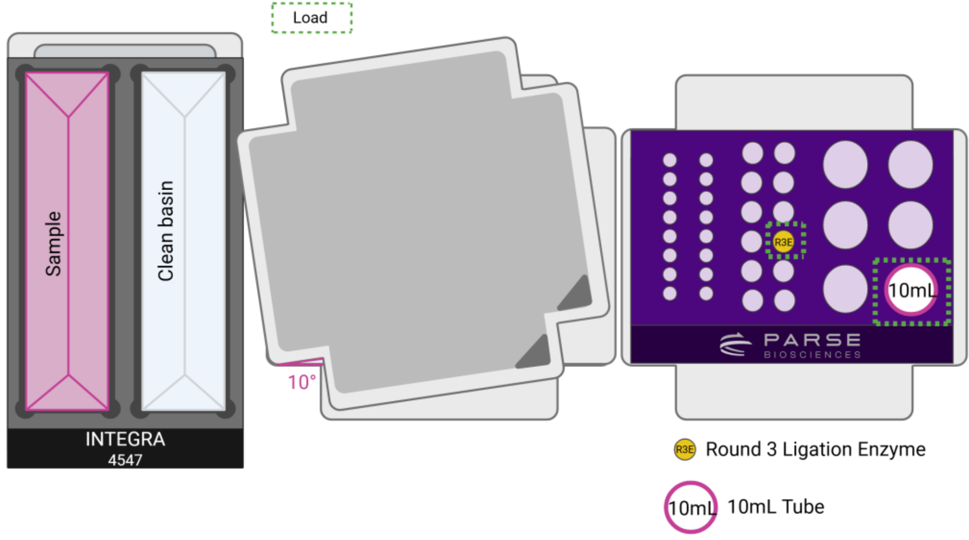
4. Load the following reagents to their respective positions on the Parse Metal Cold Block on Deck C.

   a. Column 2:

      i. pos 10: 🟡 Round 3 Ligation Enzyme.

   b. Column 3:

      i. pos 6: a clean 10 mL transportation tube.

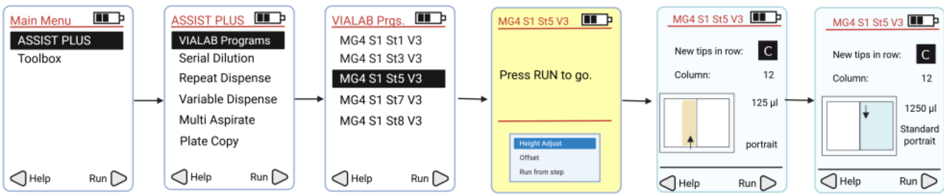


5. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

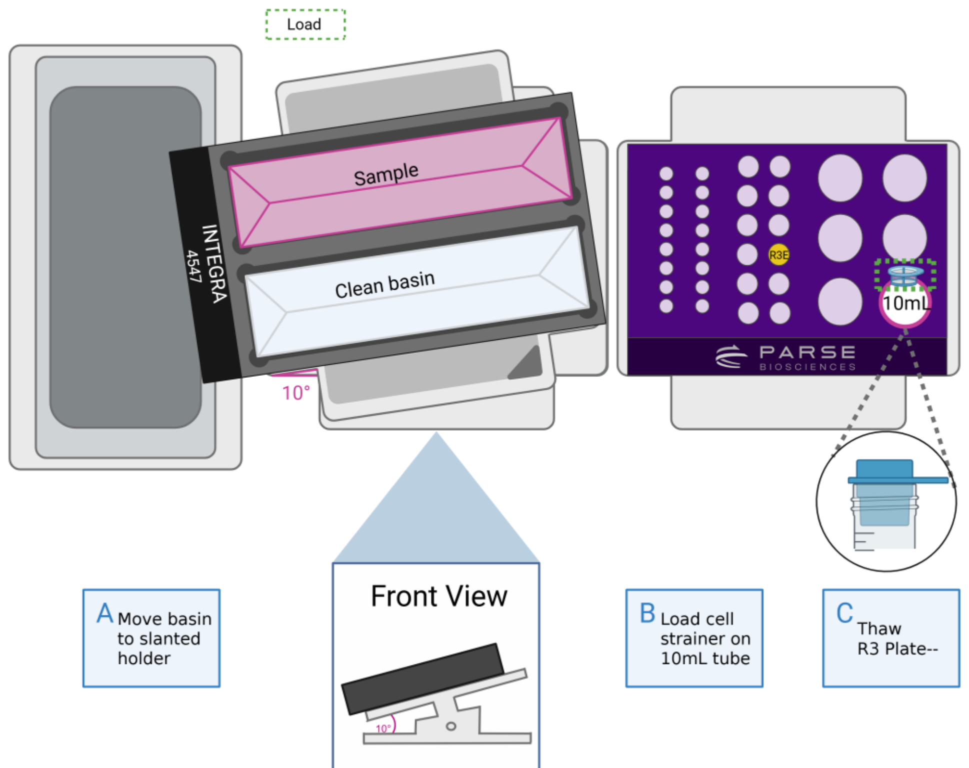
    a. Remove the VIAFLO 12-Ch 5-125 µL pipette and corresponding tip deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    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.

6. Remove the reagent caps, then select and run the program **MG4 S1 St5 V3_5** following the diagram below.



7. Press “Run” to continue the program. Follow the program prompts for manual intervention to move the basin as indicated below:



Note: When moving the dual reservoir adapter onto the slanted plate holder on Deck B, take extra care when moving the sample to avoid spills.

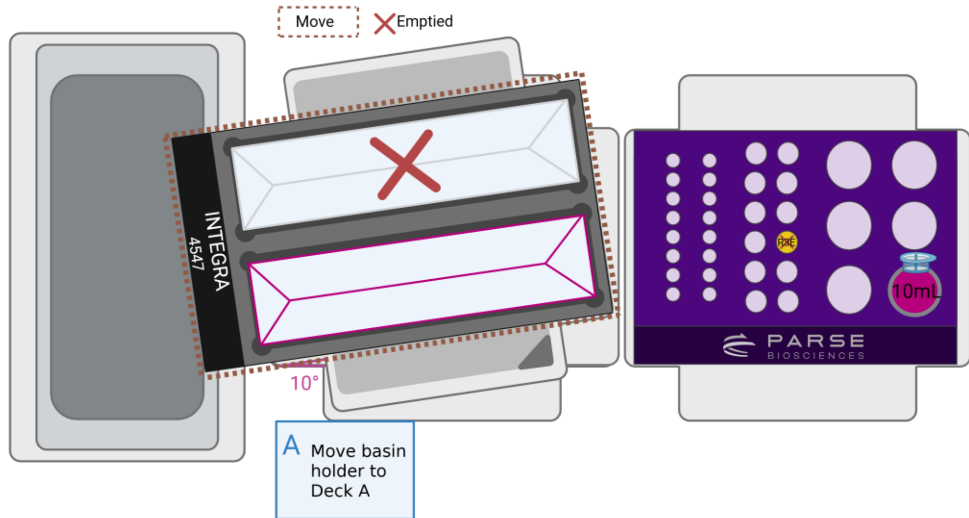
a. Move the Dual Reservoir Adapter with the sample from Deck A to the slanted plate holder on Deck B. The INTEGRA logo at the front of the Dual Reservoir Adapter faces towards the pipette tip box. The sample should now be located on the top reservoir of the Dual Reservoir Adapter.

b. Load a cell strainer so it sits inside of the 10 mL transport tube.

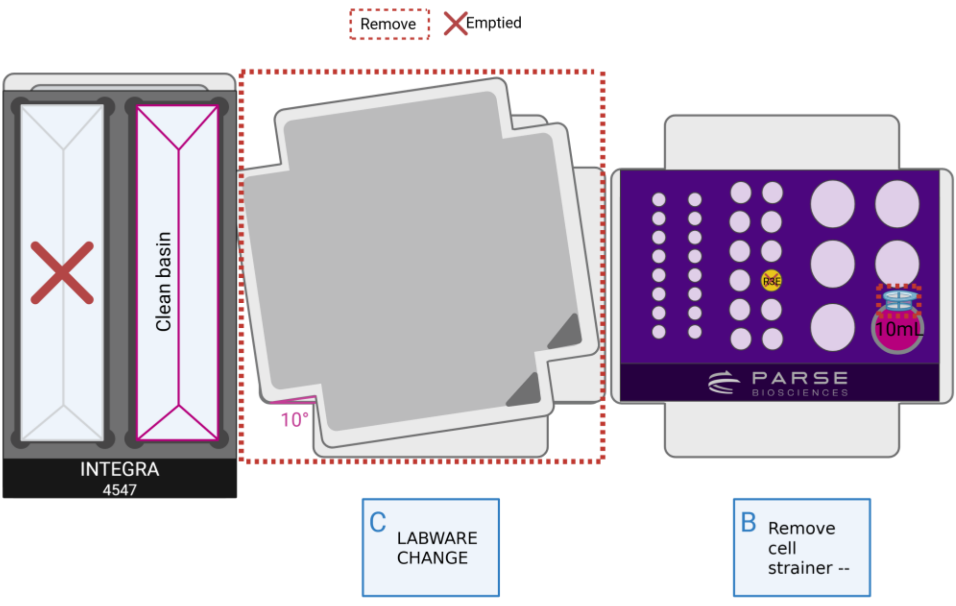
c. Thaw the Parse Round 3 Plate using the program below for later use. While the thermocycling program is running, remove a new Thermochromic PCR Cold Block with Riser from -20°C freezer and thaw at room temperature and continue to the next step.

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

8. Press "Run" to continue the program. Follow the program prompts to complete the program:



a. Move the Dual Reservoir adapter to Deck A. Ensure that the INTEGRA logo is facing the front.



b. Remove the used cell strainer from the 10 mL transport tube on Deck C.

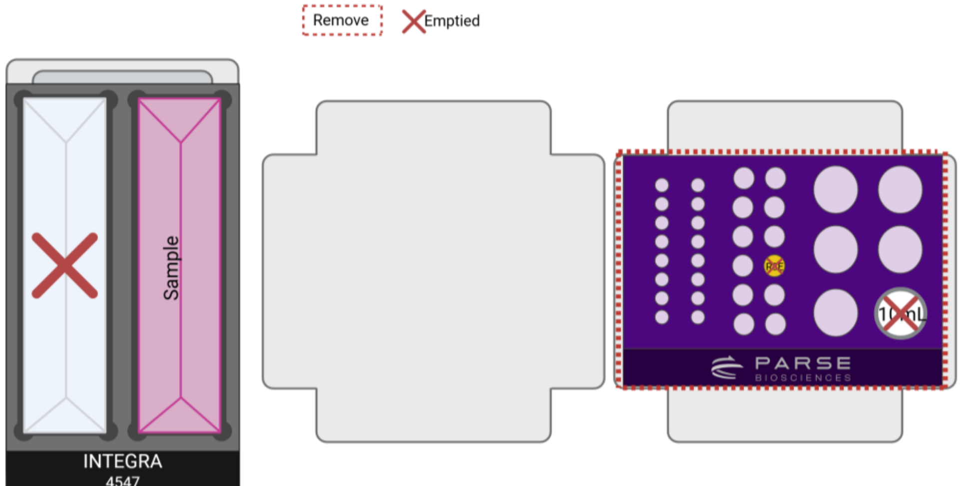
Note: There may be bubbles left on the strainer. This will not affect the results.

c. Remove Slanted Plate Holder from Deck B.

9. Press "Run" to continue the program. At the completion of the run:

   a. Keep all labware on Deck A. The sample is in the right basin liner on Deck A. Do not discard this. It will be used in the next step.

   b. Remove all labware from Deck C. Discard all used tubes on Deck C.



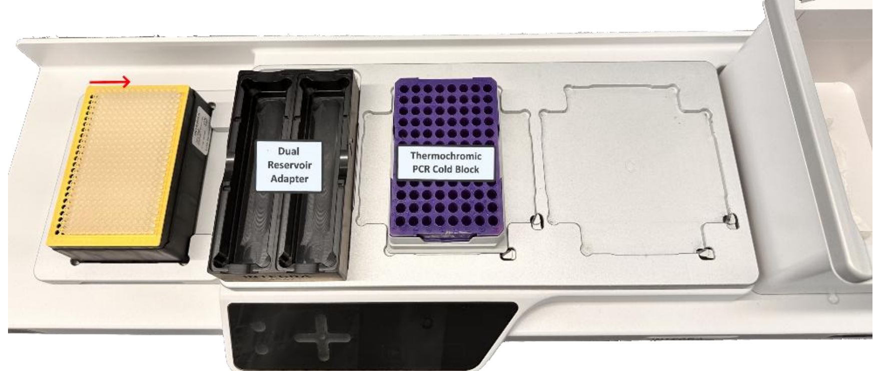
1.6. Round 3 Ligation

The program loads the Cell Suspension Mix on Deck A into the Round 3 Plate on Deck B. Following the prompts for Round 3 incubation, the program will then load Round 3 Stop Buffer into all the wells and pool all the samples together on Deck A.

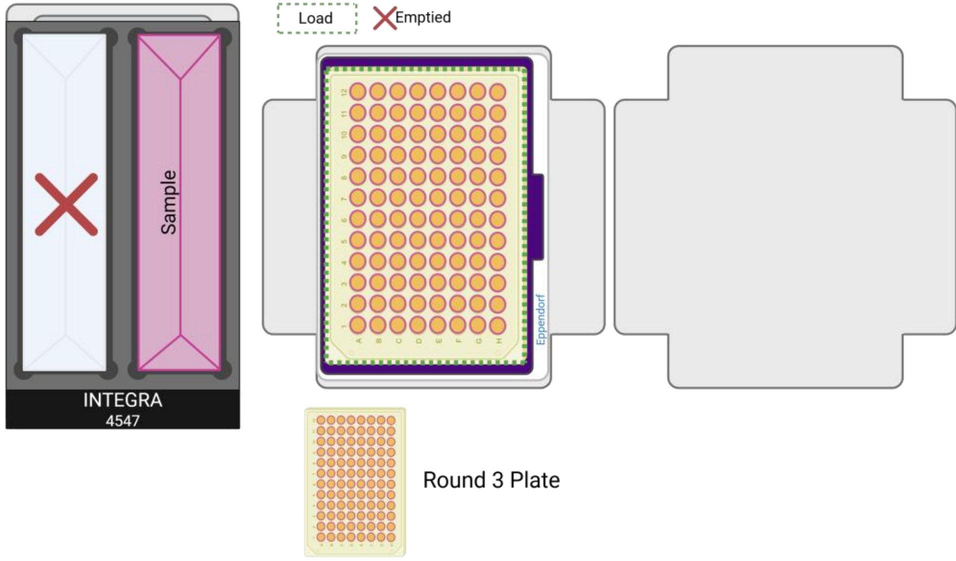
1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Component	1	
Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse	1	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	1	
INTEGRA Dual 25mL Basin Reservoir Adapter	INTEGRA Component	1	
25 mL Basin Reservoir Liners	INTEGRA	2	
25 µm, 40 µm, or 70 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA	1	
○ Round 3 Stop Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

2. Place the Thermochromic PCR Cold Block with Riser removed from the freezer in step 1.5.7c on Deck 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 and place on Deck B with A1 at the bottom left.

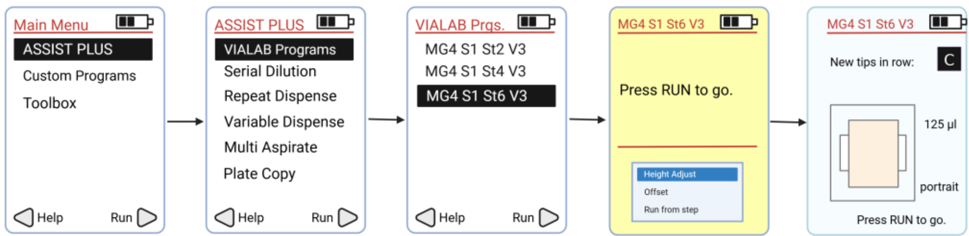


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

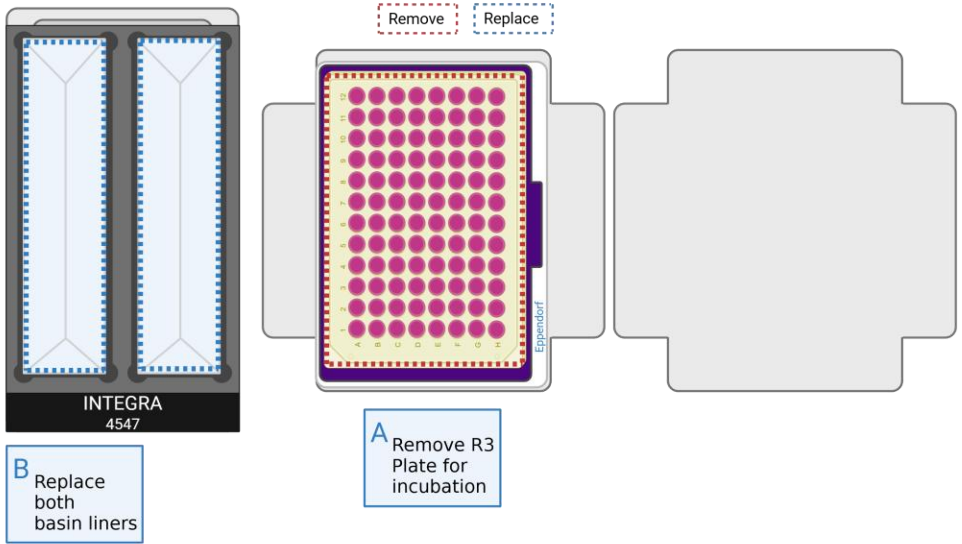
    a. Remove the D-ONE Pipetting Module 1-Ch, 5-1250 µL pipette and corresponding tip deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    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.

5. Run the program **MG4 S1 St6 V3_5** following the diagram below.



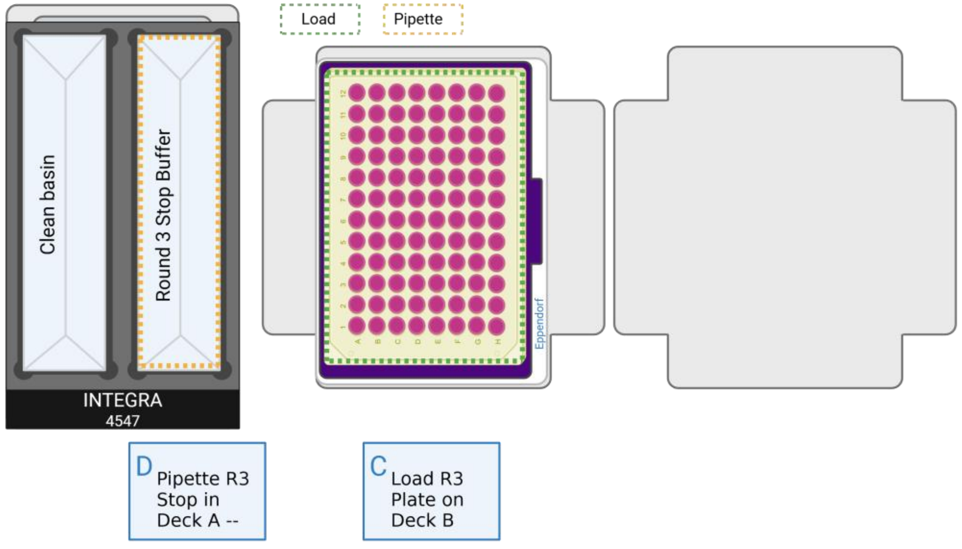
6. Press "Run" to continue the program. Follow the program prompts for manual intervention:



a. Remove the Round 3 Plate from Deck B. Reseal the Round 3 Plate with an adhesive seal, place it into a thermocycler, and run the following program.

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

b. Replace both used basin liners on Deck A with clean 25 mL basin reservoir liners.



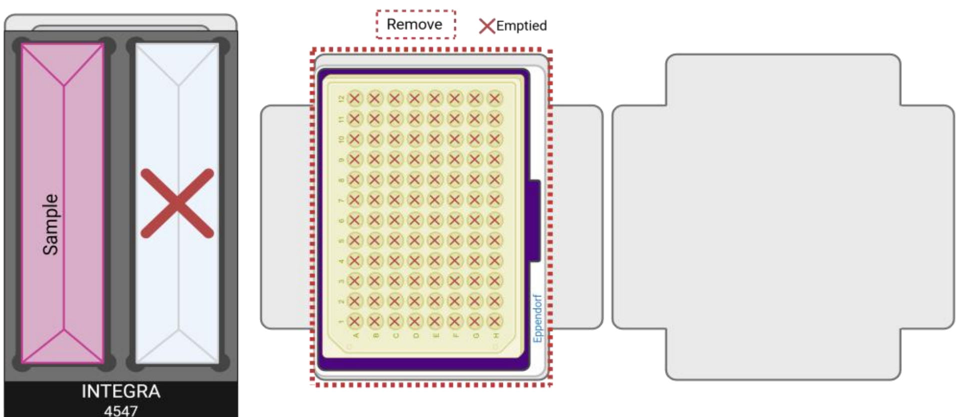
c. When the Barcoding Round 3 Thermocycling program is complete, load the Round 3 plate on the Thermochromic PCR Cold Block on Deck B with A1 oriented towards the lower left corner.

d. Briefly vortex (2-3 seconds) the ○ Round 3 Stop Buffer and ensure there is no precipitate. Pipette the total volume (~3.5 mL) to the right basin on Deck A using a P1000 set to 1000 µL.

7. At the completion of the run:

   a. Keep all the labware on Deck A. The sample is in the left basin liner on Deck A. Do not discard this. It will be used in the next step.

   b. Remove all labware from Deck B. Discard the used Round 3 Plate. Remove the Thermochromic PCR Cold Block located on Deck B and store it in the freezer.



1.7. Pre-Lysis

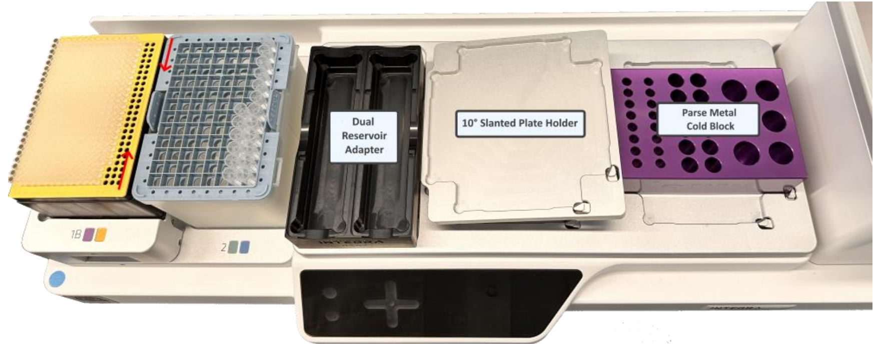
The pooled cell suspension on Deck B is strained into the 10 mL transport tube on Deck C. The Spin Additive is then added into the cells and centrifuged. Supernatant is removed; the cells are resuspended in Pre Lysis Wash Buffer, and centrifuged again. The supernatant is then removed.

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	Keep on ice when not in use.
ASSIST PLUS Slanted Plate Holder	INTEGRA Components	1	
Dual 25mL Basin Reservoir Adapter	INTEGRA Components	1	
10 mL transport tube	Consumables	1	
25 mL basin reservoir liners	INTEGRA	2	
25 µm, 40 µm, or 70 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	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.

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.

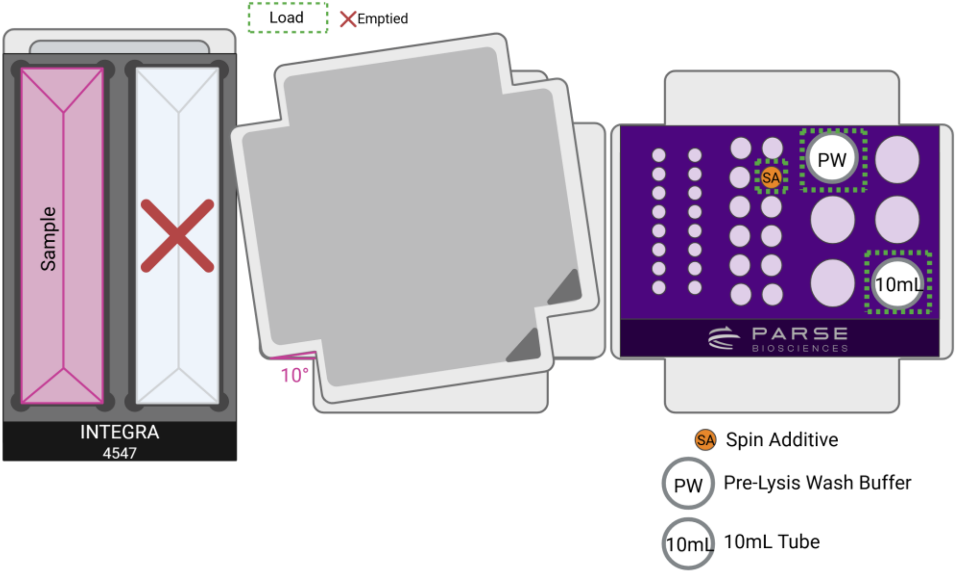
    a. Column 2:

        i. Pos 8: ● Spin Additive.

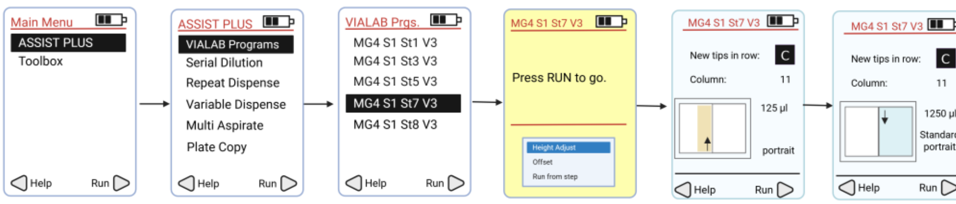
    b. Column 3:

        i. Pos 1: ○ Pre-Lysis Wash Buffer.

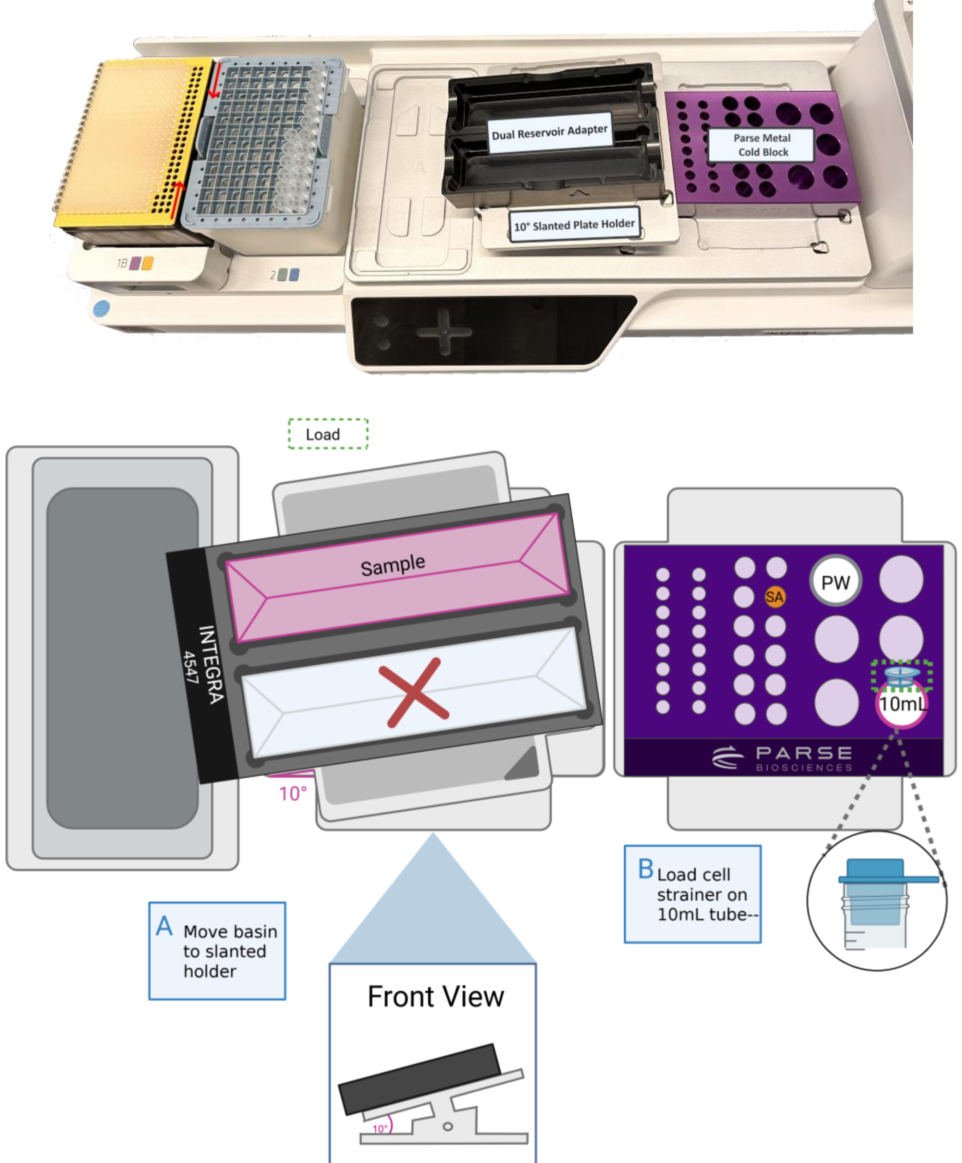
        ii. Pos 6: a clean 10 mL transport tube.



6. Remove reagent caps, select and run the program **MG4 S1 St7 V3_5** following the diagram below.



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

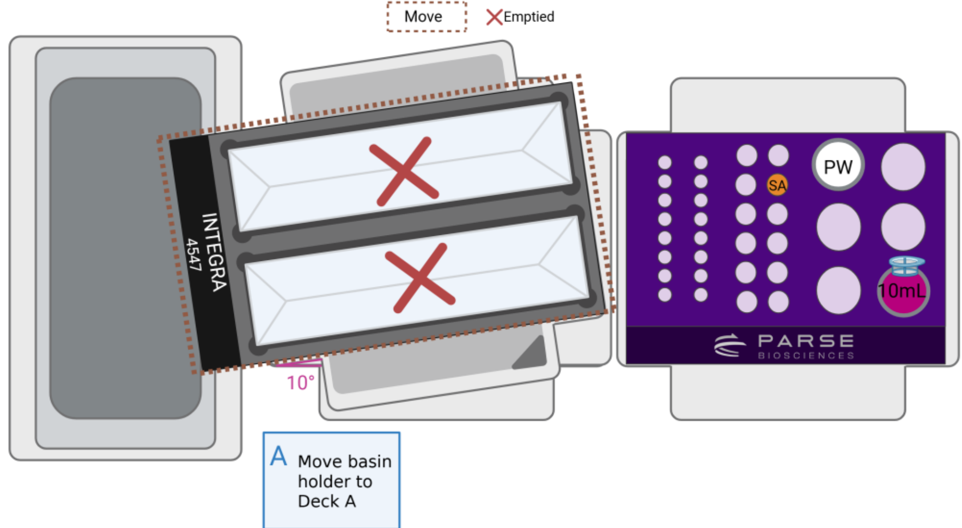


a. Move the Dual Reservoir Adapter with the sample from Deck A to the slanted plate holder on Deck B. The INTEGRA logo at the front of the Dual Reservoir Adapter faces towards the pipette tip box. The sample should now be located on the top reservoir of the Dual Reservoir Adapter.

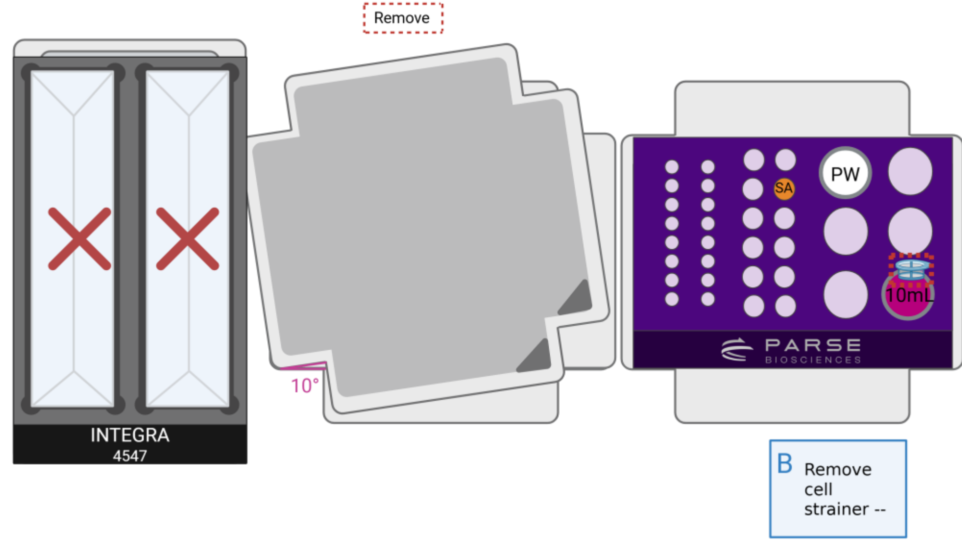
Note: When moving the dual reservoir adapter onto the slanted plate holder on Deck B, use extra care when moving the sample to avoid spills.

b. Load a cell strainer so it sits inside of the 10 mL transport tube.

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



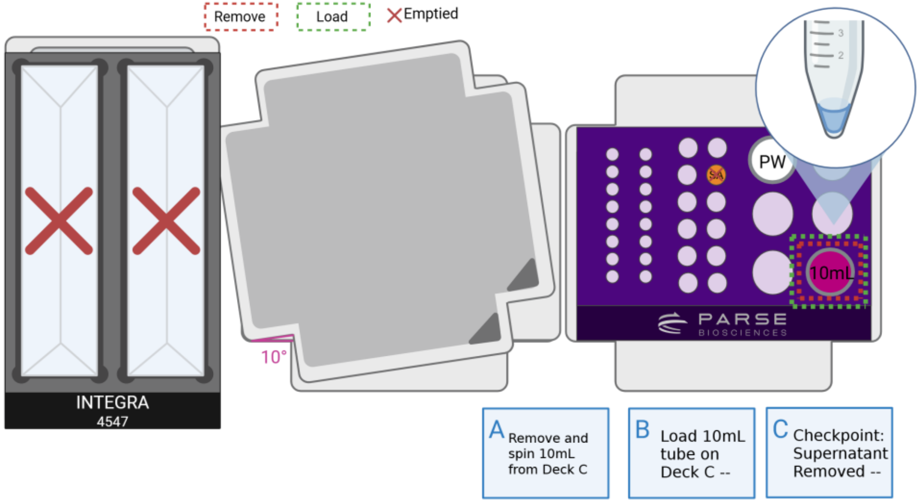
a. Move the Dual Reservoir adapter back to Deck A. Ensure that the INTEGRA logo is facing the front.



b. Remove the used cell strainer from the 10 mL transport tube on Deck C, position 6.

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

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



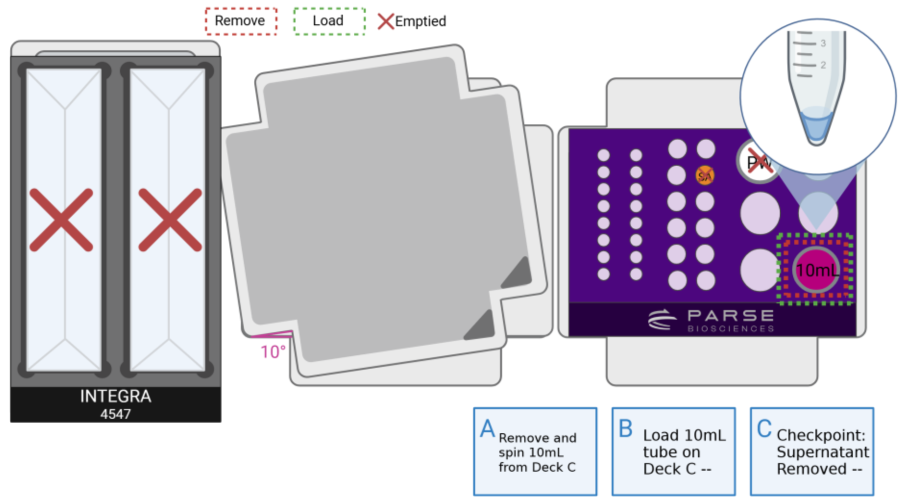
a. Cap and invert once the 10 mL transport tube containing the pooled cells/nuclei. Centrifuge the sample in a swinging bucket centrifuge cooled to 4°C for **10 minutes** at 200-500 x g.

b. 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.

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. A pellet may not be visible. Avoid disturbing the pellet—do not insert the pipette tip into the pellet area to measure the supernatant.

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



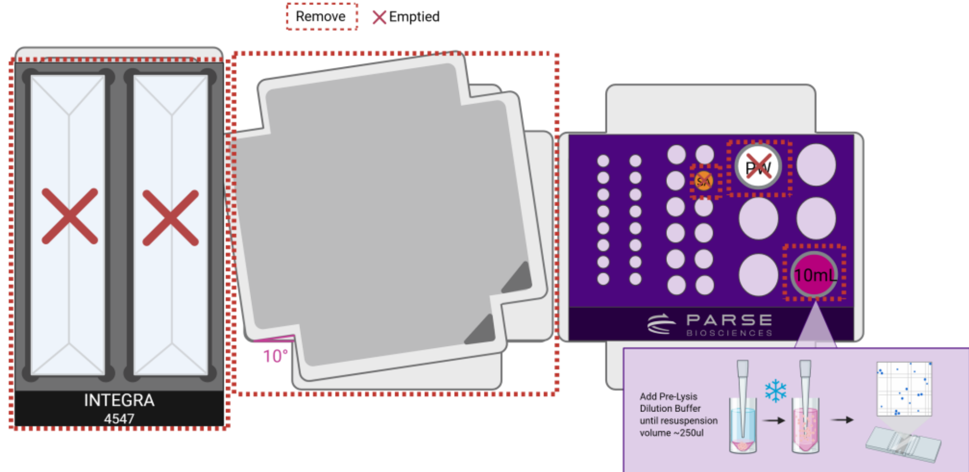
a. Cap and invert once the 10 mL transport tube containing the pooled cells/nuclei. Centrifuge the sample in a swinging bucket centrifuge cooled to 4°C for **10 minutes** at 200-500 x g.

b. 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 located on Deck C, position 6.

CRITICAL! Immediately return the 10 mL transport tube to the Parse Metal Cold Block 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. A pellet may not be visible. Avoid disturbing the pellet—do not insert the pipette tip into the pellet area to measure the supernatant.

11. At the conclusion of the run:



a. Store the 10 mL transport tube from Deck C on ice. If the remaining volume in the 10 mL transport tube is less than 100 µL, add ● Pre-Lysis Dilution Buffer for a final total volume of 250 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.

Note: Only count intact cells/nuclei. Including damaged or broken cells/nuclei can compromise data quality and will be excluded during downstream analysis.

b. Discard the used 1.5 mL and 5 mL reagent tubes on Deck C.

c. Keep the Parse Metal Cold Block on Deck C. It will be used in the next step.

d. Remove all labware on Deck A. Discard the used reservoir liners and their contents.

e. Remove all labware on Deck B.

1.8. Lysis and Sublibrary Generation

The program requires a dilution of the sample to 2500 cells/nuclei per µL with a volume of 420 µL. The program will create sixteen lysates with 62,500 cells/nuclei each. It then mixes the Lysis Master Mix and aliquots the master mix into the generated lysates.

To generate and lyse sublibraries:

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
8-count PCR strip tube	Consumables	2	
1.5 mL tube	Consumables	2	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
● 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.

2. Dilute the cells to a concentration of 2,500 cells/nuclei per µL for a total volume of at least **420 µL** using the ● Pre-Lysis Dilution Buffer in a 1.5 mL transport tube. Store this dilution on ice until ready to use.

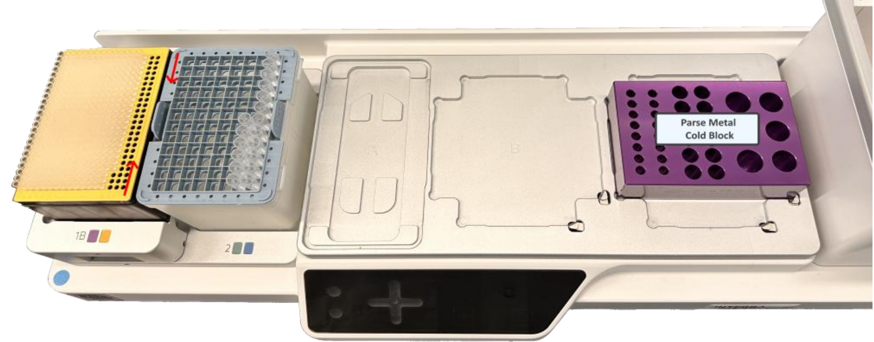
Note: If you do not have at least 1,000,000 cells/nuclei, dilute the remaining cells to 420 µL and record the amount of cells per sublibrary.

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 1,000,000 cells/nuclei across 16 sublibraries requires a minimum input of 1,050,000 cells/nuclei. If fewer than 1,050,000 cells/nuclei are

available, the dispensing can be performed manually to ensure maximum utilization of all available cells/nuclei.

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



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

    a. Column 1: Two 8-count PCR strip tubes.

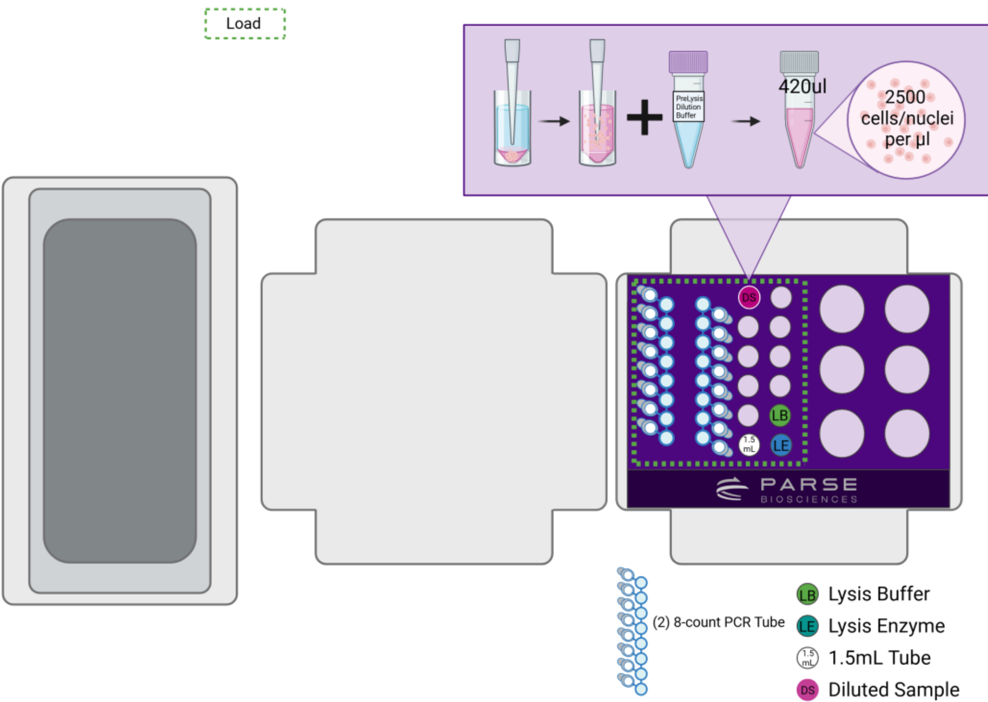
    b. Column 2:

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

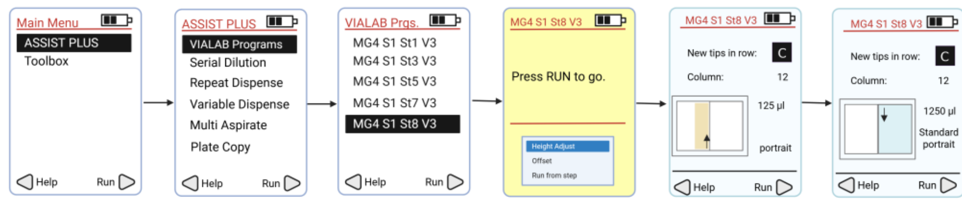
        ii. Pos 6: a clean 1.5 mL tube.

        iii. Pos 11: ● Lysis Buffer.

        iv. Pos 12: ● Lysis Enzyme.



5. Remove the reagent caps, ensure that all strip tubes are open, select and run the program **MG4 S1 St8 V3_5** following the diagram below.



6. At the conclusion of the run:

    a. Remove the PCR strip tubes from Deck C. Securely cap and vortex the 0.2 mL tube(s) for **10 seconds**. Briefly centrifuge. Place the tube(s) into a thermocycler and run the following program.

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

7. For Standard Fixation samples, freeze the lysates at -80°C or proceed to Section 2. For Low Input Fixation samples, proceed to step 8.

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

9. While still on the magnetic rack, manually transfer **55 µL** of the supernatant containing lysed cells into new 0.2 mL tubes.

10. Freeze the lysates at -80°C or proceed to Section 2.

STOP 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 8-count PCR strip tubes. 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.

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 Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
24 mm Labware Pedestal	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	Keep on ice when not in use.
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
8 Row Reservoir	INTEGRA	1	Individually wrapped consumable
300 mL Reservoir Base	INTEGRA	1	
8-count PCR strip tubes	Consumables	3	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
● Streptavidin Beads	4°C Reagents	1	Keep at room temperature.
○ Bead Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Wash Buffer 1	-20°C Reagents	1	
○ Wash Buffer 2	-20°C Reagents	1	
○ Wash Buffer 3	-20°C Reagents	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Enhancer	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
SPRI Beads	Consumables and Reagents		Equilibrate at room temperature before use.

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 D-ONE Pipetting Module 1-Ch, 5-1250 µL and the corresponding tip rack.

7. On the 24 mm Pedestal, place a clean INTEGRA 8 Row Reservoir with the 300 mL Reservoir Base.

8. Vortex, and dispense **1,420 µL** SPRI beads into a 2 mL tube.

9. Vortex ● Streptavidin Beads.

Note: Ensure that the ● Streptavidin Beads and 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.

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

    a. Deck A: clean 8 Row Reservoir.

b. Deck B, column 1, left: a clean 8-count PCR strip tube.

c. Deck B, column 2:

   i. Pos 5: Fully resuspended SPRI beads.

   ii. Pos 6: ● Binding Buffer.

   iii. Pos 11: ● Enhancer.

   iv. Pos 12: Fully resuspended ● Streptavidin Beads.

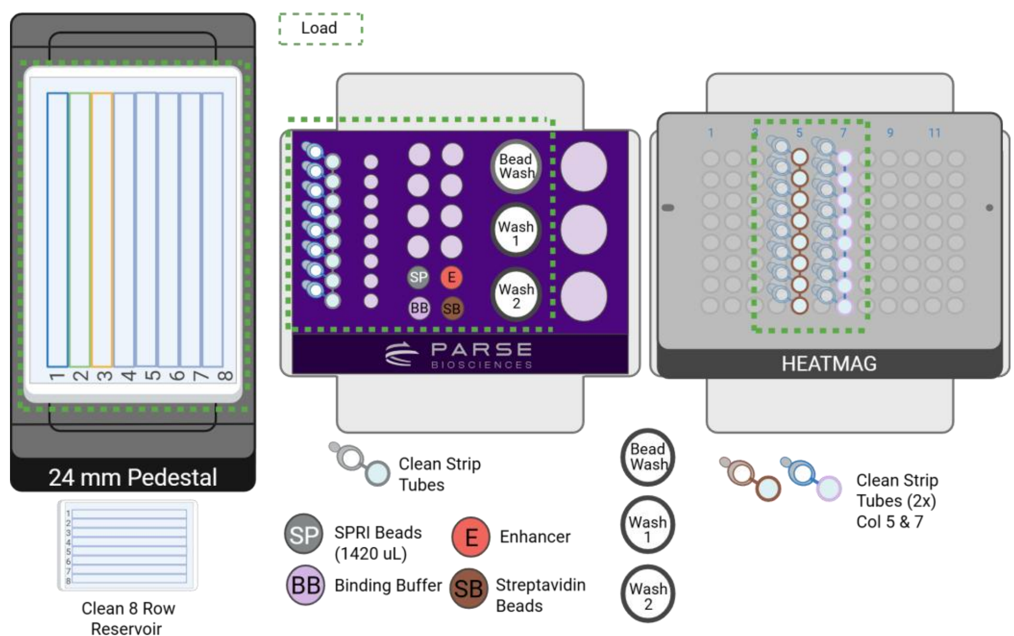
d. Deck B, column 3:

   i. Pos 1: ○ Bead Wash Buffer.

   ii. Pos 2: ○ Wash Buffer 1.

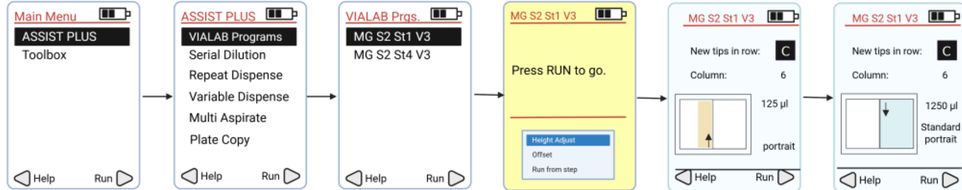
   iii. Pos 3: ○ Wash Buffer 2.

e. Deck C: Two clean 8-count PCR strip tubes with the caps facing to the left in columns 5 and 7 on the HEATMAG.



11. Remove the reagent caps and ensure that all strip tubes are open.

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



13. If continuing directly from Section 1, store lysates on ice until prompted.

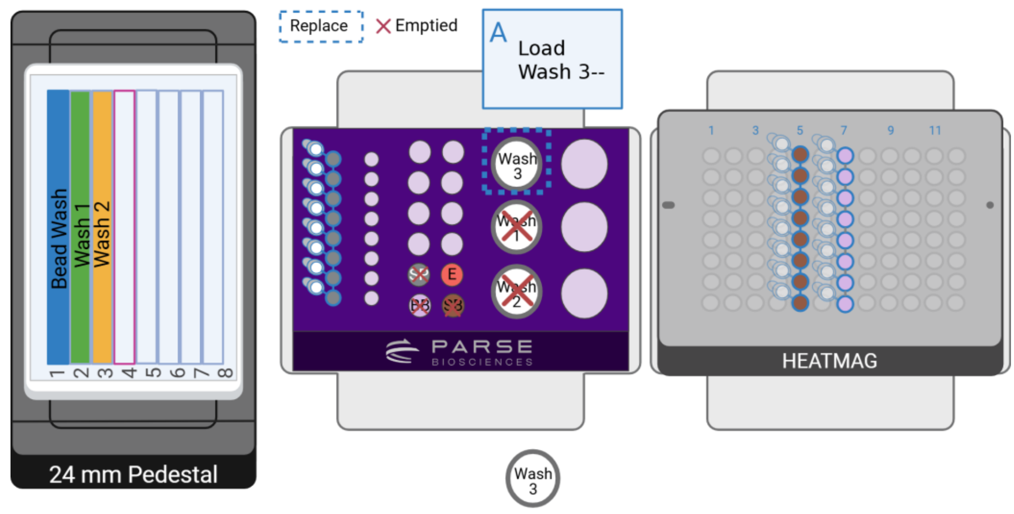
14. If lysates are previously frozen, when prompted, incubate the lysates in a thermocycler at 37°C for **5 minutes** to thaw them. Press “Run” on the pipette to continue the program while the lysates are thawing.

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

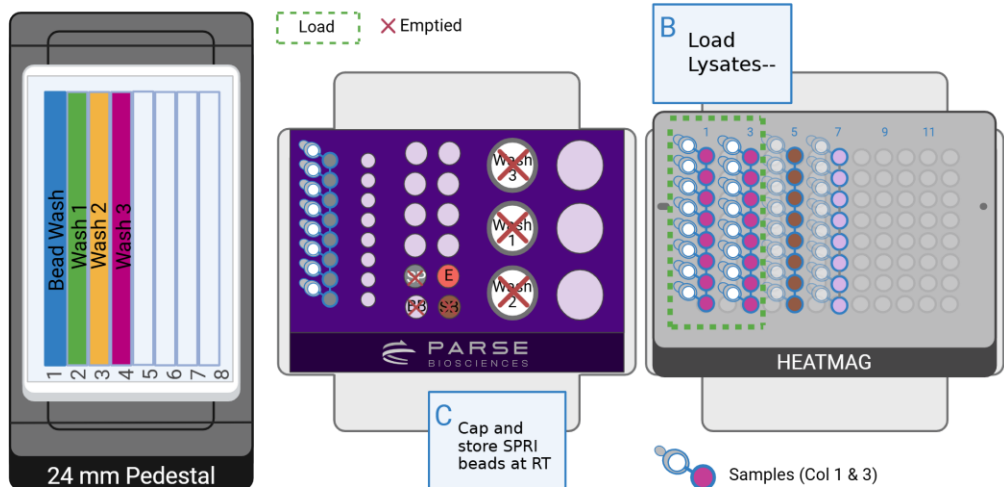
Note: When thawing the lysate, ensure there is no precipitate before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

15. When the lysates finish thawing, briefly centrifuge and store at room temperature.

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



a. Replace the ○ Bead Wash Buffer with ○ Wash Buffer 3 on Deck B, column 3, position 1.



b. Load the thawed lysates in columns 1 and 3 of the HEATMAG with 96 Well PCR Adapter. Press "Run" to continue.

Note: Ensure the 8-count PCR strip tube caps are facing the same direction. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.

c. When prompted, cap and store the PCR strip tubes on column 1 left position of the Parse Metal Cold Block on Deck B at room temperature until after cDNA cleanup. Label these tubes as SPRI beads.

18. At the conclusion of the run:

a. Verify that Streptavidin Bead volumes in column 5 of Deck C are even.

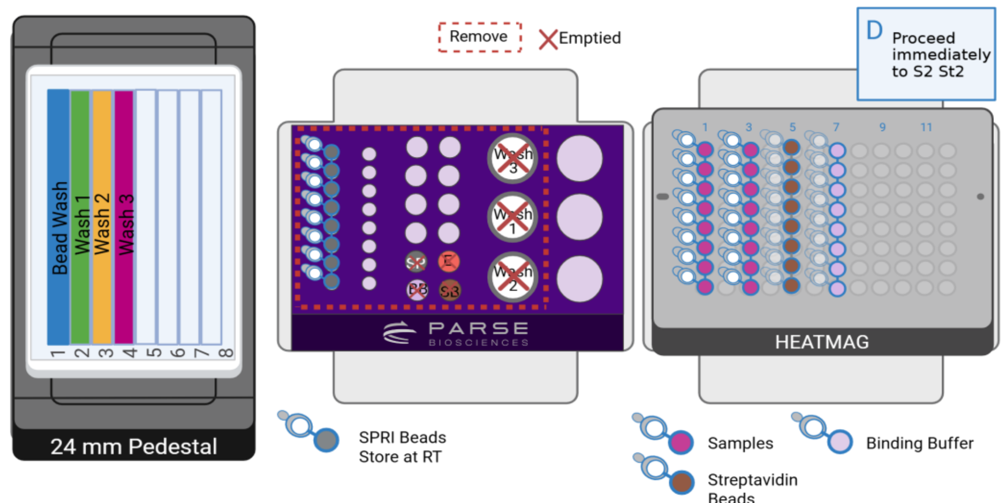
b. Verify that the Binding Buffer volumes in column 7 of Deck C are even.

c. Verify that all wash buffers have been transferred to the 8 Row Reservoir.

Note: Refer to Appendix B for specific volumes.

d. Remove and discard empty tubes on Deck B. Place the SPRI bead tubes aside for later use.

e. Proceed immediately to Section 2.2.


Remove
Emptied
D Proceed immediately to S2 St2
Bead Wash
Wash 1
Wash 2
Wash 3
1 2 3 4 5 6 7 8
24 mm Pedestal
Wash 3
Wash 1
Wash 2
HEATMAG
1 3 5 7 9 11
SPRI Beads Store at RT
Samples
Binding Buffer
Streptavidin Beads

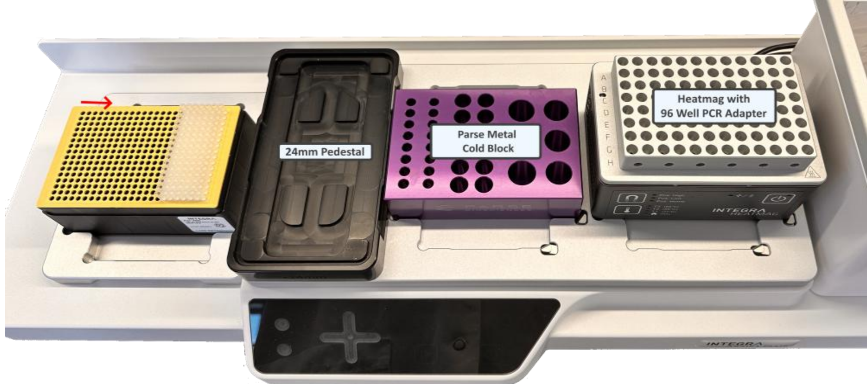
2.2. cDNA Capture

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.

To capture the cDNA:

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 Component	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	

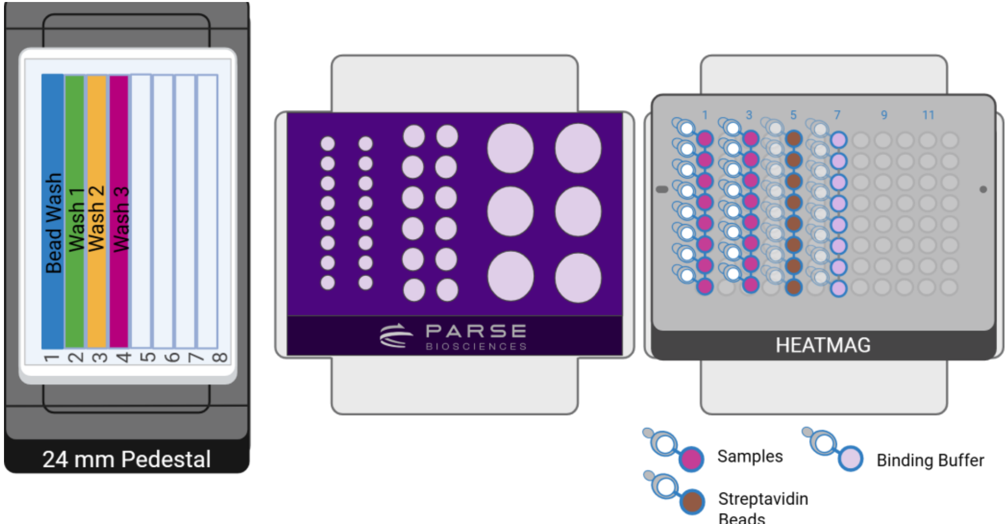


2. Load the following uncapped strip tubes (if removed from previous steps) to their respective positions on the HEATMAG with 96 Well PCR Adapter:

    a. Samples on columns 1 and 3.

    b. Streptavidin Beads on column 5.

    c. Binding Buffer on column 7.

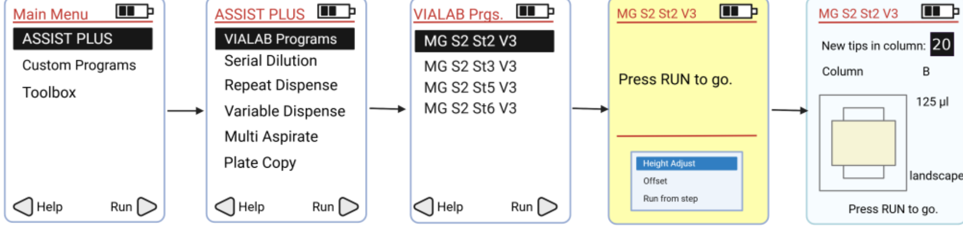


3. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    b. 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.

    c. 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. Select and run the program **MG S2 St2 V3_5** following the diagram below.



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

    a. Cover the 8 Row Reservoir to avoid contamination.

    b. Cap the sample strip tubes on Deck C columns 1 and 3.

    c. Place the strip tubes into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly.

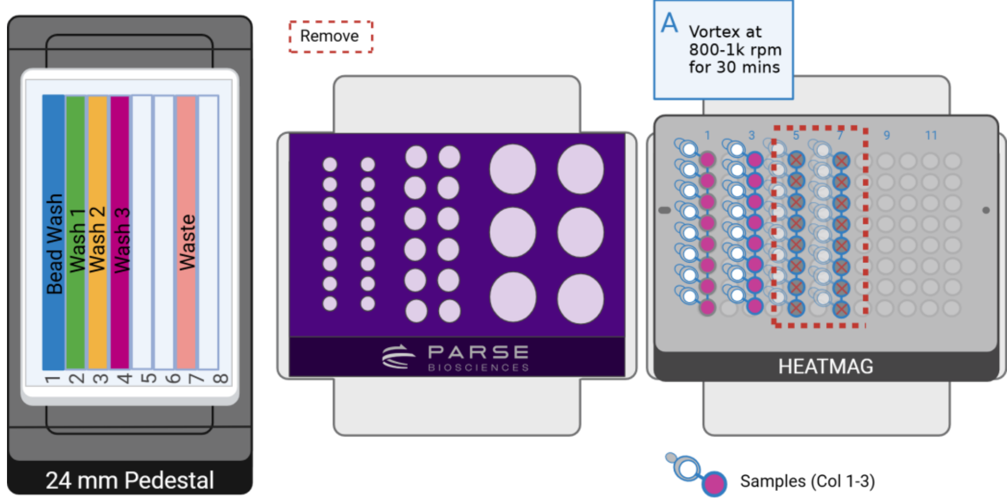
d. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex on 100% power for **1 minute**.

e. 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.

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

g. Discard the used strip tubes in columns 5 and 7 on Deck C and proceed to Section 2.3.



2.3. Streptavidin Beads Wash

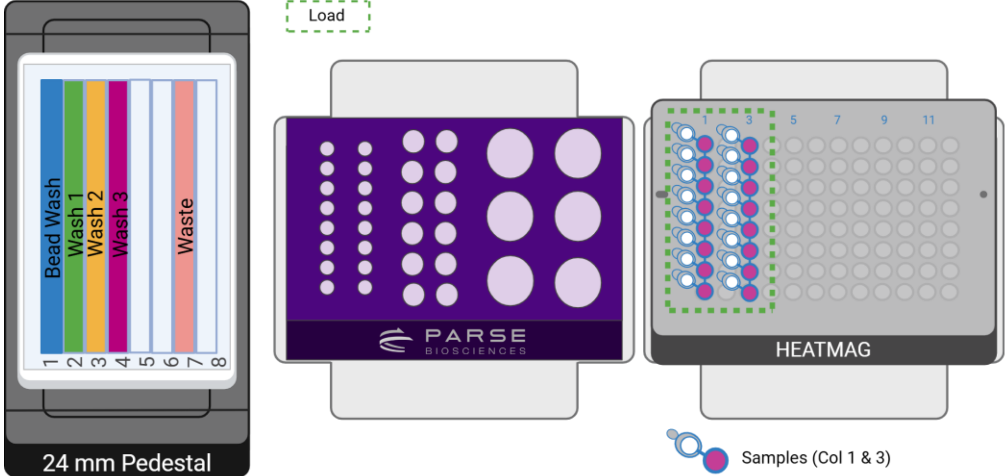
Captured cDNA samples are washed to remove cellular debris.

1. Briefly centrifuge the captured cDNA sample tubes for **30 seconds** at 100 x g at 4°C.

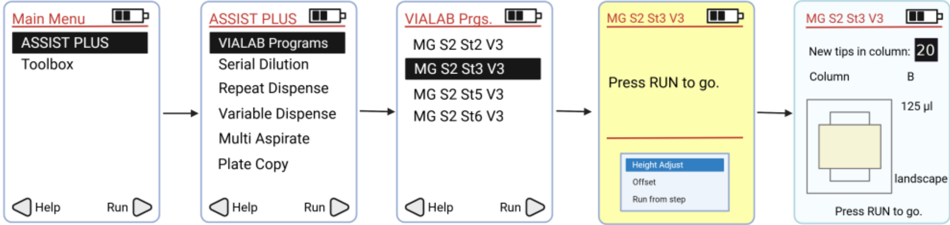
2. Uncap and place the sample tubes back on the HEATMAG with 96 Well Adapter on Deck C, columns 1 and 3.

Note: Ensure the PCR strip tube caps are facing the same direction. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.

3. Uncover the 8 Row Reservoir. The deck layout should correspond to the configuration below.



4. Select and run the program **MG S2 St3 V3_5** following the diagram below.



5. At the conclusion of the run, 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.

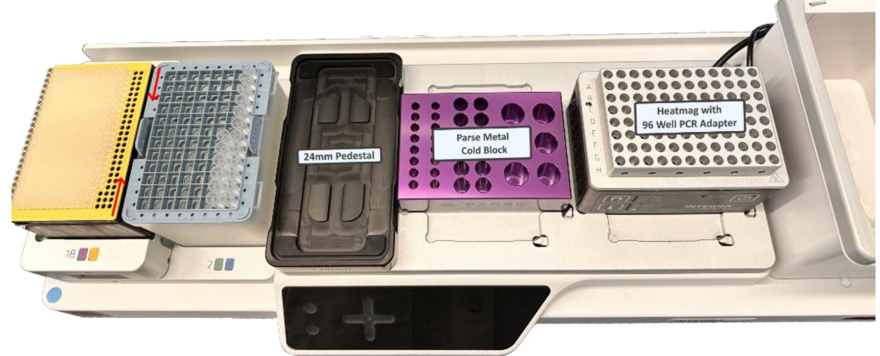
To prepare reagents:

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

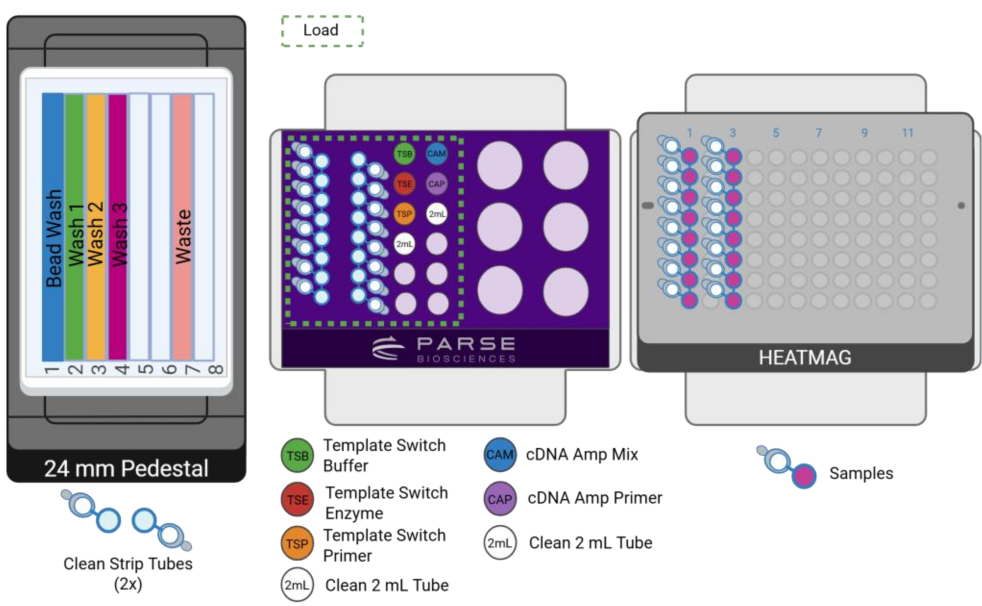
ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Component	1	
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
300 mL Reservoir Base	INTEGRA Component	1	
8-count PCR strip tubes	Consumables	2	
2 mL tubes	Consumables	2	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
● 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	

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. On Deck B, on the Parse Metal Cold Block, place the following consumables and reagents using the deck configuration below:
    a. Column 1, left and right: two clean 8-count PCR strip tubes.
    b. Column 2:
        i. Pos 1: ● Template Switch Buffer.
        ii. Pos 2: ● Template Switch Enzyme.
        iii. Pos 3: ● Template Switch Primer.
        iv. Pos 4: a clean 2 mL tube.
        v. Pos 7: ● cDNA Amp Mix.
        vi. Pos 8: ● cDNA Amp Primers
        vii. Pos 9: a clean 2 mL tube.

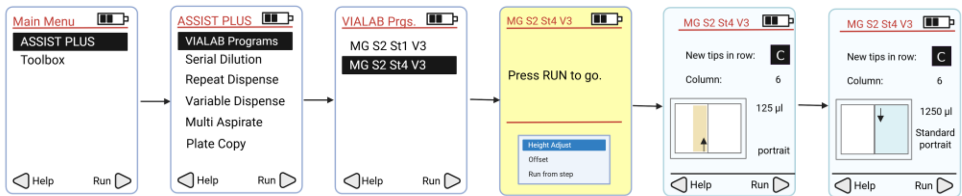


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    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.

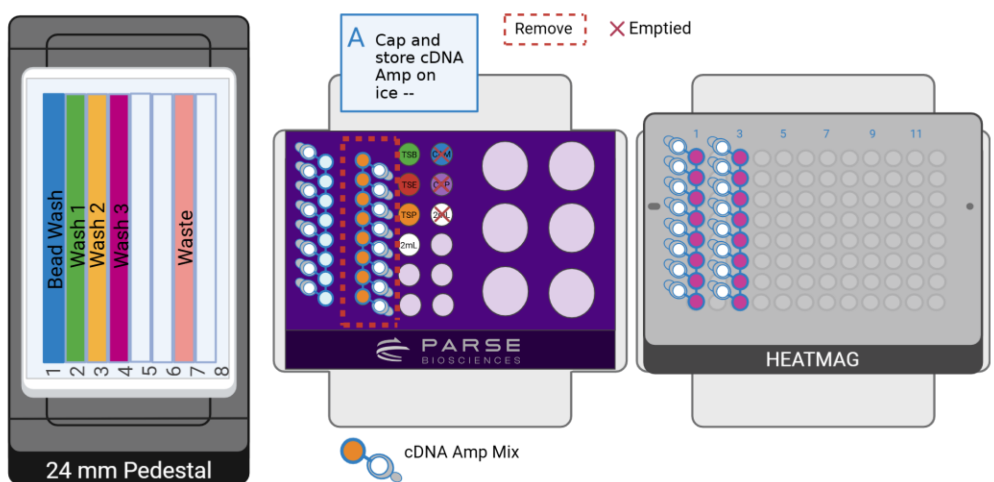
    b. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure the tip deck and tip boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lids prior to starting the program.

5. Remove the reagent caps, select and run the program **MG S2 St4 V3_5** following the diagram below.



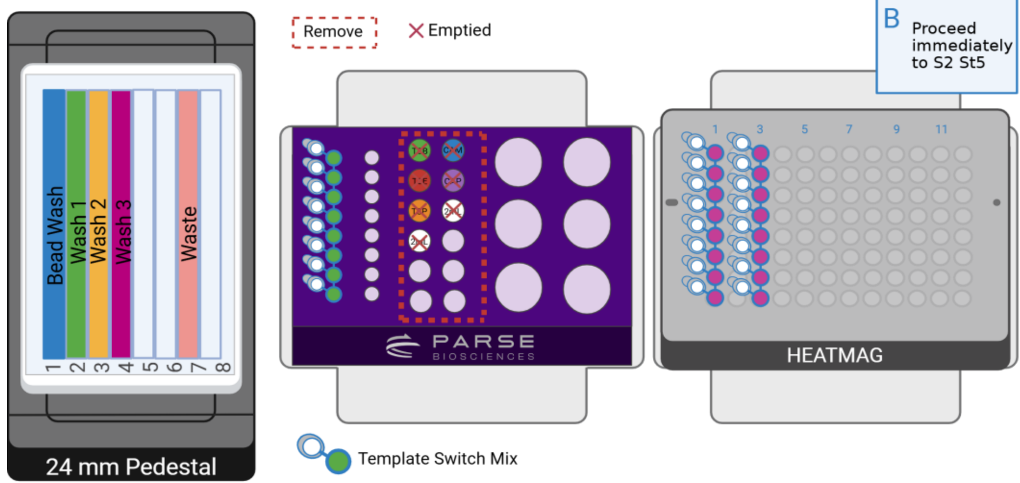
6. Press "Run" to continue the program. Follow the program prompts for manual intervention:

    a. Cap, label, and store the cDNA Amplification Master Mix dispensed in the right PCR strip tube on Deck B, column 1, right side on ice. Verify the volume is even. Press "Run" to continue.



7. At the conclusion of the run:

    a. Verify that the volume of the Template Switch Master Mix in the left PCR strip tube on Deck B, column 1 left is even (~110 µL). Keep it on this position as it will be used in the next step.



    b. Remove and discard used 1.5 mL and 2 mL reagent tubes on Deck B, column 2.

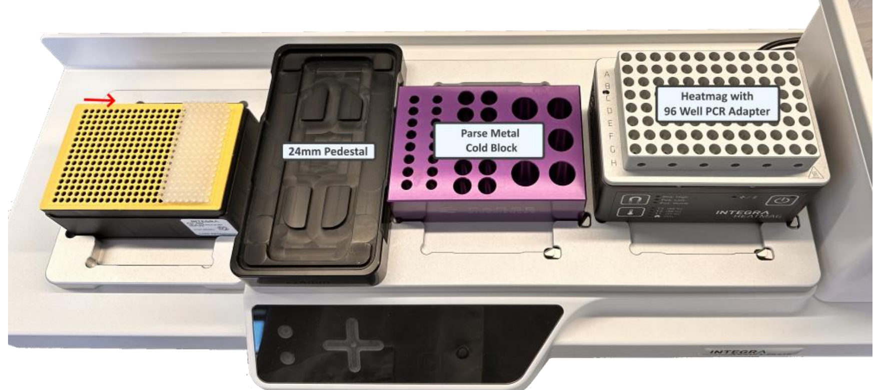
2.5. Template Switch and cDNA Amplification

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.

To perform template switch:

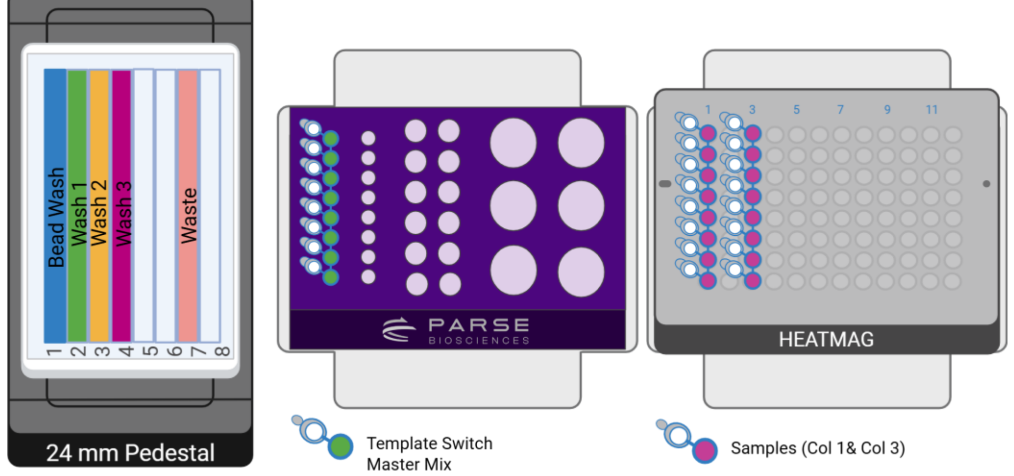
1. Gather the following items and set up the deck indicated:

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



2. Ensure the following are loaded following the deck configuration below:
    a. Deck B, column 1, left: 8-count PCR strip tube with Template Switch Master Mix.
    b. Deck C, columns 1 and 3: Samples on the HEATMAG with 96 Well PCR Adapter.

Note: Ensure the PCR strip tube caps are facing the same direction. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.

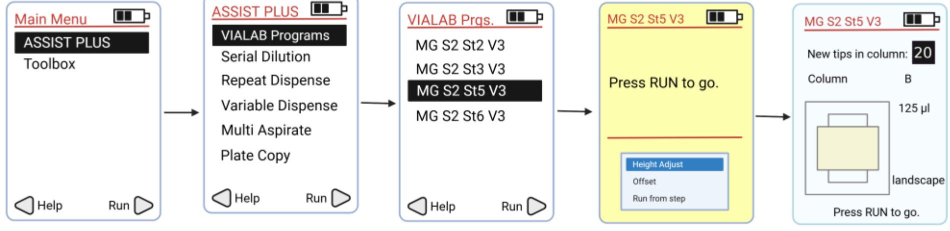


3. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

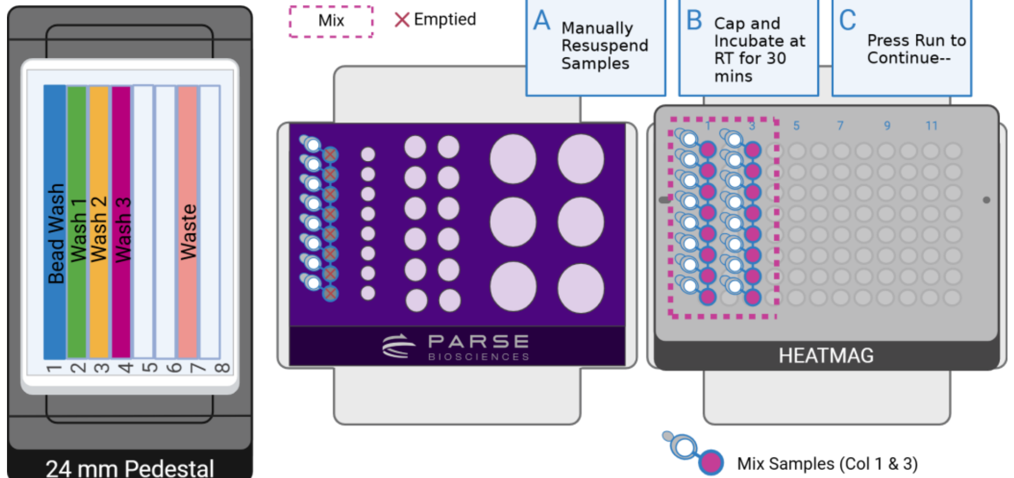
   a. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

   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.

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



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



a. Remove the samples from the deck and manually mix to fully resuspend the streptavidin pellet in the Template Switch Master Mix. Avoid introducing bubbles.

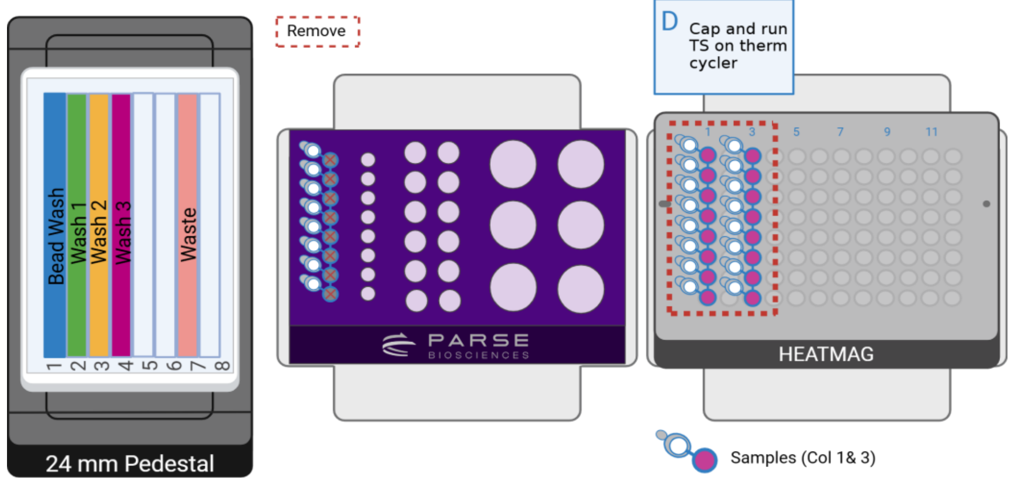
Note: The Template Switch Master Mix is viscous, which can make resuspending the beads challenging. Do not directly touch the bead pellet with the pipette tip, as the beads may stick to it.

b. Cap the sample strip tubes on Deck C column 1. Incubate the cDNA samples on Deck C, columns 1 and 3 at room temperature for **30 minutes**. Add a PCR plate seal to the reagent reservoir on Deck A during the 30 minute incubation.

c. After the 30 minute incubation, uncap the sample strip tube caps and remove the plate seal from the reservoir. Press "Run" to continue the program.

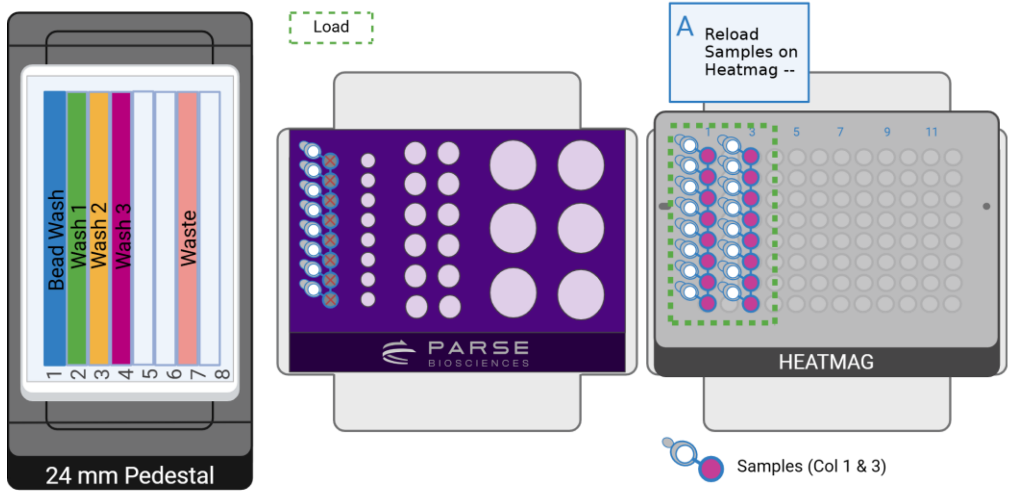
Note: Ensure the PCR strip tube caps are facing the same direction. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.

6. When prompted, remove the samples from the Deck C, columns 1 and 3 and place them into a thermocycler. Run the following program.



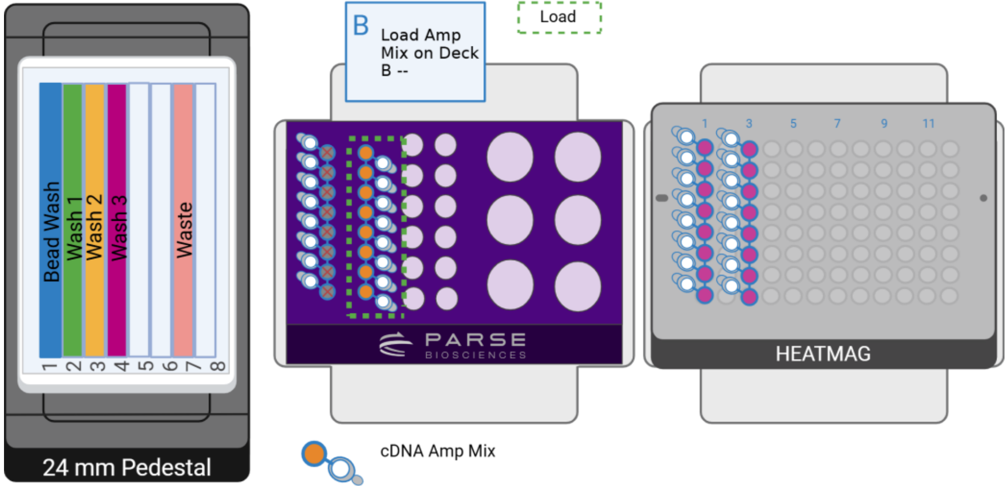
TEMPLATE SWITCH		
Run Time	Lid Temperature	Sample Volume
60 min	70°C	100 µL
Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

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

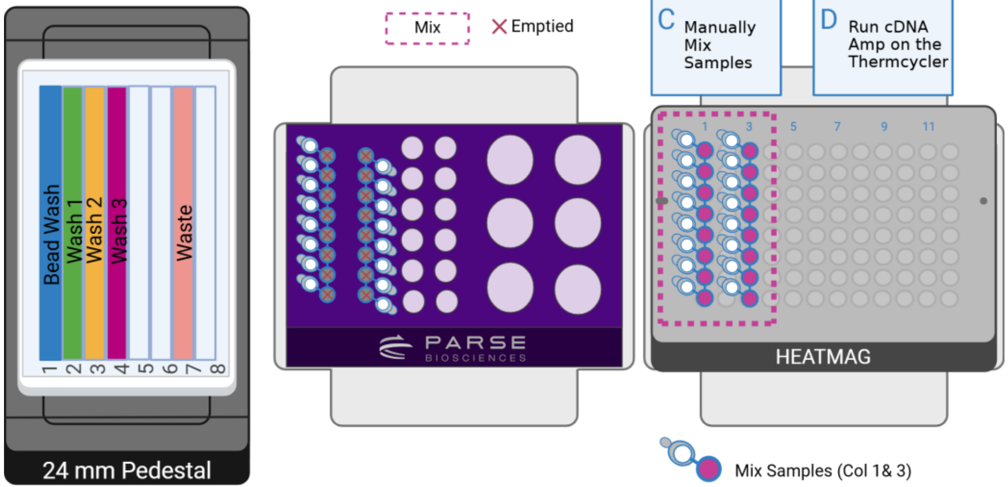


a. When the Template Switch thermocycling program has completed, remove the cover from the 8 Row reservoir on Deck A and reload the samples on the HEATMAG on Deck C columns 1 and 3.

Note: Ensure the PCR strip tube caps are facing the same direction to the left. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.



b. Place the cDNA Amplification Master Mix from section 2.4.6 back into Deck B, column 1, right. Press "Run" to continue the program.



c. Remove the samples from the deck and manually mix to fully resuspend the streptavidin pellet in the cDNA Amp Mix. Avoid introducing bubbles.

d. Use the guidelines below to amplify the cDNA samples.

8. 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
200-1,000	11	13	12
1,000-2,000	9	11	10
2,000-6,000	7	9	8
6,000-12,500	6	8	7
12,500-25,000	4	6	5
25,000-62,000	3	5	4

9. When prompted, place the sample strip tube into thermocycler and run the following program.

cDNA AMPLIFICATION			
Run Time	Lid Temperature	Sample Volume	
50-70 min	105°C	100 µL	
Step	Time	Temperature	Cycles
1	3 min	95°C	1
2	20 sec	98°C	5
3*	45 sec	65°C*	
4	3 min	72°C	
5	20 sec	98°C	Variable, see above
6*	20 sec	67°C*	
7	3 min	72°C	
8	5 min	72°C	1
9	Hold	4°C	1

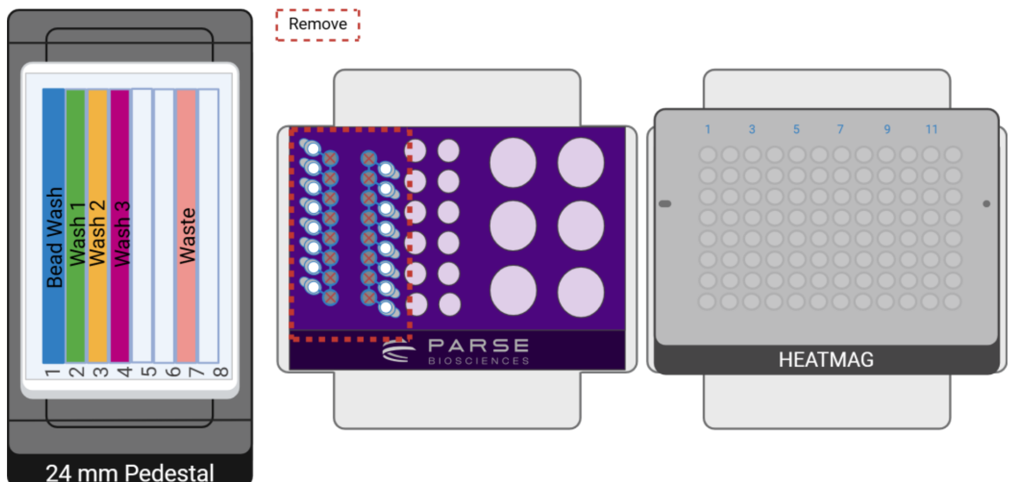
CRITICAL! If processing sublibraries with different numbers of cells/nuclei, they should be amplified in separate thermocyclers according to the recommendations above.

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. We recommend covering the 8 Row Reservoir with a PCR plate seal during this time to reduce contamination.



Safe stopping point: Amplified cDNA can be stored at 4°C for up to 18 hours.

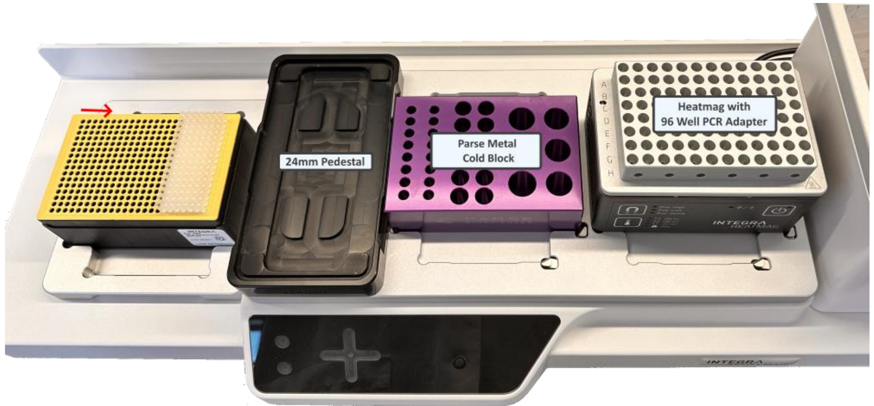
2.6. Post-Amplification Purification

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

To purify the cDNA:

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 Component	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Component	1	
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
300 mL Reservoir Base	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	
8-count PCR strip tubes	Consumables	4	
Ethanol	Consumables and Reagents		
Nuclease free water	Consumables and Reagents		
SPRI Beads	Consumables and Reagents		



2. Prepare at least **8 mL** 85% ethanol with nuclease-free water.

3. With a P1000 pipette set at 1,000 µL, add:

    a. **8 mL** 85% Ethanol in lane **5** of the 8 Row Reservoir on Deck A.

    b. **3.5 mL** nuclease free water in lane **6** of the 8 Row Reservoir on Deck A.

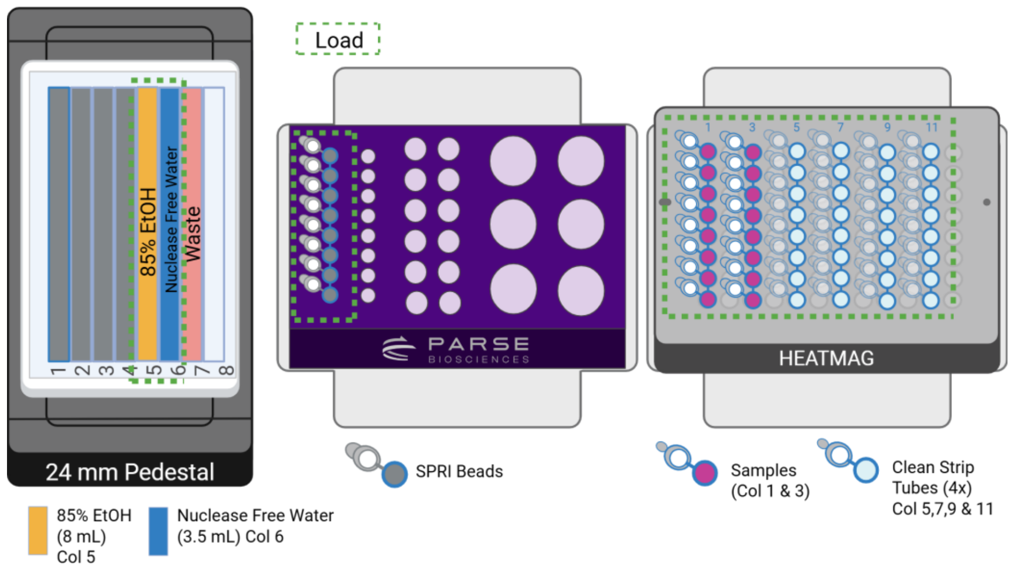
4. Place the dispensed SPRI beads in strip tubes from Section 2.1.17c on Deck B, column 1 (left) on the Parse Metal Cold Block. If the beads have settled, they should be manually resuspended prior to loading them on the Parse Metal Cold Block.

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

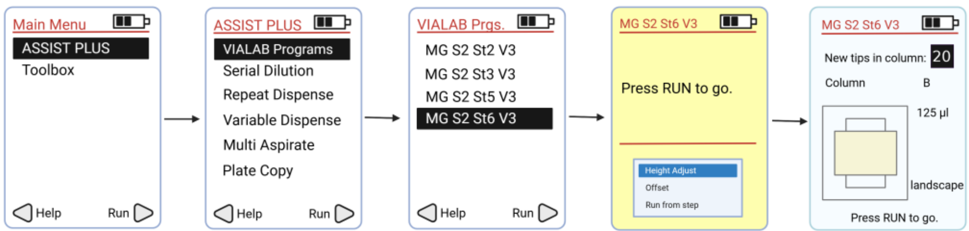
5. Once the cDNA Amplification thermocycler program is complete, place the sample strip tubes in columns 1 and 3 on the HEATMAG with 96 Well Adapter on Deck C.

Note: Ensure the PCR strip tube caps are facing the same direction. Push the caps wide open to avoid interference with the pipette's tips.

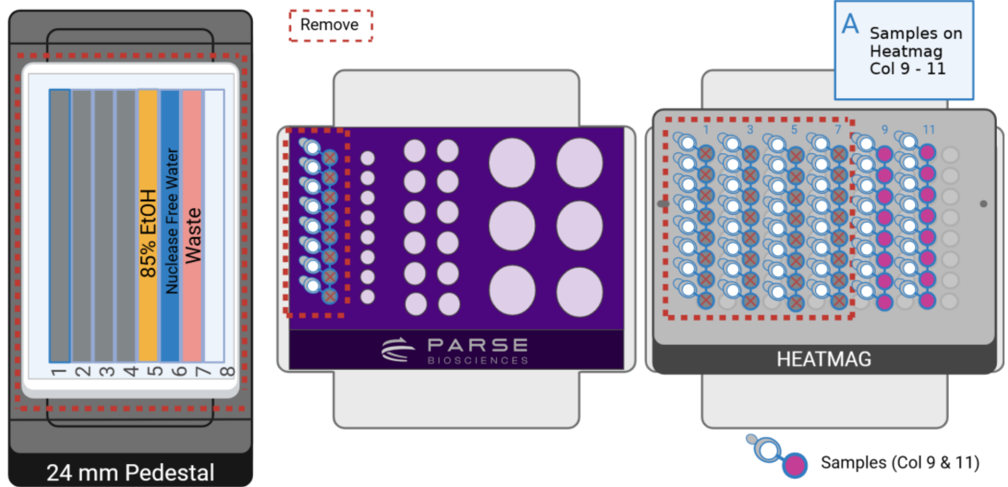
6. Place 4 new 8-count PCR strip tubes in columns 5, 7, 9, and 11 on Deck C. The deck layout should correspond to the Deck Configuration below.



7. Select and run program **MG S2 St6 V3_5** following the diagram below.



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



   a. Final cDNA libraries are on the HEATMAG with 96 Well Adapter in columns 9 and 11. Remove and discard the empty strip tubes and the 8 row reservoir.

Safe stopping point: Amplified cDNA 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.

To quantify the cDNA:

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.

STOP Safe stopping point: purified cDNA 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.

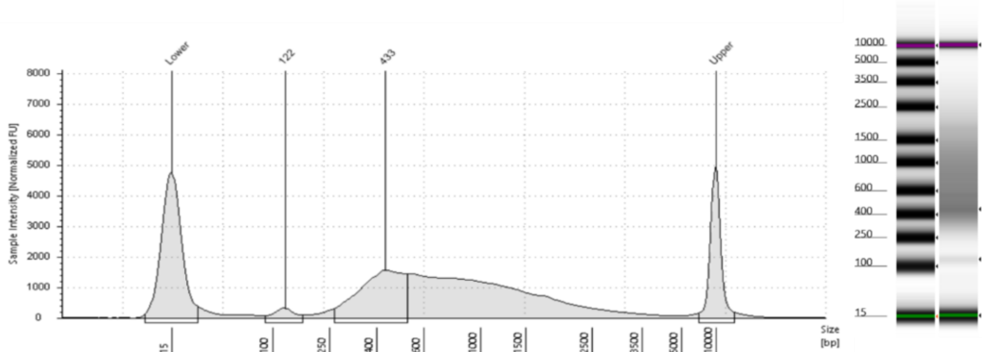


Figure 8: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

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

3.0. cDNA Normalization (Optional)

Prior to starting Section 3, cDNA can optionally be normalized to ensure that all sublibraries fall within the same amplification condition. It is advantageous to have all sublibraries use the same amplification condition because Section 3 processing occurs in a 96 well PCR plate instead of PCR strip tubes. The program uses nuclease free water on Deck B2 to normalize amplified cDNA from tube strip(s) on Deck B.

If choosing NOT to normalize amplified cDNA:

Manually prepare Diluted cDNA in columns 1 and 2 of a Semi-Skirted 96 Well PCR Plate as follows to a final volume of 35 µL and store on ice. Store any remaining purified cDNA at -20°C.

DILUTED cDNA	
Purified cDNA	10 µL
Nuclease-free water	25 µL
Total Volume	35 µL

To normalize amplified cDNA:

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 Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
Nuclease free water	Reagents and Consumables		

2. If frozen, thaw the amplified cDNA and store it on ice.

3. Download the Parse Biosciences Evercode WT Mega Integra Normalization file. The most current version can be found on the Parse Biosciences Customer Support Suite.

4. Obtain recorded cDNA concentrations from Section 2.7.

5. Fill out the following cells of the Parse Biosciences Evercode Mega/WT Integra Normalization v1.0.xlsm. Target sample volume should be around 10 µL (Figure 9).

    a. Sample

    b. Source Well

    c. Concentration (ng/µL)

    d. Library Input (ng)

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

Parse Biosciences
700 Dexter Ave
Suite 600
Seattle, WA 98109

Support Suite: support.parsebiosciences.com
Email: support@parsebiosciences.com
WT Mega - Version 2.0

Evercode WT cDNA Normalization Loading Table

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

This sheet should be filled out prior to starting Section 3.

Step	Instructions
1	Ensure Macros are enabled.
4	Input your sample names.
6	Input source wells location.
6	Input destination well location.
6	Input cDNA concentration in ng/uL.
7	Input total ng library prep input.
8	CRITICAL: Ensure Sample Volume (uL) is between 4 to 25 uL. Larger sample volume leads to higher sublibrary complexity.
9	Open the "Integra Loading Table" sheet. Click on the "Generate a cDNA Normalization Worklist for Import into VIALAB" to generate the worklist file.

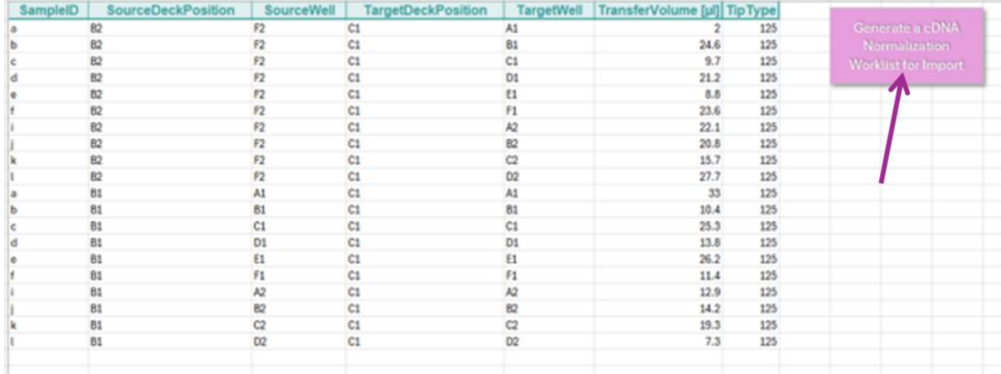
Number of PCR Cycles	
cDNA Input (ng)	PCR Cycles
10 - 24	13
25 - 49	12
50 - 99	11

Number of PCR Cycles	
cDNA Input (ng)	PCR Cycles
100 - 299	10
300 - 999	8
1,000 or more	7

Sample	Source Well	Destination Well	Conc. (ng/uL)	Library Input (ng)	Sample Volume (uL)	Diluent Volume (uL)
a	A1	A1	10.28	339	33.0	2.0
b	B1	B1	9.64	100	10.4	24.6
c	C1	C1	3.96	100	25.3	9.7
d	D1	D1	7.26	100	13.8	21.2
e	E1	E1	3.82	100	26.2	8.8
f	F1	F1	8.78	100	11.4	23.6
g	G1	G1				
h	H1	H1				
i	A2	A2	7.74	100	12.9	22.1
j	B2	B2	7.02	100	14.2	20.8
k	C2	C2	5.18	100	19.3	15.7

Figure 9: Evercode WT Mega cDNA normalization loading table.

6. Navigate to the “INTEGRA Loading Table” tab on the excel sheet, and click on “Generate a cDNA Normalization Worklist for Import”. Save the generated CSV file (called Section3NormWTWorksheet_xxxxxxx_xxxxxxx.csv) (Figure 10).



SampleID	SourceDeckPosition	SourceWell	TargetDeckPosition	TargetWell	TransferVolume [µl]	TipType
a	B2	F2	C1	A1	2	125
b	B2	F2	C1	B1	24.6	125
c	B2	F2	C1	C1	9.7	125
d	B2	F2	C1	D1	21.2	125
e	B2	F2	C1	E1	8.8	125
f	B2	F2	C1	F1	23.6	125
i	B2	F2	C1	A2	22.1	125
j	B2	F2	C1	B2	20.8	125
k	B2	F2	C1	C2	15.7	125
l	B2	F2	C1	D2	27.7	125
a	B1	A1	C1	A1	33	125
b	B1	B1	C1	B1	10.4	125
c	B1	C1	C1	C1	25.3	125
d	B1	D1	C1	D1	13.8	125
e	B1	E1	C1	E1	26.2	125
f	B1	F1	C1	F1	11.4	125
i	B1	A2	C1	A2	12.9	125
j	B1	B2	C1	B2	14.2	125
k	B1	C2	C1	C2	19.3	125
l	B1	D2	C1	D2	7.3	125

Figure 10: Generated cDNA normalization worklist.

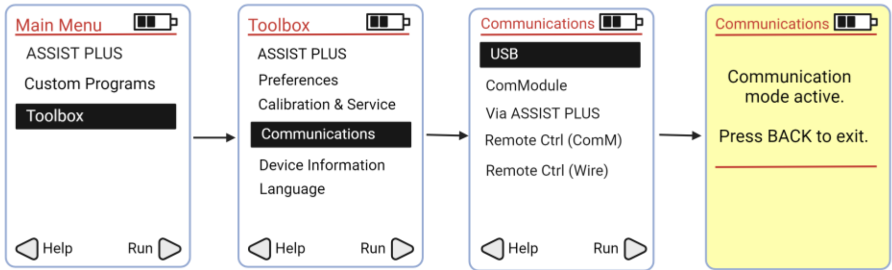
7. Open the VIALAB program **MG S3 St0 V3 DONE_5** and navigate to the “Method” section. In the “02 Worklist”, under the “Worklist and Volumes” tab, import the “Section3NormWTWorksheet_xxxxxxx_xxxxxxx.csv” worklist file generated in the previous step. The "Import" button is located in the upper left of the Worklist and Volumes tab (Figure 11).

Figure 11: VIALAB worklist generation for diluent volumes using 02 Worklist.

8. Save and transfer your program to your D-ONE Pipetting Module (1-Ch, 5-1250 µL) as follows:

    a. Connect your computer with the D-ONE Pipette using Communication/Charging.

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



    c. In the VIALAB software on your computer, select “Transfer”.

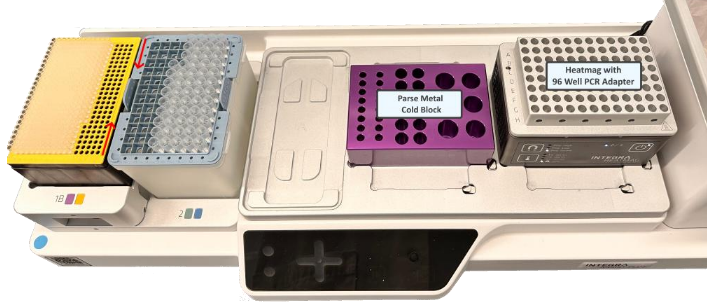
    d. Confirm that the pipette has been recognized by the software. Click "Send to pipette". An audible signal will indicate the successful upload of the **MG S3 St0 V3_5** program to the D-ONE Pipette.

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

    f. A program named **MG S3 St0 V3_5** will be found on your pipette.

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

9. Set up the deck following the deck configuration below.

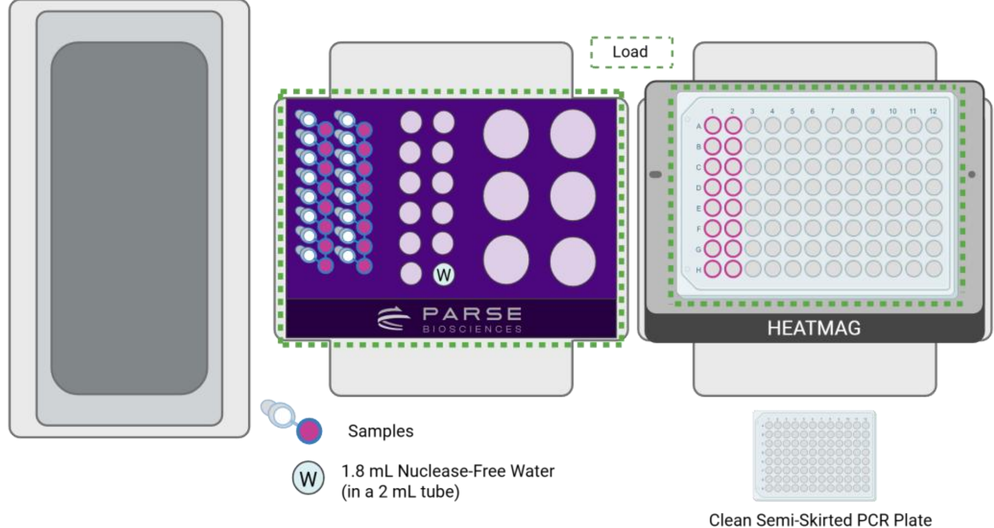


10. Set up the deck layout Deck Configuration below:

a. Briefly vortex and centrifuge the amplified cDNA and place on the Parse Metal Cold block on Deck B, column 1, left and right.

b. Fill a clean 2 mL tube with 1.8 mL nuclease-free water and place on the Parse Metal Cold Block on Deck B, column 2 position 12.

c. Place a clean semi-skirted plate on the HEATMAG with 96 Well Adapter on Deck C.

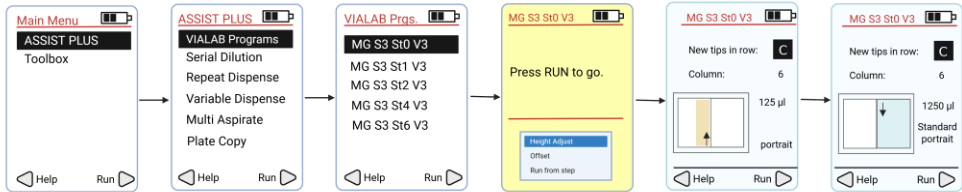


11. If needed, change the pipette:

    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 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.

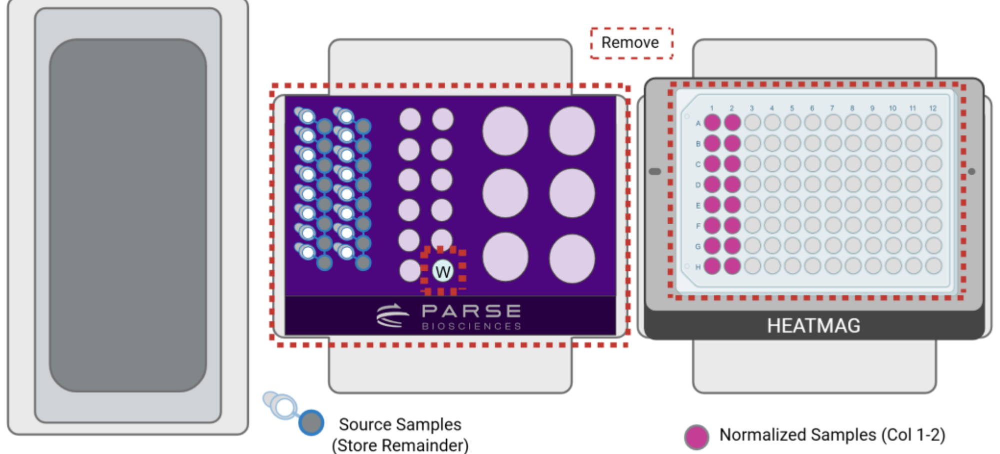
12. Select and run program **MG S3 St0 V3_5** following the diagram below.



13. At the conclusion of the run:

a. The remaining cDNA samples from Deck B, column 1 left can be stored at -20°C.

b. Store the normalized cDNA sample plate on Deck C on ice.



3.1. SPRI Bead Plating

The SPRI beads are aliquoted to be used throughout all of Section 3. Aliquots can then be quickly accessed via multichannel pipette when needed. SPRI beads should be kept at room temperature.

1. Gather the following components and reagents:

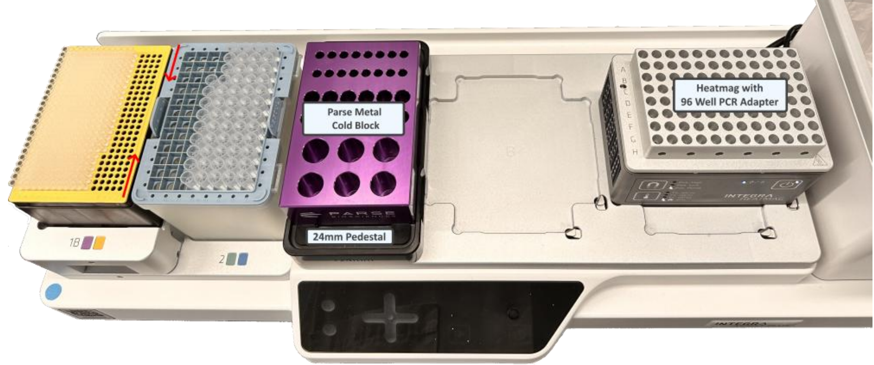
ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Component	1	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
2 mL tube	Consumables	2	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
SPRI Beads	Consumables and Reagents		

2. If not connected already, 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.

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

4. If not connected already, connect the HEATMAG on Deck C.

5. Move the Parse Metal Cold Block onto the 24 mm Labware pedestal on Deck A. The deck layout should correspond to the configuration below:

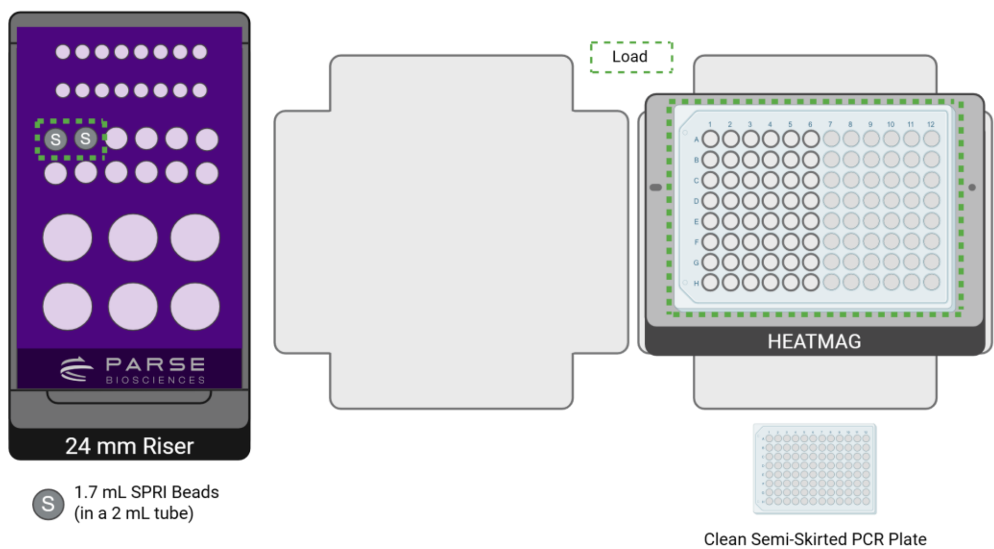


6. Ensure the following are loaded, using the deck configuration below:

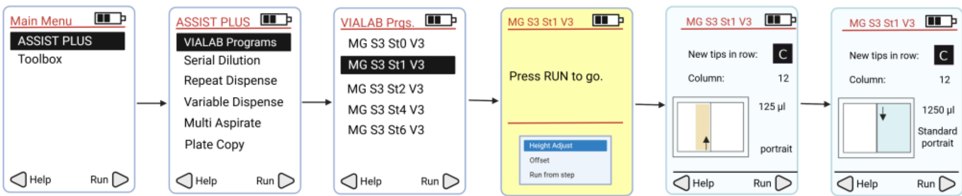
   a. Deck A column 1:

      i. Pos 5 and 6: two 2 mL tubes with **1,700 µL** fully resuspended SPRI beads each. If the beads have been sitting overnight, they should be manually resuspended prior to loading them on the Parse Metal Cold Block.

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



7. Select and run the program **MG S3 St1 V3_5** following the diagram below.



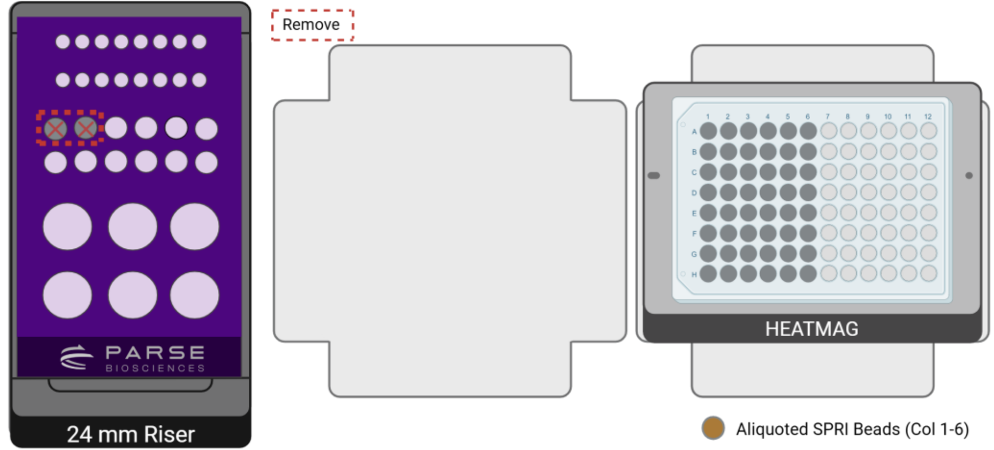
8. Wait for the program to complete.

9. Start thawing the Thermochromic PCR Cold Block with Riser stored at -20°C that will be used in the next step.

10. At the conclusion of the run:

    a. Leave the D-ONE pipette connected to the instrument. If proceeding immediately to Section 3.2, leave the plate with beads on the HEATMAG. Otherwise, remove the plate with beads and keep it aside at room temperature.

    b. Discard the used tubes on Deck A. Remove and store the Parse Metal Cold Block on ice.



3.2. Fragmentation Mix Creation and Plating

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

1. Gather the following components and reagents:

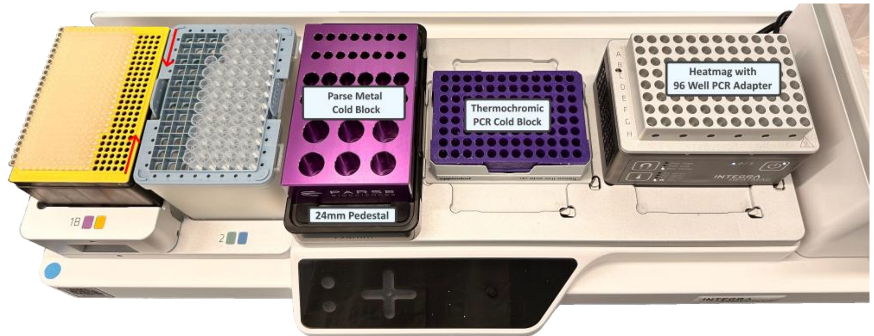
ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse	1	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse	1	
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
1.5 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.

2. 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.

3. If not done already, remove both Thermochromic PCR Cold Block with Riser from -20°C and thaw at room temperature for **10 minutes**.

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

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



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

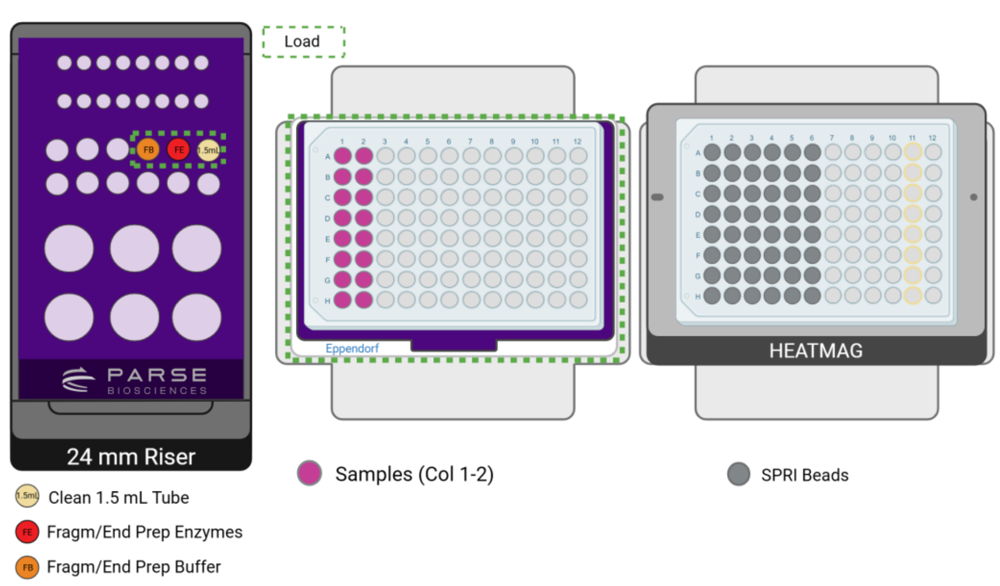
    a. Deck A, column 1:

        i. Pos 1: a clean 1.5 mL tube.

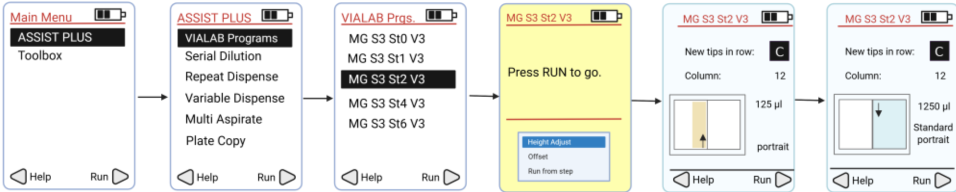
        ii. Pos 2: ● Fragm/End Prep Enzymes.

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

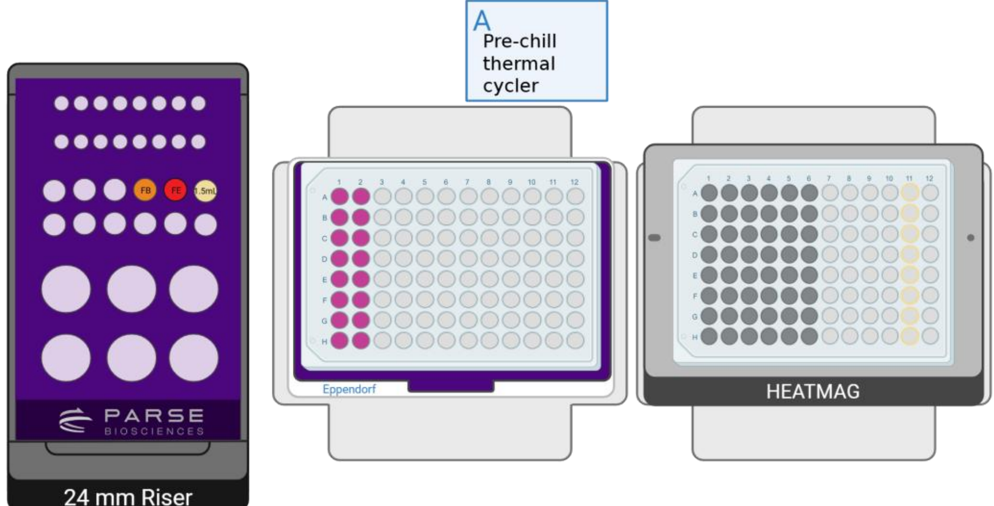
    b. Transfer the cDNA sample plate stored on ice from Section 3.0 to the Thermochromic PCR Cold Block on Deck B with A1 in the upper left corner, pressing firmly to ensure the plate is fully seated.



7. Remove the reagent caps, then select and run the program **MG S3 St2 V3_5** following the diagram below.



8. Follow the program prompts for manual intervention:



a. 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 3.3.6. Press "Run" on the pipette to continue.

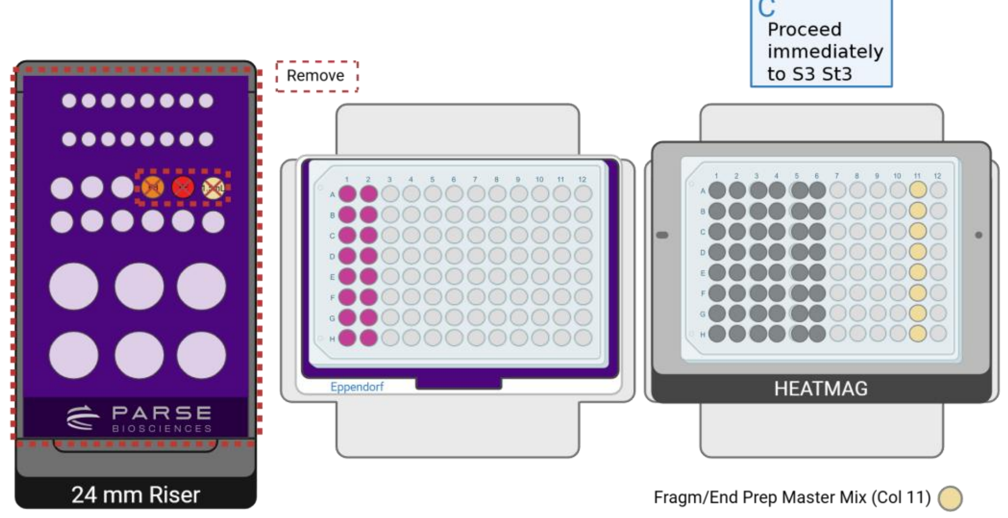
FRAGMENTATION AND END PREP		
Run Time	Lid Temperature	Sample Volume
40 min	70°C	50 µL
Step	Time	Temperature
1	Hold	4°C
2	10 min	32°C
3	30 min	65°C
4	Hold	4°C

9. 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 3.3.



3.3. Fragmentation Mix Addition and Post-Fragmentation SPRI Cleanup

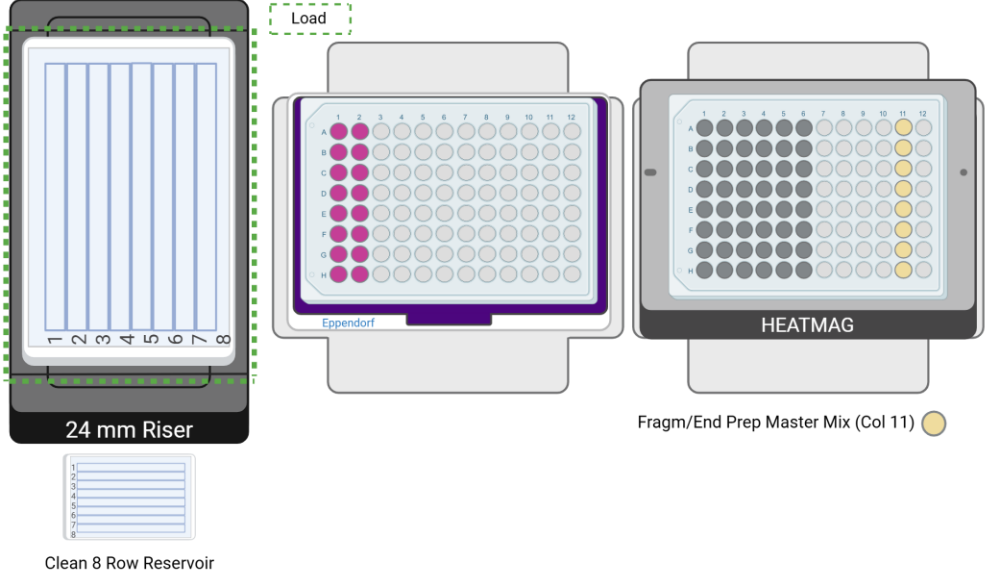
The program uses the VOYAGER multichannel pipette to add Fragmentation Mix to sublibraries. After the user runs the Fragmentation thermal cycling program, sublibraries are loaded back onto the ASSIST PLUS and a double-sided SPRI cleanup is carried out.

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, 5-1250 µL	INTEGRA Component	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	1	
24 mm Labware Pedestal	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse	2	Pull the Thermochromic PCR Cold Block with riser from the -20°C freezer. Thaw 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 Component	1	
300 mL Reservoir Base	INTEGRA	1	
8 Row Reservoir	INTEGRA	1	
125 µL Tip Rack	INTEGRA	1	
Ethanol	Consumables and Reagents		
Nuclease-free water	Consumables and Reagents		

2. Prepare at least **8 mL** of 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.

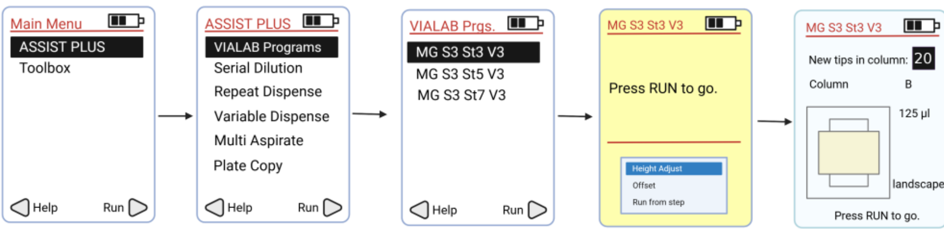


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

   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.

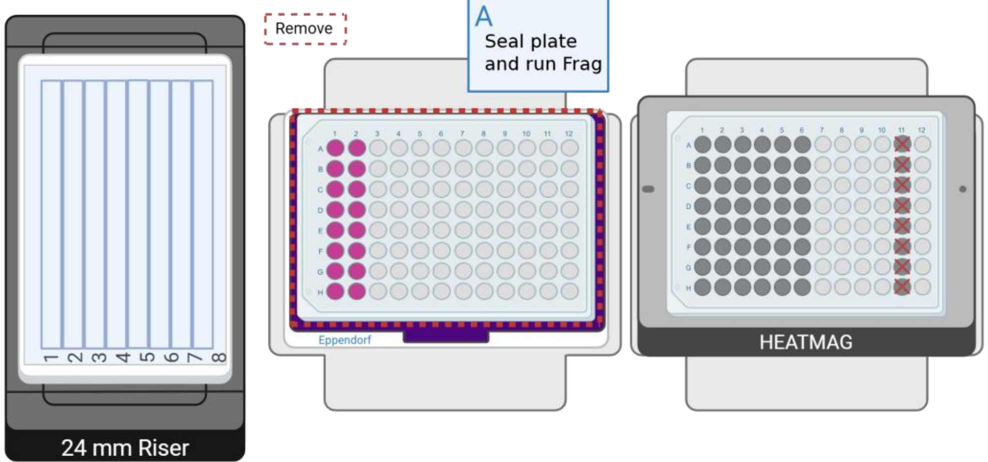
   b. Replace it with VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Ensure the Tip Deck and tip box are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

5. Select and run the program **MG S3 St3 V3_5** following the diagram below.



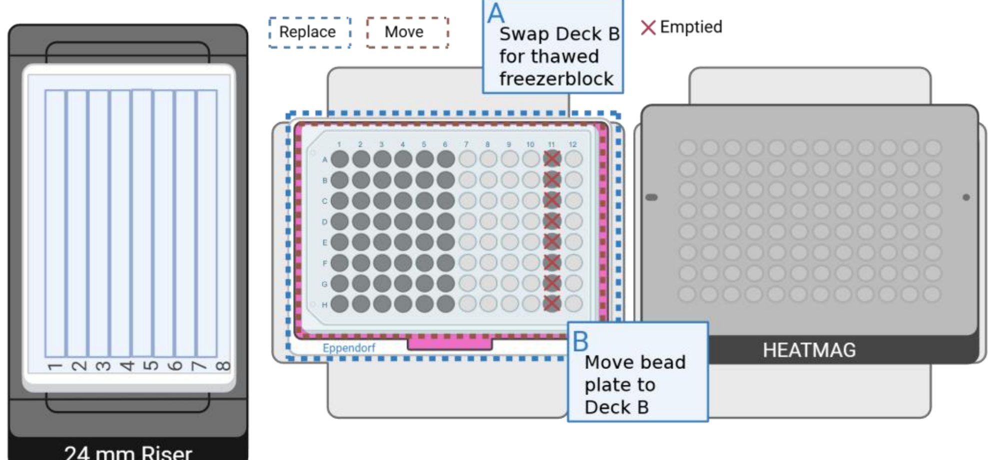
6. Press "Run" to continue the program. Follow the program prompts for manual intervention:

    a. Remove the plate from Deck B and seal with a PCR plate seal. Place the sealed plate into the pre-cooled thermocycler from Section 3.2.8a.



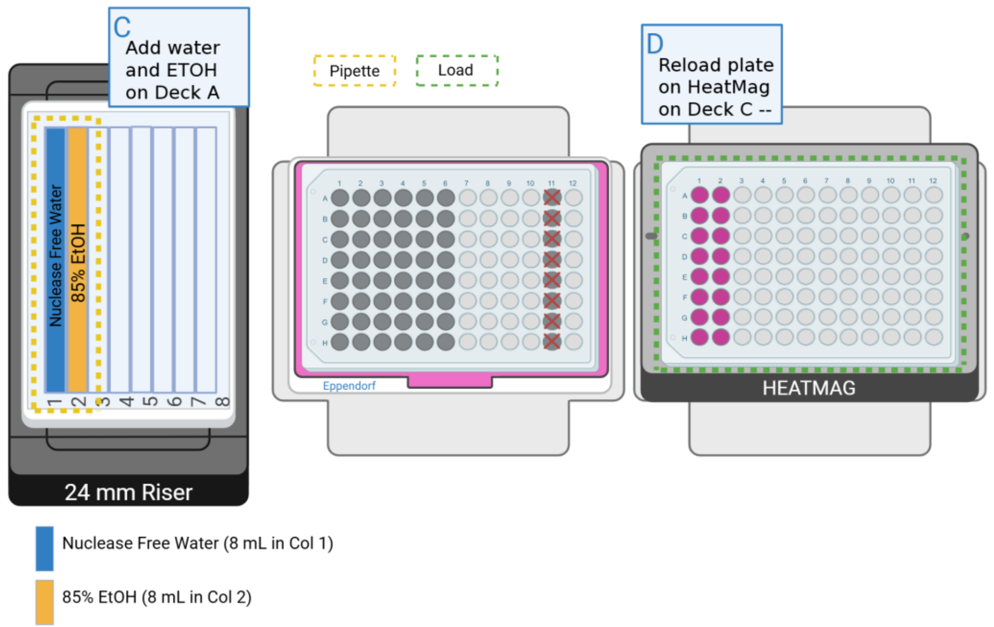
7. Ensure the thermocycler is cool prior to use and start the Fragmentation and End Prep program in the thermocycler precooled in Section 3.2.8a. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and End Prep program.

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 with the one that has been brought to room temperature. A fully thawed Thermochromic PCR Cold block should look pink in color. Press "Run" 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 P1000 pipette set to 1,000 µL add:

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

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

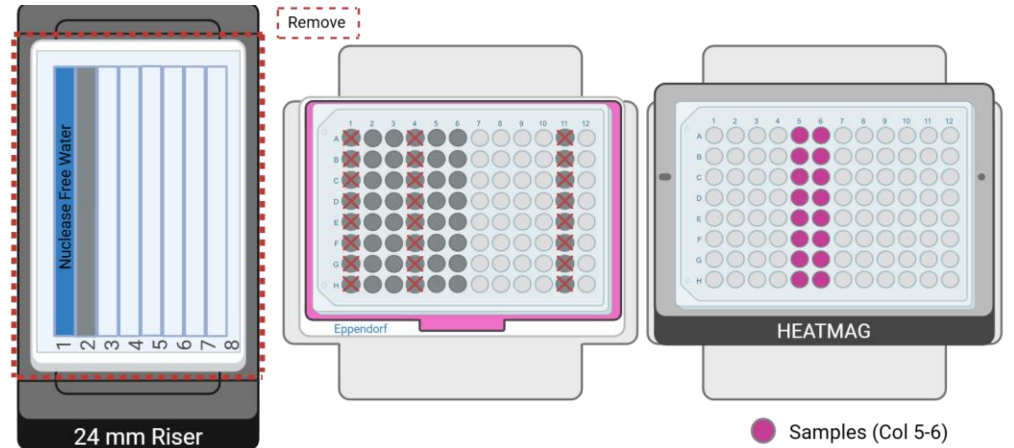
d. When Fragmentation has completed and when prompted, load the sublibrary plate onto the HEATMAG on Deck C. Ensure the nuclease free water and the 85% Ethanol are evenly distributed within their rows.

9. At the conclusion of the run:

a. Cover with a PCR plate seal and remove the 8 Row Reservoir on Deck A. 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 3.4.



Safe stopping point: The size-selected fragmented and end prepped DNA can be stored at 4°C for up to 18 hours or at -20°C for up to 2 weeks.

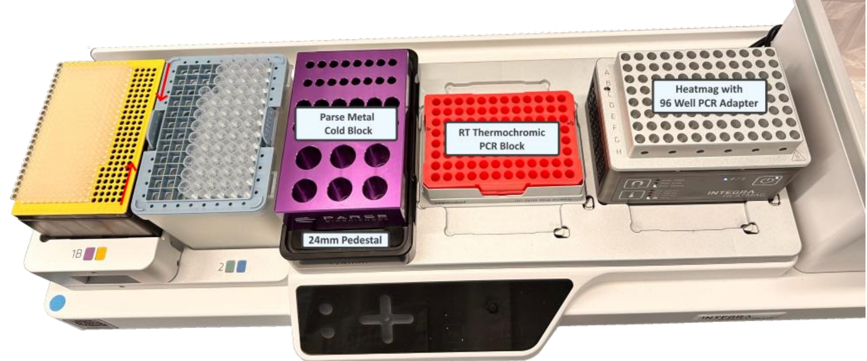
3.4. Ligation Mix Creation and Plating

The program prepares the Ligation Mix in a clean 1.5 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 Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	Parse	1	
24 mm Labware Pedestal	INTEGRA Component	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 Component	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	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:

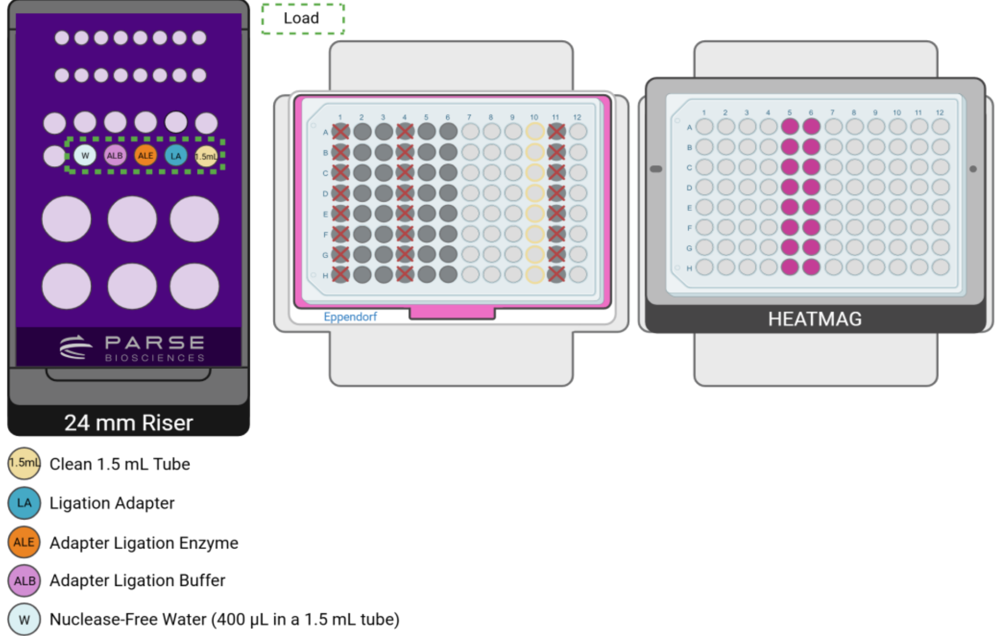
        i. Pos 7: a clean 1.5 mL tube.

        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 **400 µL** of nuclease free water.

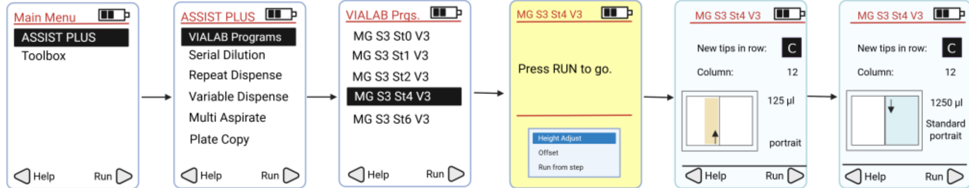


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

    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.

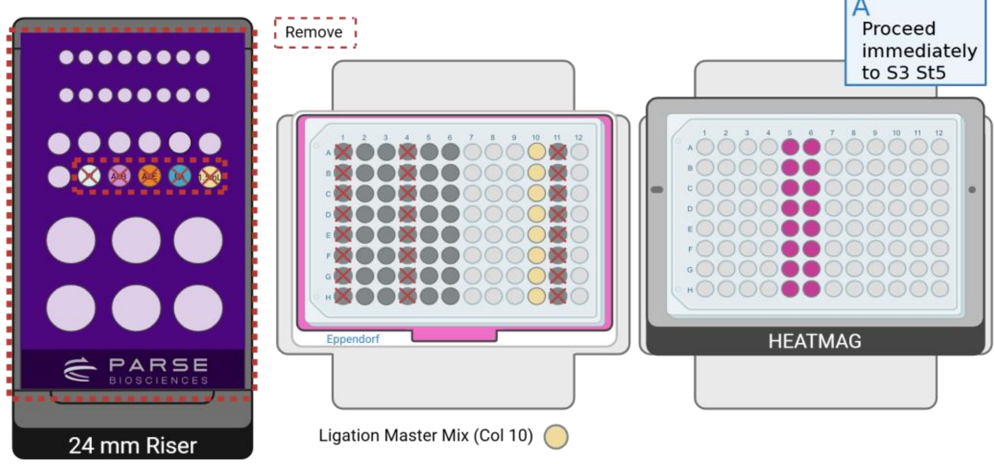
5. Remove the reagent caps, select and run the program **MG S3 St4 V3_5** following the diagram below.



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 3.5.



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

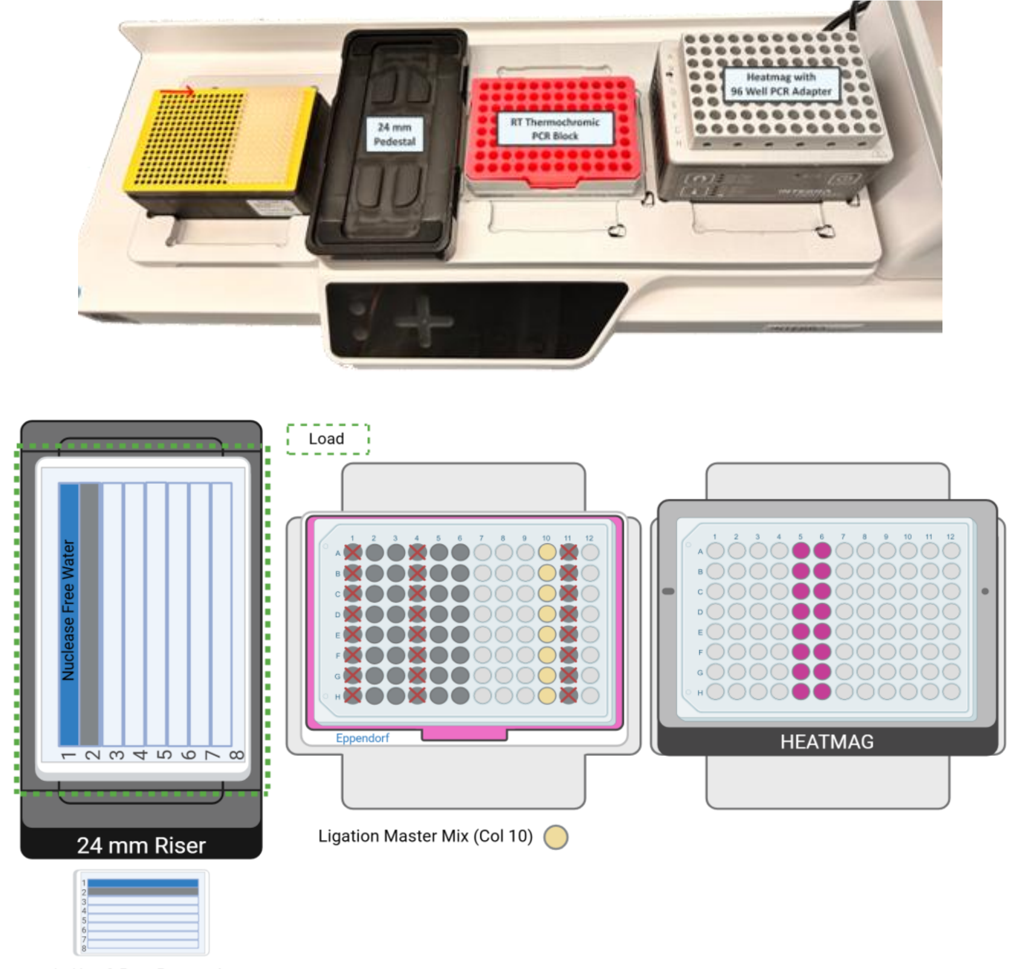
The program uses the VOYAGER multichannel pipette to add Ligation Mix to sublibraries. After the user runs the Ligation thermocycling program, sublibraries are loaded back onto the ASSIST PLUS and a single-sided SPRI cleanup is carried out.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	1	
24 mm Labware Pedestal	INTEGRA Component	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 Component	1	
300 mL Reservoir Base	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	
8 Row Reservoir	INTEGRA	1	
Ethanol	Consumables and Reagents		
Nuclease-free water	Consumables and Reagents		

2. Prepare at least **8 mL** of 85% ethanol with nuclease free water.

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

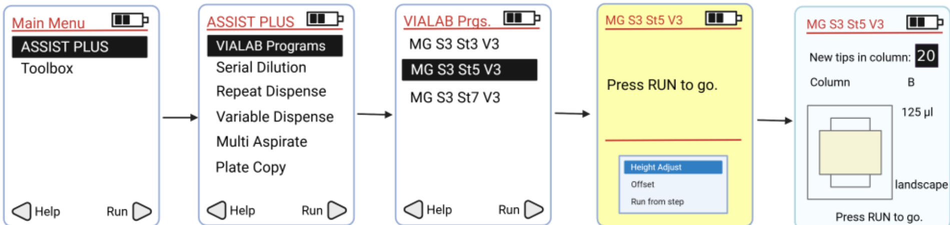


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

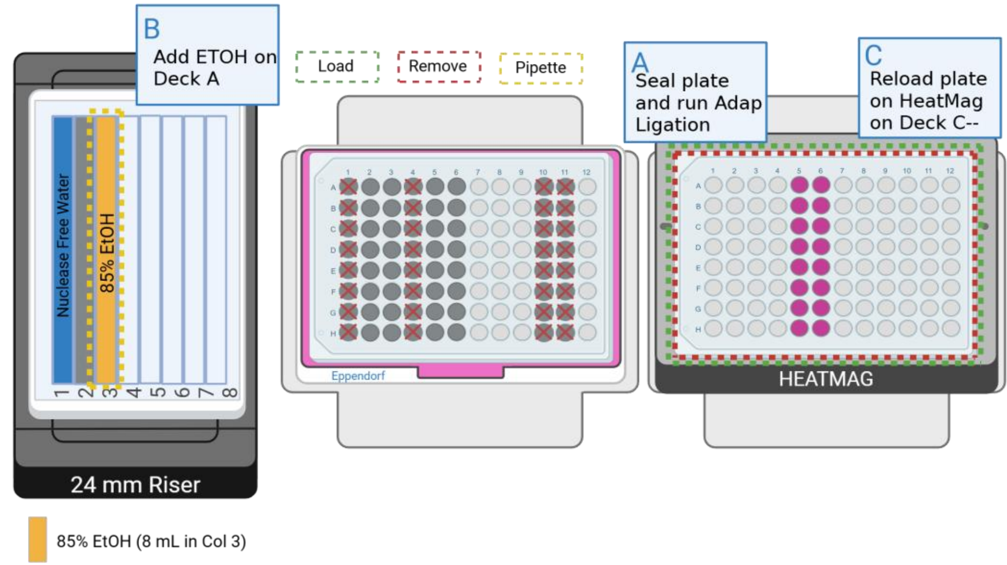
    a. Remove the D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Ensure it is disconnected by pressing the back button until the main menu is displayed.

    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.

5. Navigate to the VIALAB programs on the Voyager Pipetting Module, select and run the program **MG S3 St5 V3_5** following the diagram below.



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



a. Seal the 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 program is still running.

ADAPTER LIGATION		
Run Time	Lid Temperature	Sample Volume
15 min	30°C*	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.

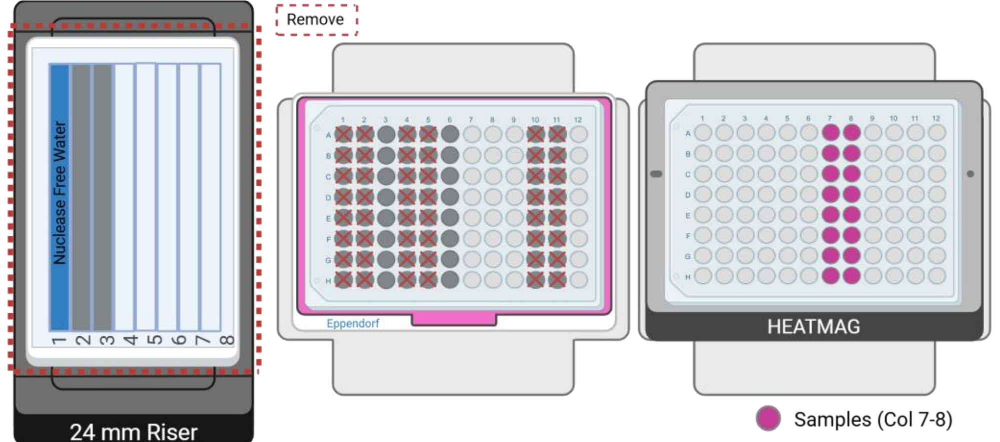
c. With a P1000 pipette set to 1000 µL, add **8 mL** 85% Ethanol to lane **3** of the 8 Row Reservoir on Deck A.

d. Upon thermocycling completion reload the sample plate onto the HEATMAG with 96 Well Adapter located on Deck C and remove the seal.

7. At the conclusion of the program:

   a. Cover with a PCR plate seal and remove the reagent reservoir on Deck A. Store at room temperature for later use.

   b. Keep all labware on Decks B and C. Proceed to Section 3.6.



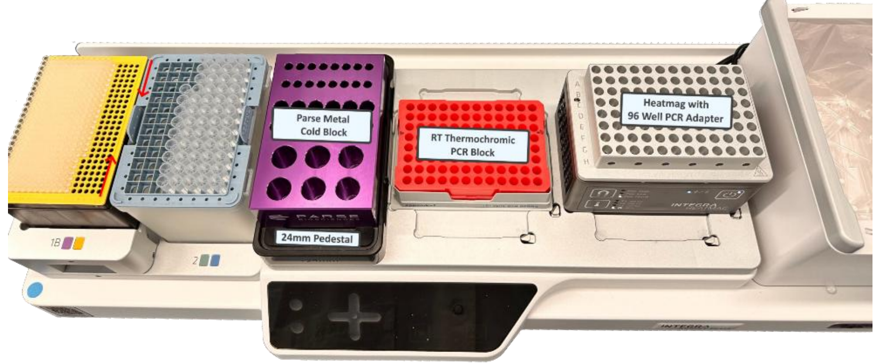
3.6. Barcoding Round 4

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.

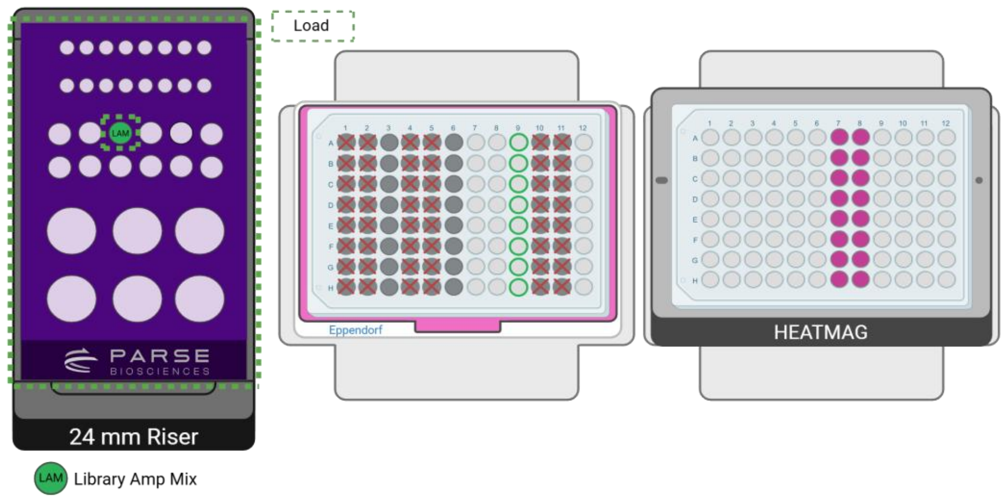
1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	1	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	1	
Parse Metal Cold Block	INTEGRA Component	1	
24 mm Labware Pedestal	INTEGRA Component	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 Component	1	
125 µL Tip Rack	INTEGRA	1	
1250 µL Tip Rack	INTEGRA	1	
● 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. Place the ● Library Amp Mix in the Parse Metal Cold Block, column 1, position 4. The deck layout should correspond to the configuration below.

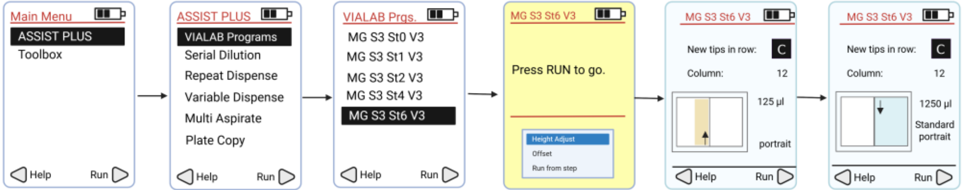


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

   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.

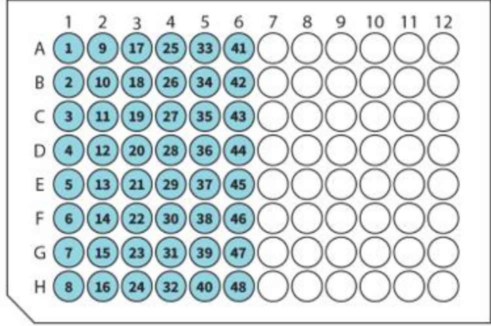
5. Uncap the reagents caps, select and run the program **MG S3 St6 V3_5** following the diagram below.



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

7. Wipe the surface of the 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. With a multichannel P20, manually pierce the seal of the chosen wells of the UDI Plate - WT.

10. **When prompted**, with a multichannel P20 and new tips, manually mix by pipetting 5x then immediately transfer **4 µL** from a chosen unused well of the UDI Plate - WT to its corresponding sample well in columns 7 and 8 on Deck C.

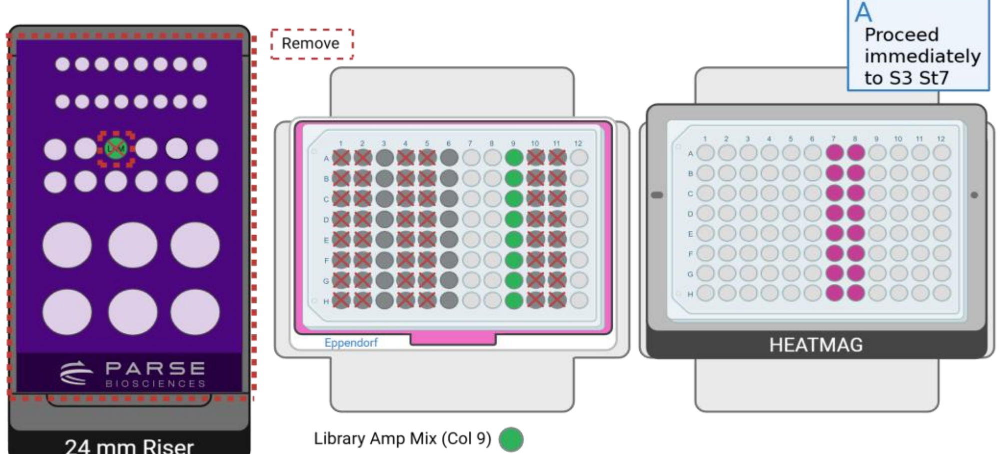
CRITICAL! Only transfer primers from 1 well of the UDI Plate - WT to 1 well of adapter ligated DNA.

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

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

    a. Remove all labware from Deck A.

b. Proceed immediately to Section 3.7.



3.7. Library Amp Mix Addition and Size Selection

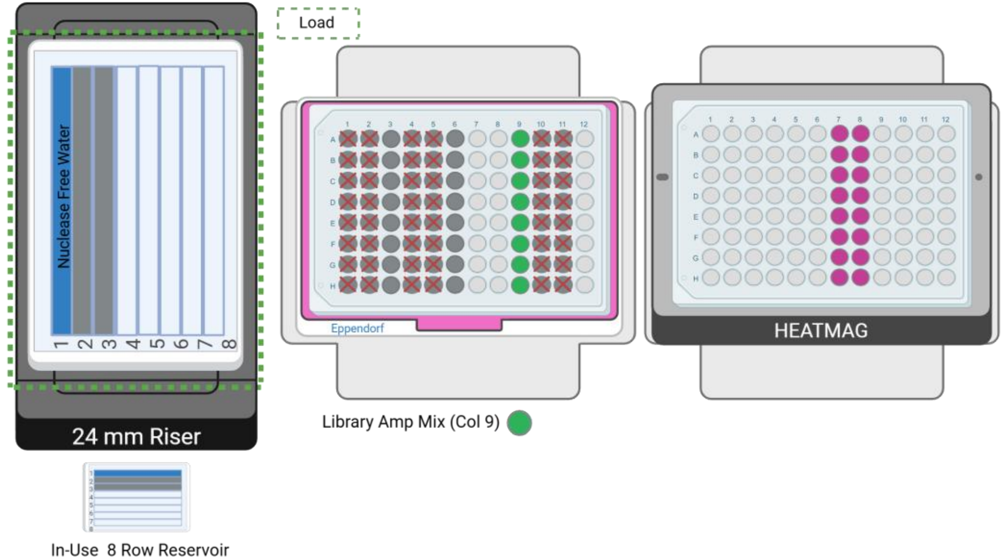
The program uses the VOYAGER multichannel pipette to add Library Amp Mix to the sublibraries. After the user runs the Indexing PCR thermocycling program, sublibraries are loaded back onto the ASSIST PLUS and a double-sided SPRI cleanup is carried out.

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Component	1	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	1	
24 mm Labware Pedestal	INTEGRA Component	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 Component	1	
HEATMAG with 96 Well Adapter	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA	1	
8 Row Reservoir	INTEGRA	1	
Ethanol	Consumables and Reagents		
Nuclease-free water	Consumables and Reagents		

2. Prepare **8 mL** of 85% ethanol with nuclease-free water.

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

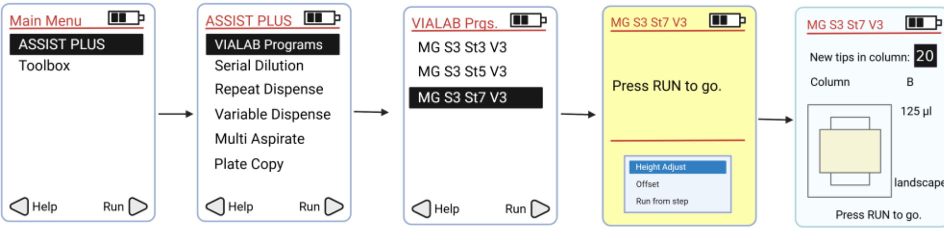


4. Change the pipette that is connected to the INTEGRA ASSIST PLUS:

   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.

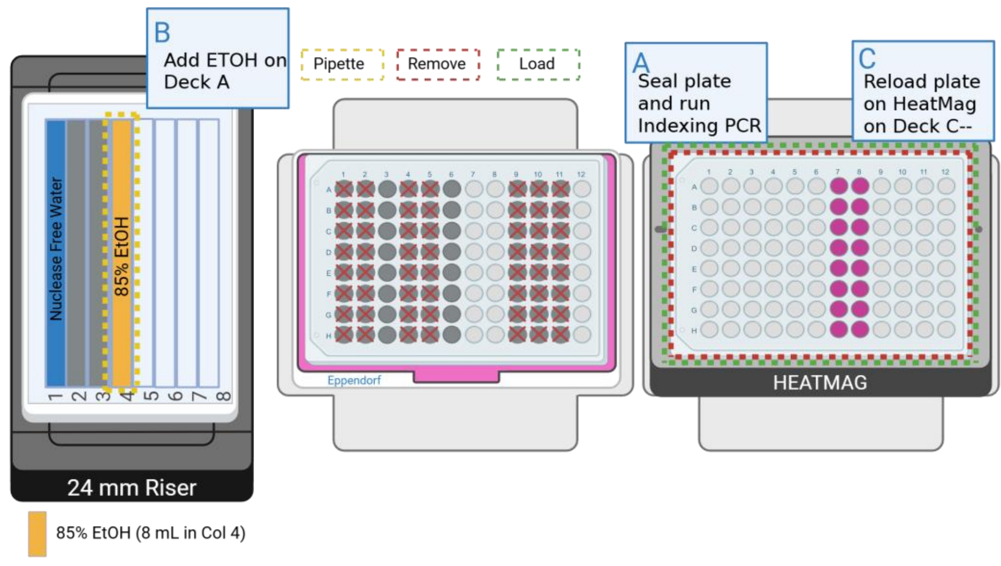
5. Select and run the program **MG S3 St7 V3_5** following the diagram below.



6. While the program is running, determine the number of PCR cycles required for the Indexing PCR based on the amount of cDNA added to the fragmentation and end prep reaction.

NUMBER OF PCR CYCLES	
cDNA Input (ng)	PCR Cycles
10-24	13
25-49	12
50-99	11
100-299	10
300-999	8
1,000 or more	7

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



a. Remove the sublibrary plate from the INTEGRA ASSIST PLUS, seal the sample plate on Deck C and place it into a thermocycler. Run the following program. When the Indexing PCR thermocycling program is complete, press "Run" to continue.

INDEXING PCR			
Run Time	Lid Temperature	Sample Volume	
~30 min	105°C	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	
5	5 min	72°C	1
6	Hold	4°C	1

b. With a P1000 pipette, add **8 mL** 85% Ethanol to row **4** of the 8 Row Reservoir on Deck A. Press "Run" to continue.

c. Reload the sample plate onto the HEATMAG with 96 Well Adapter on Deck C.

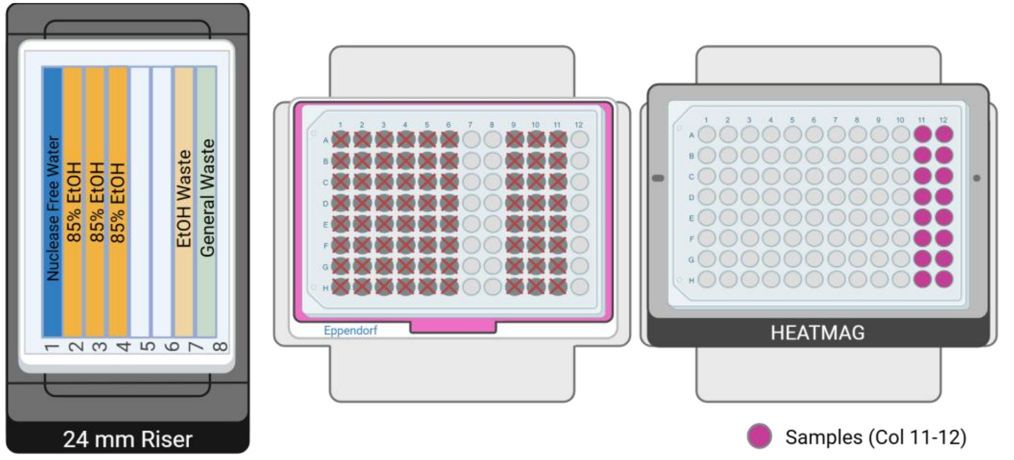
8. At the conclusion of the run:

   a. The sequencing libraries will be in columns 11 and 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 content.

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

STOP Safe stopping point: Sequencing libraries can be stored at -20°C for up to 3 months.



Nuclease Free Water
85% EtOH
85% EtOH
85% EtOH
EtOH Waste
General Waste
1
2
3
4
5
6
7
8
24 mm Riser
Eppendorf
HEATMAG
Samples (Col 11-12)

3.8. 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.

To quantify the sequencing libraries:

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.

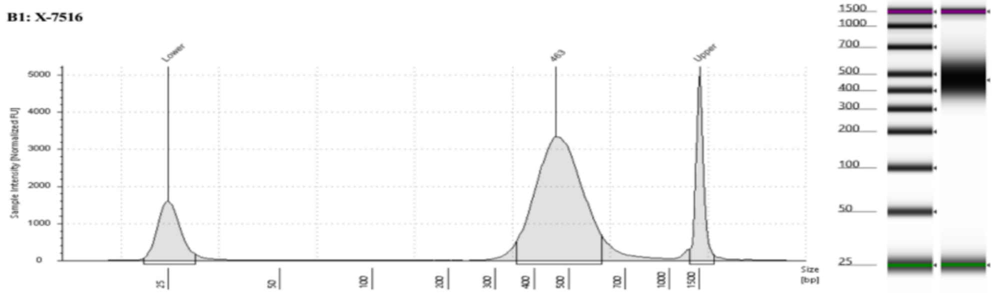


Figure 12: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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 is 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.

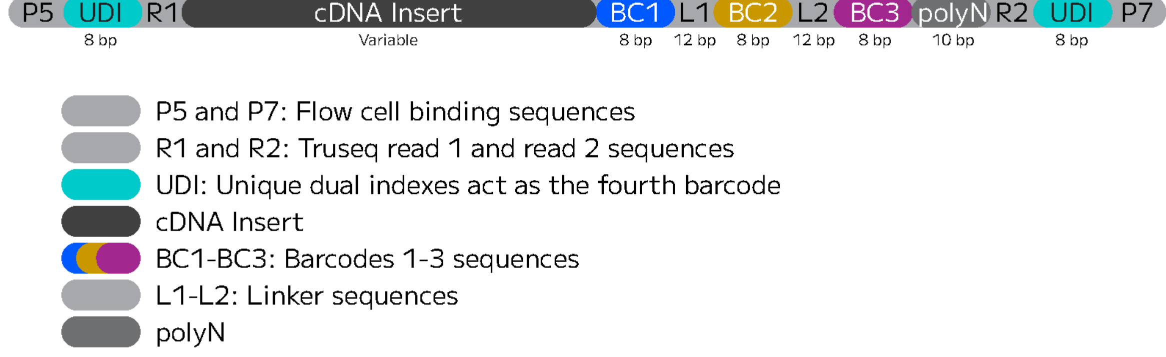


Figure 13: Whole transcriptome sequencing library structure.

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

For the sequencing BCR libraries please refer to the Evercode BCR User Manual.

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 Set Up for Standard Fixation Samples

MG4 S1 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Mega384DilutionWorksheetPlate upload	30 min
3	Prompt to replace dilution plates.	

Section 1.2 Loading and Pooling the Round 1 Plate

MG4 S1 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Thaw Sample Plate B Prompt	
3	Transfer Sample into Dilution Plate	4 min 39 sec
4	Add diluted sample into the R1 plate A.	4 min 52 sec
5	Prompt to load sample set B plates	
6	Thaw Sample Plate C Prompt	
7	Transfer Sample into Dilution Plate	5 min 9 sec
8	Add diluted sample into the R1 plate B	4 min 52 sec
9	Prompt to load sample set B plates	
10	Thaw Sample Plate D prompt	
11	Transfer Sample into Dilution Plate	5 min 9 sec
12	Add diluted sample into the R1 plate C	4 min 52 sec
13	Prompt to load sample set D	

STEPS	ACTION	DURATION
14	Transfer Sample into Dilution Plate	5 min 9 sec
15	Add diluted sample into the R1 plate D	4 min 52 sec
16	Therm R1 plate D prompt--	
17	Prompt to load R1 Plates A&B	
18	Plate A Pool	2 min 55 sec
19	Plate A pool 2nd pass	1 min 30 sec
20	Plate B Pool	2 min 50 sec
21	Plate B pool 2nd pass	1 min 32 sec
22	Prompt to replace plates	
23	Plate C Pool	2 min 56 sec
24	Plate C pool 2nd pass	1 min 30 sec
25	Plate D Pool	2 min 50 sec
26	Plate D pool 2nd pass	1 min 32 sec

Section 1.3 Round 2 Ligation Preparation

MG4 S1 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Plate A: Transfer 1 SBO	1 min 14 sec
3	Plate A: Transfer 0 SBO	24 sec
4	Plate B: Transfer 1 SBO	1 min 19 sec
5	Plate B: Transfer 0 SBO	28 sec
6	Plate C: Transfer 1 SBO	1 min 14 sec
7	Plate C: Transfer 0 SBO	24 sec

STEPS	ACTION	DURATION
8	Plate D: Transfer 1 SBO	1 min 19 sec
9	Plate D: Transfer 0 SBO	28 sec
10	Add Spin Additive	1 min 58 sec
11	Make Round 2 Ligation Master Mix	2 min 47 sec
12	Mix	35 sec
13	Distribute	43 sec
14	Add R2 Lig Enzy	31 sec
15	Mix R2 Ligation MM	40 sec
16	Mix R2 Ligation MM	40 sec
17	Mix R2 Ligation MM	40 sec
18	Prompt to add strain tubes	
19	Prompt to re-load sample tubes	
20	Tube 1 Remove Supernatant	22 sec
21	Tube 1 Remove Supernatant	14 sec
22	Tube 1 Remove Supernatant	21 sec
23	Tube 1 Remove Supernatant	27 sec
24	Tube 2 Remove Supernatant	24 sec
25	Tube 2 Remove Supernatant	17 sec
26	Tube 2 Remove Supernatant	24 sec
27	Tube 2 Remove Supernatant	29 sec
28	Tube 3 Remove Supernatant	22 sec
29	Tube 3 Remove Supernatant	15 sec
30	Tube 3 Remove Supernatant	22 sec
31	Tube 3 Remove Supernatant	27 sec

STEPS	ACTION	DURATION
32	Tube 4 Remove Supernatant	24 sec
33	Tube 4 Remove Supernatant	17 sec
34	Tube 4 Remove Supernatant	24 sec
35	Tube 4 Remove Supernatant	29 sec
36	Resuspend Sample A	54 sec
37	Plate A: Strain	41 sec
38	Resuspend Sample B	55 sec
39	Plate B: Strain	43 sec
40	Prompt to remove cell strainers	
41	Repeat Dispense Plate A	1 min 7 sec
42	Repeat Dispense Plate B	1 min 4 sec
43	Prompt to replace strain tubes	
44	Resuspend Sample C	54 sec
45	Plate C: Strain	42 sec
46	Resuspend Sample D	55 sec
47	Plate D: Strain	43 sec
48	Prompt to remove cell strainers	
49	Repeat Dispense Plate C	1 min 2 sec
50	Repeat Dispense Plate D	1 min 5 sec

Section 1.4 Round 2 Ligation

MG4 S1 St4 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Large volume mix Row 1 of sample	49 sec

STEPS	ACTION	DURATION
3	Samp in 1st half of R2 plate	2 min 45 sec
4	Large volume mix Row 2 of sample	46 sec
5	Samp in 2nd half of R2 plate	2 min 26 sec
6	Prompt for Round 2 Plate incubation	
7	Prompt to replace Round 2 plate back on Deck after incubation	
8	Prompt to add Round 2 stop to basin	
9	Add R2 Stop mix	4 min 43 sec
10	Prompt to incubate for Round 2 stop	
11	Prompt to load Round 2 plate back on deck after incubation	
12	First Pass Pool R2 Plate	2 min 50 sec
13	Second Pass Pool R2 Plate	1 min 21 sec

Section 1.5. Round 3 Ligation Preparation

MG4 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	

STEPS	ACTION	DURATION
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

MG4 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	
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

MG4 S1 St7 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	“Move Basin Holder to Deck B” message	

STEPS	ACTION	DURATION
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
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

MG4 S1 St8 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	Aliquot 25 µL of sample	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	"remove PCR tubes from Deck C" message	

Section 2.1. Reagent Plating

MG S2 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Dispense Streptavidin Beads (88 µL/well)	2 min
3	Dispense SPRI Beads (160 µL/well)	1 min
4	Dispense Binding Buffer (110 µL/well)	2 min
5-7	Dispense Bead Wash Buffer (entire volume)	1 min
8	"Thaw Lysates" message	
9-12	Dispense Wash Buffer 1 (entire volume)	1.5 min
13-17	Dispense Wash Buffer 2 (entire volume)	1.5 min
18	"Load Wash 3–" - message	
19-22	Dispense Wash Buffer 3 (entire volume)	1.5 min
23	"Load Lysates–" message	
24	Enhancer Addition	9 min
25	"Cap and store SPRI beads at RT" message	

STEPS	ACTION	DURATION
26	“Proceed immediately to S2 St2” message	

Section 2.2. cDNA Capture

MG S2 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Raise magnet	
3-4	Mix Lysates	1 min
5-7	Remove Streptavidin Bead Supernatant (entire volume)	3 min
8-15	1st Bead Wash (100 µL/well)	4.5 min
16-23	2nd Bead Wash (100 µL/well)	4.5 min
24-31	3rd Bead Wash (100 µL/well)	4.5 min
32-33	Add Binding Buffer and Mix (110 µL/well)	3 min
34-37	Add Streptavidin Beads to Sample (50 µL/well)	1 min
38	“Vortex at 800-1k rpm for 30 mins” message	

Section 2.3. Streptavidin Beads Wash

MG S2 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-6	Supernatant Removal (entire volume)	3.5 min
7-15	1st Wash 1 (120 µL/well)	8 min
16-24	2nd Wash 1 (120 µL/well)	8 min
25-32	Wash 2 (120 µL/well)	8 min

STEPS	ACTION	DURATION
33-34	Wash 3 (120 µL/well)	1 min
35	"Proceed immediately to S2 St4" message	

Section 2.4. Master Mixes Preparation

MG S2 St4 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-5	cDNA Amp Mix Prep: Enzyme: 1050 µL Primer: 1050 µL	2.5 min
6	Dispense cDNA Amp Mix to Strip Tubes (230 µL/well)	1 min
7	"Cap and store cDNA Amp on ice–" message	
8-13	Template Switch Mix Prep: Buffer: 1710 µL (total) Enzyme: 92.4 µL Primer: 26.2 µL	5 min
14	Dispense Template Switch to Strip Tubes (220 µL/well)	1.5 min
15	"Proceed immediately to S2 St5" message	

Section 2.5. Template Switch and cDNA Amplification

MG S2 St5 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-6	Remove Wash 3 Supernatant (entire volume)	4 min
7-10	Adds Template Switch Mix to Samples (100 µL/well)	4.5 min

STEPS	ACTION	DURATION
11	“Manually Mix Samples” message	
12	“Seal and incubate at RT for 30 mins” message	
13	“Reload samples on Deck C–” message	
14-17	Mix samples	2 min
18	“Cap and run TS on thermocycler” message	
19	“Reload samples on Heatmag–” message	
20-24	Remove Template Switch Supernatant (entire volume)	3.5 min
25-27	Wash 3 (120 µL/well)	1.5 min
28	“Load Amp Mix on Deck B–” message	
29-32	Remove Wash 3 Supernatant (entire volume)	2.5 min
33-36	Add cDNA Amp Mix to Sample (100 µL/well)	4.5 min
37	“Manually Mix Samples” message	
38	“Run cDNA Amp on the Thermocycler” message	

Section 2.6. Post-Amplification Purification

MG S2 St6 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-3	Mix Samples	1 min
4	Activate Magnet	
5-6	Mix SPRI Beads	2 min
7-9	Transfer Sample (Col 1 & 3) to Tubes (Col 5 & 7) on Heatmag (90 µL/well)	2 min
10-15	Add SPRI Beads to Samples (72 µL/well)	9.5 min
16-22	Remove Supernatant (entire volume)	6 min

STEPS	ACTION	DURATION
23-33	1st EtOH Wash (120 µL/well total)	4.5 min
34-45	2nd EtOH Wash (120 µL/well total)	6.5 min
46-57	Elution (25 µL/well)	15 min
58	“Samples on Heatmag Column 9-11” message	

Section 3.0. cDNA Normalization

MG S3 St0 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Normalize samples	12.5 min

Section 3.1. SPRI Bead Plating

MG S3 St1 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Plate out beads into columns 1 & 3 (50 µL/well)	2 min
3	Plate out beads into column 2 (90 µL/well)	1 min
4	Plate out beads into columns 4 & 6 (50 µL/well)	2 min
5	Plate out beads into column 5 (90 µL/well)	1 min

Section 3.2. Fragmentation Mix Creation and Plating

MG S3 St2 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Pre-chill thermal cycler message	

STEPS	ACTION	DURATION
3	Create Fragmentation Mix	4 min
4	Plate out Fragmentation Mix into column 11 (30 µL/well)	2 min
5	Proceed to S3 St3 message	

Section 3.3. Fragmentation Mix Addition and Post-Fragmentation SPRI Cleanup

MG S3 St3 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-3	Stamp Fragmentation Mix into samples (15 µL/well)	1.5 min
4-8	Deck loading messages	
9	Volume change	
10	Ensure magnet is deactivated	
11-12	Mix SPRI beads	2 min
13-14	Add SPRI beads to samples (30 µL/well)	2 min
15	5 minute bead incubation	5 min
16	Activate magnet	
17	2 minute bead immobilization	2 min
18-19	Transfer supernatant (75 µL/well)	1 min
20	Deactivate magnet	
21-22	Add SPRI beads to samples (10 µL/well)	2 min
23	5 minute bead incubation	5 min
24	Activate magnet	
25	3 minute bead immobilization	3 min
26	Volume change	

STEPS	ACTION	DURATION
27-28	Discard supernatant	1.5 min
29-32	Ethanol addition 1 (180 µL/well total)	2 min
33	1 minute ethanol incubation	1 min
34-37	Discard ethanol	3 min
38-41	Ethanol addition 2 (180 µL/well total)	2 min
42	1 minute ethanol incubation	1 min
43-46	Discard ethanol	3 min
47	Deactivate magnet	
48-49	Resuspend beads in water (50 µL/well)	30 sec
50-55	Offset mixing to ensure full resuspension	2 min
56	5 min bead incubation	5 min
57	Activate Magnet	
58	2 minute bead immobilization	2 min
59-60	Transfer eluate (50 µL/well)	1 min
61	Deactivate magnet	

Section 3.4. Ligation Mix Creation and Plating

MG S3 St4 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Create Ligation Mix	6 min
3	Slow mix to reduce volume stuck in tip	18 sec
4	Plate out Ligation Mix into column 10 (104 µL/well)	1 min

STEPS	ACTION	DURATION
5	Proceed to S3 St5 message	

Section 3.5. Ligation Mix Addition and Post-Ligation SPRI Cleanup

MG S3 St5 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-3	Stamp Ligation Mix into samples (50 µL/well)	2 min
4-6	Deck loading messages	
7	Ensure magnet is deactivated	
8-9	Mix SPRI beads	1.5 min
10-11	Add SPRI beads to samples (80 µL/well)	3 min
12	5 minute bead incubation	5 min
13	Activate magnet	
14	5 minute bead immobilization	5 min
15	Volume change	
16-19	Discard supernatant	2.5 min
20-23	Ethanol addition 1 (180 µL/well total)	2 min
24	1 minute ethanol incubation	1 min
25-28	Discard ethanol	3.5 min
29-32	Ethanol addition 2 (180 µL/well total)	2 min
33	1 minute ethanol incubation	1 min
34-37	Discard ethanol	3.5 min
38	Air dry delay	1 min
39	Deactivate magnet	
40-41	Resuspend beads in water (23 µL/well)	1.5 min

STEPS	ACTION	DURATION
42	5 minute bead incubation	5 min
43	Activate magnet	
44	2 minute bead immobilization	2 min
45-46	Transfer eluate (21 µL/well)	1 min
47	Deactivate magnet	

Section 3.6. Barcoding Round 4

MG S3 St6 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2	Plate out Amplification Mix into column 9 (55 µL/well)	1 min
3	Add UDIs message	
4	Proceed to S3 St 7 message	

Section 3.7. Library Amp Mix Addition and Size Selection

MG S3 St7 V3_5

STEPS	ACTION	DURATION
1	Initial Volumes	
2-3	Stamp Amplification Mix into samples (25 µL/well)	1.5 min
4-6	Deck loading messages	
7	Ensure magnet is deactivated	
8-11	Mix SPRI beads	2 min
12-13	Add SPRI beads to samples (30 µL/well)	2 min
14	5 minute bead incubation	5 min

STEPS	ACTION	DURATION
15	Activate magnet	
16	2 minute bead immobilization	2 min
17-18	Transfer supernatant (75 µL/well)	1 min
19	Deactivate magnet	
20-21	Add SPRI beads to samples (10 µL/well)	2 min
22	5 minute bead incubation	5 min
23	Activate magnet	
24	3 minute bead immobilization	3 min
25	Volume change	
26-27	Discard supernatant	1.5 min
28-31	Ethanol addition 1 (180 µL/well total)	2 min
32	1 minute ethanol incubation	1 min
33-36	Discard ethanol	3.5 min
37-40	Ethanol addition 2 (180 µL/well total)	2 min
41	1 minute ethanol incubation	1 min
42-45	Discard ethanol	3.5 min
46	Deactivate magnet	
47-48	Resuspend beads in water (20 µL/well)	30 sec
49-54	Offset mixing to ensure full resuspension	1.5 min
55	5 minute bead incubation	5 min
56	Activate magnet	
57	2 minute bead immobilization	2 min
58-59	Transfer eluate (20 µL/well)	1 min
60	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	August 2025
1.1	Section 3.7.7b: Corrected ETOH volume	August 2025
1.2	Section 1.8: Added Low Fixation Samples beads removal steps	October 2025

Appendix E: Acknowledgements

All graphics were created with BioRender.com.

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