User Manual

Version 1.2

Evercode™ TCR Mega with INTEGRA ASSIST PLUS Barcoding Workflow

For use with

ECIT2500 (Human)

ECIT1510 (Mouse)

INTEGRA ASSIST PLUS

Legal Notices

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Overview

Workflow

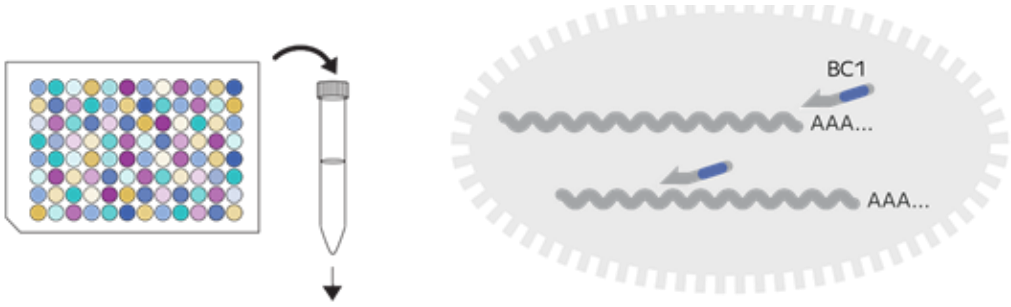
The Evercode combinatorial barcoding workflow is now compatible with the INTEGRA ASSIST PLUS to enable large-scale single cell RNA-Seq experiments through a robust, semi-automated process.

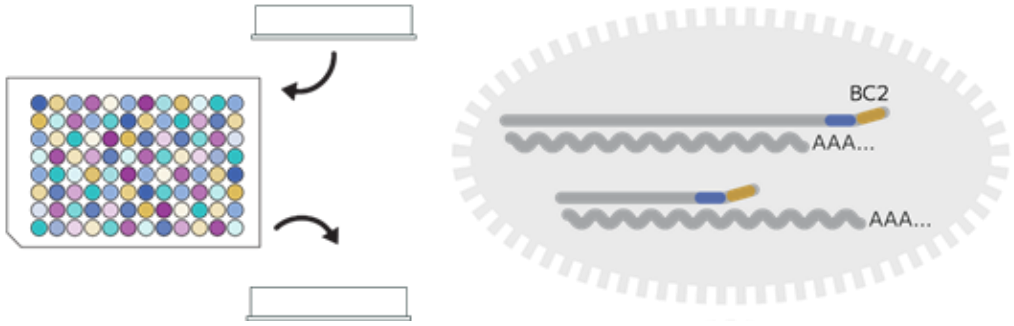
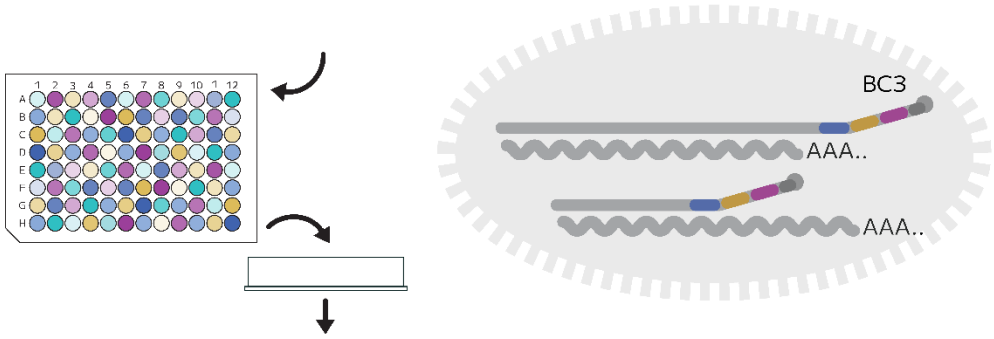
Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode TCR Mega can profile up to 1,000,000 cells across up to 96 different biological samples or experimental conditions. Evercode fixation kits fix and permeabilize cells so they act as individual reaction compartments. This eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell is uniquely labeled. The four rounds of barcoding can yield a vast number of possible barcode combinations, which is more than sufficient to uniquely label up to 1,000,000 cells while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads that share the same four barcode combinations to a single cell.

Evercode TCR expands capabilities of scRNA-seq by simultaneously capturing T-cell receptor (TCR) information. A sufficient sample size is crucial to detecting and tracking rare clones. Discover up to 1,000,000 T cells, identify phenotypes and their paired TCR sequences.

By integrating the Evercode Mega assay kits with the ASSIST PLUS platform, the semi-automated workflow performs sample normalization and combinatorial barcoding through a series of reverse transcription, ligation, and library preparation steps.

The table below provides a high-level overview of the automated barcoding workflow.

Subsections 1.1 - 1.3: Round 1 Barcoding	
1	 The program loads fixed cells into the 96 wells of the Round 1 Plate. RNA is reverse transcribed into cDNA via oligo dT and random hexamer primers, appending a well-specific barcode to specific samples. Cells are then pooled and resuspended.
Subsections 1.4 - 1.5: Round 2 Barcoding	

2	 Pooled cells are loaded into the Round 2 Plate. An adapter with a well-specific second barcode is ligated to the first barcode on the generated cDNA. Cells are then pooled and resuspended.
Subsections 1.6-1.7: Round 3 Barcoding	
3	 Pooled cells are loaded into the Round 3 Plate. A third barcode, which also contains an Illumina Truseq Read 2 sequence and biotin, is ligated to the second barcode on the generated cDNA. Cells are then pooled and resuspended.
Subsection 1.8: Lysis and Sublibrary Generation	
4	 Pooled cells are split across 16 sublibraries and lysed.

This recommended protocol is intended to be followed when performing the Evercode Mega assays on the INTEGRA ASSIST PLUS liquid handler. The protocol replaces "Section 1. *In Situ* Cell Barcoding" (subsections 1.1 through 1.5) of the standard Evercode TCR Mega User Guide. Upon completing Section 1 of this protocol, proceed to "Section 2. cDNA Capture and Amplification", of the standard Evercode User Guide.

Parse Biosciences and INTEGRA are continuing development and will soon release more semi-automated protocols that will provide comprehensive support for Fixation in addition to Section 2 and Section 3 of the Evercode Whole Transcriptome workflow.

Important Guidelines

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

Comprehensive guidance on optimizing the complete standard Evercode TCR Mega workflow is provided in the Evercode TCR Mega User Guide. For further information on the experimental workflow, please contact support@parsebiosciences.com. Please contact support-us@integra-biosciences.com for any questions regarding workflow automation or the INTEGRA ASSIST PLUS instrument.

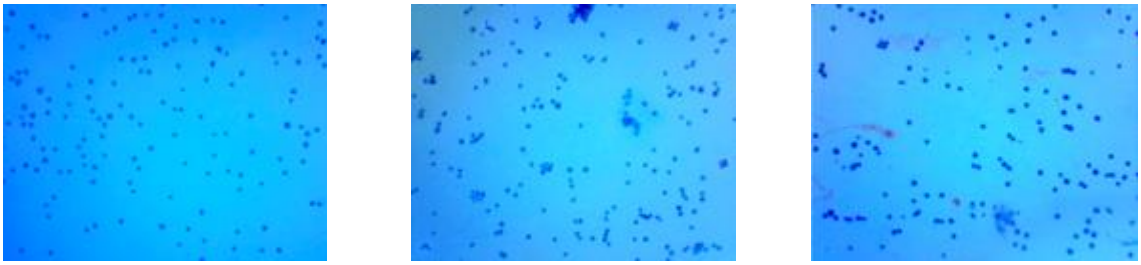
Sample Input

- This protocol begins with cells previously fixed with an Evercode Cell Fixation v3 kit. It is also compatible with samples fixed with Evercode Cell Fixation v2 kit.
- When working with mouse T cells, use the Evercode Cell Fixation (Mouse TCR) kit, which includes a murine RNAse Inhibitor.
- Even if samples were counted before freezing, we strongly recommend counting cells again after thawing to account for any changes in cell 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 are loaded in the Round 1 Plate, and counting accuracy at this stage is critical to recover the desired number of cells 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 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 counts should be recorded in the Sample Loading Table, and any remaining cell material in the thawed counting aliquot should be discarded.
- Once fixed samples have been thawed, they should not be refrozen.

Cell 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.
- When first using Evercode kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- Examples of trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells 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.

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 any Evercode assay User Guide.

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

Optimizing Cell Recovery

- It is critical to thoroughly resuspend the cells after centrifugation throughout the protocol. Resuspend by repeatedly pipetting until no clumps are visible. Due to cell adherence to tubes, carefully pipette along the bottom and sides of tubes to minimize loss.

- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.

- Only use recommended polypropylene consumables, as polystyrene tubes will lead to substantial sample loss.

- When using an Evercode assay kit for the first time, we recommend retaining supernatants after each centrifugation step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.

Sample Loading Table

- Ensure that the Parse Evercode WT INTEGRA Mega 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 Evercode WT INTEGRA Mega Sample Loading Table (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.

- Proceed as-is. The INTEGRA pipette will dispense the highest possible number of cells based on the existing concentration. This may lead to reduced library yields for samples with particularly low concentrations.

Plate Sealing

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

Sample Concentrations

- The recommended cell concentration is between 3,000 cells per µL. If the cell concentration is too high, dilute the sample with the Pre-Lysis Dilution Buffer to the desired concentration. Ensure you save at least **500 µL** of Pre-Lysis Dilution Buffer for step 1.8.2.

PCR Freezer Block

- The PCR freezer block changes color from dark purple to bright pink (too warm) when the block temperature exceeds 7°C. If 70% of the block is bright pink, replace the PCR freezer block with a fully frozen dark purple block.
- To maintain optimal performance and minimize temperature fluctuations, store the PCR Freezer Blocks in a -20°C freezer when not in use.
- Tip pinching may occur when using a fully frozen PCR freezer block for a plate pooling step. To minimize tip pinching, a fully frozen PCR freezer 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 freezer block, apply firm pressure on the PCR plates until they are securely seated. Avoid touching the top of the open wells.

INTEGRA ASSIST PLUS Pipetting Programs

- Ensure that the VIALAB program is installed on the same computer that will be used to communicate with the D-ONE Pipetting Module.

- Ensure that Evercode workflow script calibration had been confirmed prior to running the INTEGRA + Parse workflow. This can be done by running the Evercode WT with INTEGRA ASSIST PLUS Calibration Scripts available on the Customer Support Suite.

- Ensure that the .csv worklists generated via the Parse Evercode WT INTEGRA MG Sample Loading Table(the "DiluentVolumes.csv" worklist and the "SampleVolumes.csv" worklist) 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.

- When uploading a new worklist to the VIALAB program, all pipetting settings will be automatically reset to standard default settings and must be readjusted to the correct settings specified for that worklist. For images and details of correct pipette settings for each worklist, see Appendix A.

Deck Loading

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

Parse Reagents

The Evercode TCR Mega with INTEGRA ASSIST PLUS includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents. Store -20°C, PN HTG100 or MTG100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	HTG101 or MTG101	Green semi-skirted 96 well plate	1
	Round 2 Plate	MG102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	MG104	5 mL tube	1
	Sample Dilution Buffer	MG105	2 mL tube	1
	Round 2 Ligation Buffer	MG106	5 mL tube	1
	Round 2 Ligation Enzyme	MG107	1.5 mL tube	1
	Round 2 Stop Buffer	MG108	2 mL tube	1
	Round 3 Stop Buffer	MG109	5 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	1
	Round 3 Ligation Enzyme	MG111	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	MG112	2 mL tube	1
	Lysis Enzyme	MG113	1.5 mL tube	1
	Bead Wash Buffer	MG114	5 mL tube	1
	Wash Buffer 1	MG115	5 mL tube	1
	Wash Buffer 2	MG116	5 mL tube	1
	Capture Enhancer	MG117	1.5 mL tube	1
	Binding Buffer	MG118	1.5 mL tube	1
	Wash Buffer 3	MG119	5 mL tube	1
	Template Switch Buffer	MG120	2 mL tube	1
	Template Switch Enzyme	MG121	1.5 mL tube	1
	Template Switch Primer	MG122	1.5 mL tube	1
	cDNA Amp Mix	MG123	1.5 mL tube	1
	cDNA Amp Primers	HTG124 or MTG124	1.5 mL tube	1
	Fragm/End Prep Buffer	MG125	1.5.mL tube	1
	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
Lig Adapter	Ligation Adapter	MG127	1.5 mL tube	1
Adap Lig Buffer	Adapter Ligation Buffer	MG128	1.5 mL tube	1
Adap Lig Enzy	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
Library Amp Mix	Library Amp Mix	MG130	1.5 mL tube	1
Rec PCR 1	Receptor PCR Mix 1	HTG131 or MTG131	1.5 mL tube	1
Rec PCR 2	Receptor PCR Mix 2	HTG132 or MTG132	1.5 mL tube	1
HT Primer or MT Primer	HT Primer or MT Primer	HTG133 or MTG133	1.5 mL tube	1

4°C Reagents. Store 4°C, PN HTG200 or MTG200

LABEL	ITEM	PN	FORMAT	QTY
Spin Add	Spin Additive	MG201	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

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.

ITEM	PN	QTY
Thermochromic PCR Cold Block	NTAC1102	3
Thermochromic PCR Cold Block Riser	NTAC1103	3
Parse Cold Block 1	NTAC1101	1

INTEGRA Components

The following are required INTEGRA ASSIST PLUS components needed to run Section 1. *In situ* Cell Barcoding (1.1 to 1.5) of the Parse Biosciences assay on the INTEGRA ASSIST PLUS platform.

ITEM	ITEM TYPE	PN	QTY
Pipette Communication Module for VIAFLO Pipette	Accessory	4221	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
ASSIST PLUS Base Unit	Main	4505	1
Communication/Charging Cable for VIAFLO	Accessory	4226	1

INTEGRA-Provided Consumables

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

ITEM	ITEM TYPE	PN	QTY
8 Row Reagent Reservoirs, Partitioned (32 mL/row) with SUREFLO design	INTEGRA-Provided	6373	1
25 mL basin reservoir liners	INTEGRA-Provided	4316	1
1250 µL pipette tips (Sterile/Filter/Low Retention)	INTEGRA-Provided	6545	1
125 µL pipette tips (Sterile/Filter/Low Retention)	INTEGRA-Provided	6565	1

Consumables

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

Consumables

ITEM	SUPPLIER	PN	QTY
Sterilized 40 µm mini cell strainer	DiagnoCine	FNK-HT-AMS-14002	2
10 mL transport tubes	GlobeScientific™	6102S	2
1.5 mL tubes	GENESEE SCIENTIFIC CORP	21-257	2
PCR strip tubes (bag of 12)	USA SCI	1402-4700	1
Semi skirted plates (clear)	Thermo Fisher Scientific®	E951020362	2

Equipment

ITEM	SUPPLIER	PN	NOTES
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. 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.
Trypan Blue	Various Suppliers	Varies	Or alternative dyes to assess cell viability, such as AO/PI.

Section 1: Automation Setup

Section 1.1. Sample Normalization

Prior to setting up the Automation workflow, count the cells to assess quality and concentration of the fixed sample(s).

Fixed cells should be diluted to a concentration of 3,000 cells per µL.

After adjusting the sample(s) to the recommended dilution range, download the Sample Loading Table MACRO (Section 1.1.2), which will be used as reference for allocating the fixed cells into the 96-well PCR plate, ready for the protocol to start.

The program uses the sample dilution buffer on Deck A to normalize fixed samples from plate format on Deck C into intermediate dilution plate on Deck B.

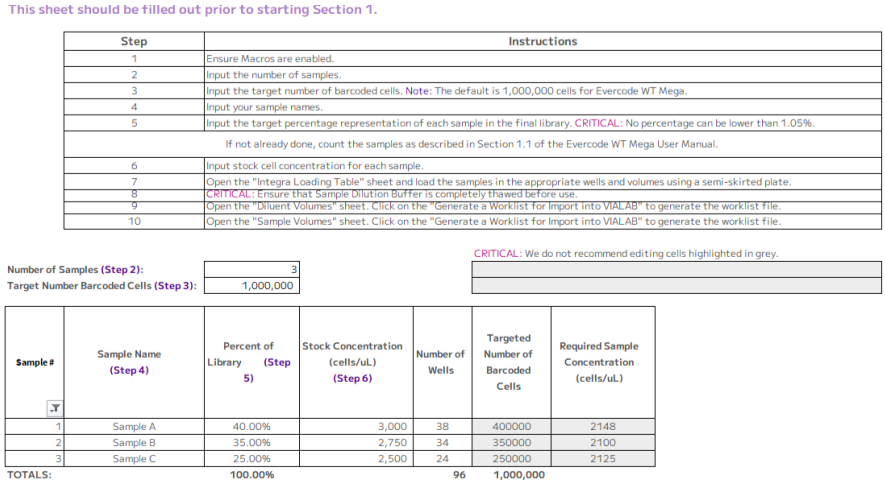
1. Gather the following components and reagents:

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	
24 mm Labware Pedestal	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse-Provided	2	Pull the Freezer Block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	2	
Parse Cold Block 1	Parse-Provided	1	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate	Consumables	2	
● Sample Dilution Buffer	-20°C Reagents (Parse Reagents)	2 mL tube	Thaw at room temperature then store on ice. Mix by inverting 3x.
▭ Round 1 Plate	-20°C Reagents (Parse Reagents)	1	Place directly on ice.

2. Download the Parse Evercode WT INTEGRA Mega Sample Loading Table. The most current version of the Evercode WT INTEGRA Mega Sample Loading Table can be found on the Parse Biosciences Customer Support Suite.

3. Start with the Sample Loading Table tab of the worksheet. Per the instructions in the worksheet, input number of samples (Figure 1).

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



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

Step	Instructions
1	Ensure Macros are enabled.
2	Input the number of samples.
3	Input the target number of barcoded cells. Note: The default is 1,000,000 cells for Evercode WT Mega.
4	Input your sample names.
5	Input the target percentage representation of each sample in the final library. CRITICAL: No percentage can be lower than 1.05%.
	If not already done, count the samples as described in Section 1.1 of the Evercode WT Mega User Manual.
6	Input stock cell concentration for each sample.
7	Open the "Integra Loading Table" sheet and load the samples in the appropriate wells and volumes using a semi-skirted plate.
8	CRITICAL: Ensure that Sample Dilution Buffer is completely thawed before use.
9	Open the "Diluent Volumes" sheet. Click on the "Generate a Worklist for Import into VIALAB" to generate the worklist file.
10	Open the "Sample Volumes" sheet. Click on the "Generate a Worklist for Import into VIALAB" to generate the worklist file.

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

Number of Samples (Step 2): 3
Target Number Barcoded Cells (Step 3): 1,000,000

Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/uL) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/uL)
1	Sample A	40.00%	3,000	38	400000	2148
2	Sample B	35.00%	2,750	34	350000	2100
3	Sample C	25.00%	2,500	24	250000	2125
TOTALS:		100.00%		96	1,000,000	

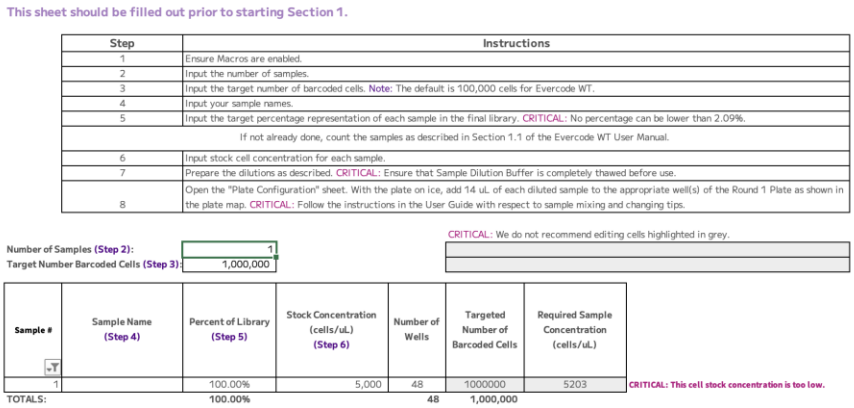
Figure 1: Evercode WT Mega Sample Loading Table.

4. While minimizing time on ice, count the number of cells in each sample with a hemocytometer or alternative cell counting device. Record the cell count.

5. Enter Target Number of Barcoded Cells, Sample Name, Percent of Library and Stock Concentration for corresponding samples (Figure 2).

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

Note: If the cell concentration is too high, we recommend diluting the sample with the Pre-Lysis Dilution Buffer. See the "Important Guidelines" section for details.



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

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

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

Number of Samples (Step 2): 1
Target Number Barcoded Cells (Step 3): 1,000,000

Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/uL) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/uL)	
1		100.00%	5,000	48	1000000	5203	CRITICAL: This cell stock concentration is too low.
TOTALS:		100.00%		48	1,000,000		

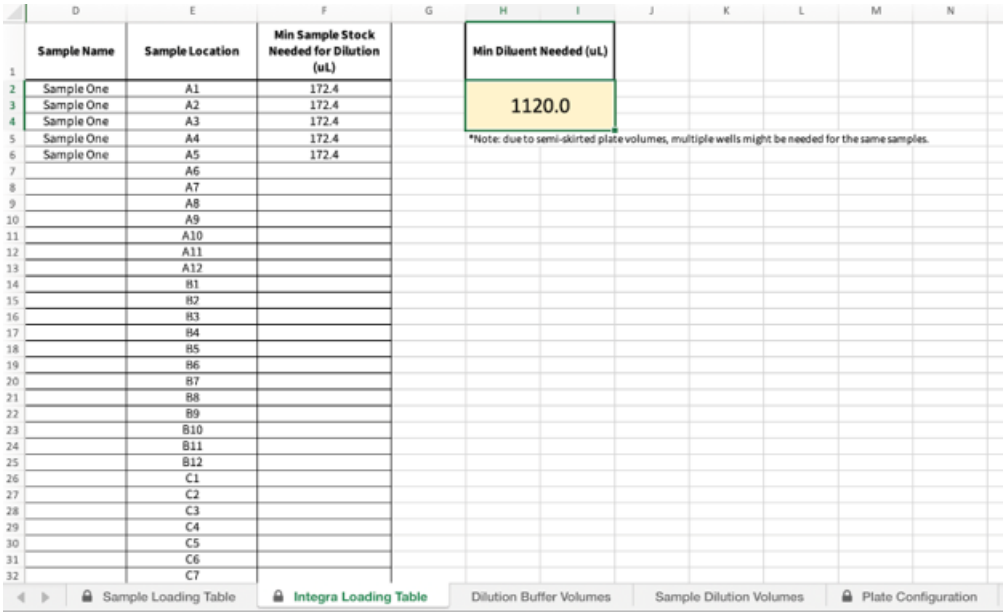
Figure 2: Example error message, noting that the sample stock concentration is too low.

6. Navigate to the “INTEGRA Loading Table” tab and check that the Minimum Diluent Needed (µL) does not exceed 1,800 µL (Figure 3).

7. Referring to the "INTEGRA Loading Table" tab, manually load fixed samples into the directed "Sample Location" well into a new Eppendorf semi-skirted plate. Note that any individual sample may require loading into more than one “Sample Location” well. This is the sample stock plate.

8. Store the sample stock plate on ice for later use.

Note: Example: Sample 1 is loaded into Sample Location A1 with a minimum volume of 172.4 µL. More sample volume (up to a maximum of 200 µL total sample volume) can be loaded to reduce bubbles during mixing.



	Sample Name	Sample Location	Min Sample Stock Needed for Dilution (uL)
2	Sample One	A1	172.4
3	Sample One	A2	172.4
4	Sample One	A3	172.4
5	Sample One	A4	172.4
6	Sample One	A5	172.4
7		A6	
8		A7	
9		A8	
10		A9	
11		A10	
12		A11	
13		A12	
14		B1	
15		B2	
16		B3	
17		B4	
18		B5	
19		B6	
20		B7	
21		B8	
22		B9	
23		B10	
24		B11	
25		B12	
26		C1	
27		C2	
28		C3	
29		C4	
30		C5	
31		C6	
32		C7	

Min Diluent Needed (uL): 1120.0

*Note: due to semi-skirted plate volumes, multiple wells might be needed for the same samples.

Sample Loading Table | Integra Loading Table | Dilution Buffer Volumes | Sample Dilution Volumes | Plate Configuration

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

9. Navigate to the “Diluent Buffer Volumes” tab and click on the purple box labeled “Generate a Worklist for Import into VIALAB”. Save the generated CSV file (called “DiluentVolumes.csv”) for later use (Figure 4).

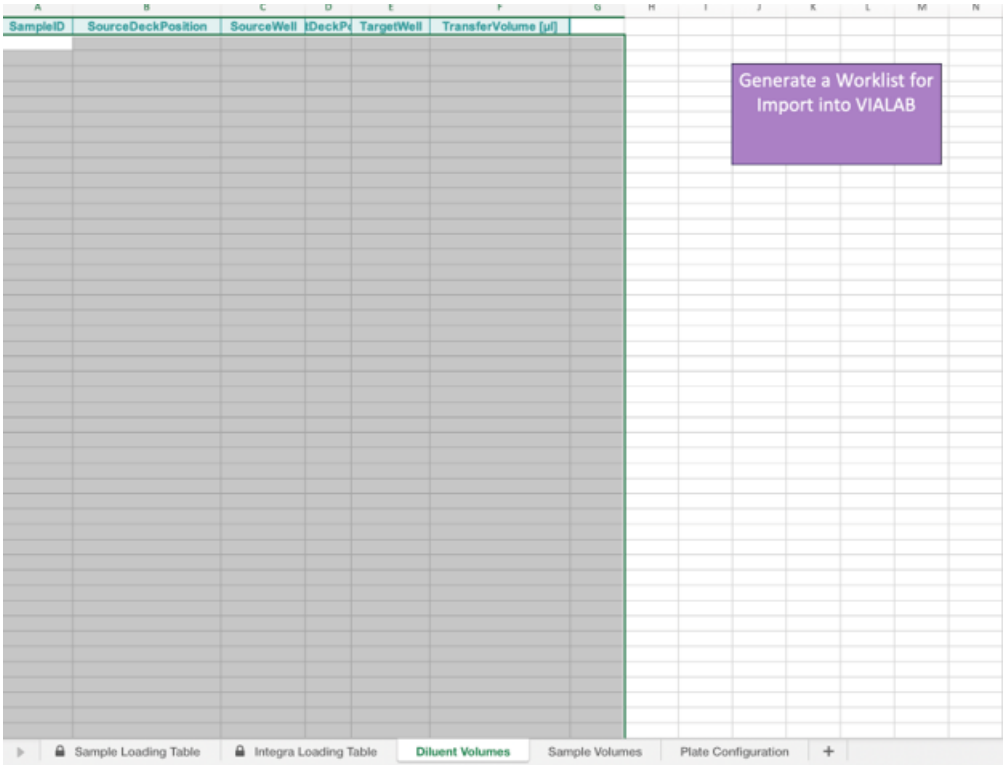


Figure 4: Diluent Buffer Volumes tab for generating a VIALAB worklist.

10. Navigate to the “Sample Dilutions Volumes” tab and click on the purple box labeled “Generate a Worklist for Import into VIALAB”. Save the generated CSV file (called “SampleVolumes.csv”) for later use (Figure 5).

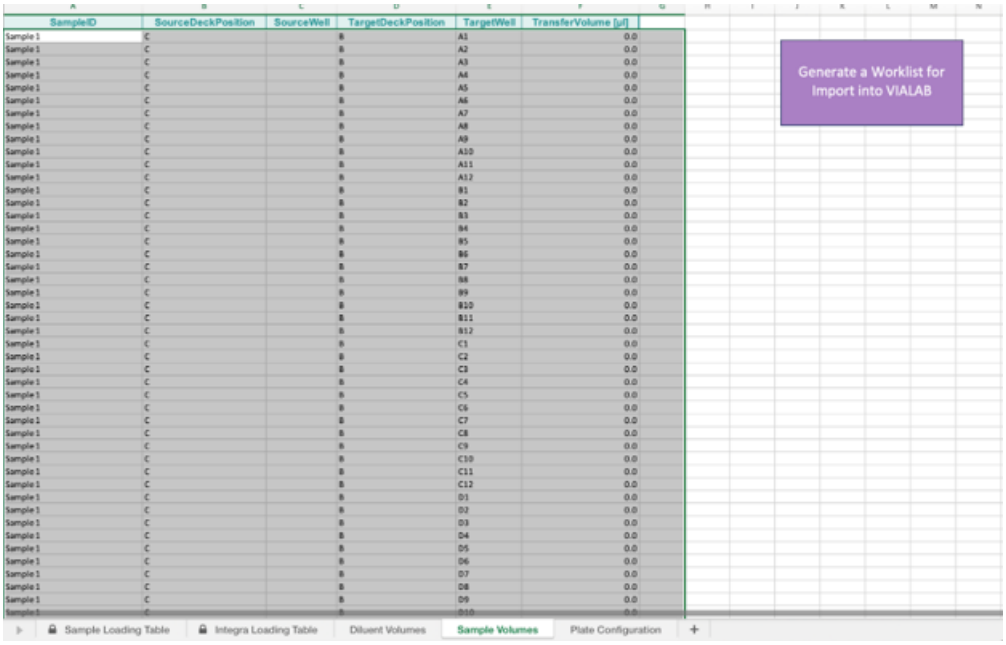


Figure 5: Sample Dilutions Volumes tab for generating a VIALAB worklist.

11. Navigate to the "Plate Configuration" tab to visualize the final sample location and orientation within the 96 well plate format (Figure 6).

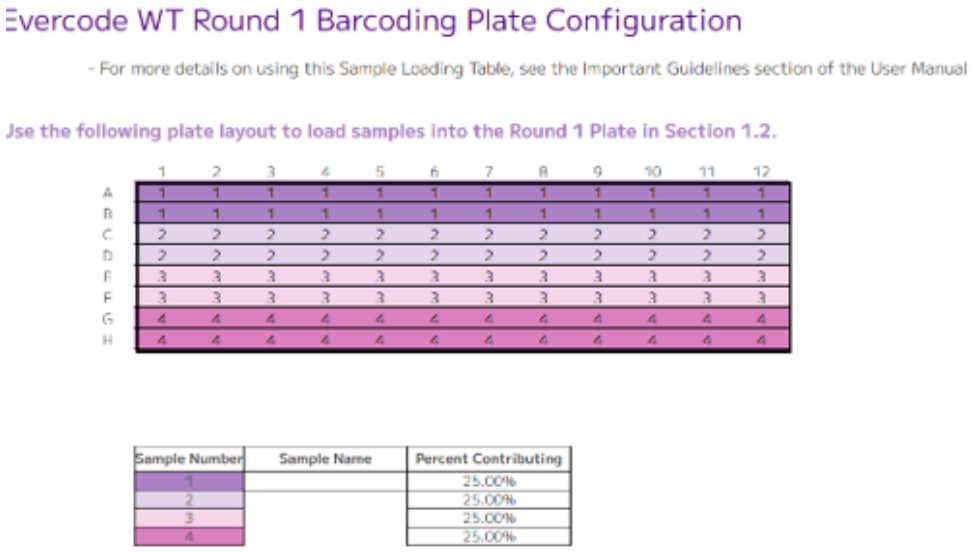


Figure 6: Plate Configuration tab visualizes the sample locations and orientations.

12. Open the VIALAB program **MG S1 St1 DONE V3** and navigate to the "Method" section. In the "02 Worklist", under the "Worklist and Volumes" tab, upload the "DiluentVolume.csv" worklist file generated in Step 9 using the "Import" button (Figure 7).

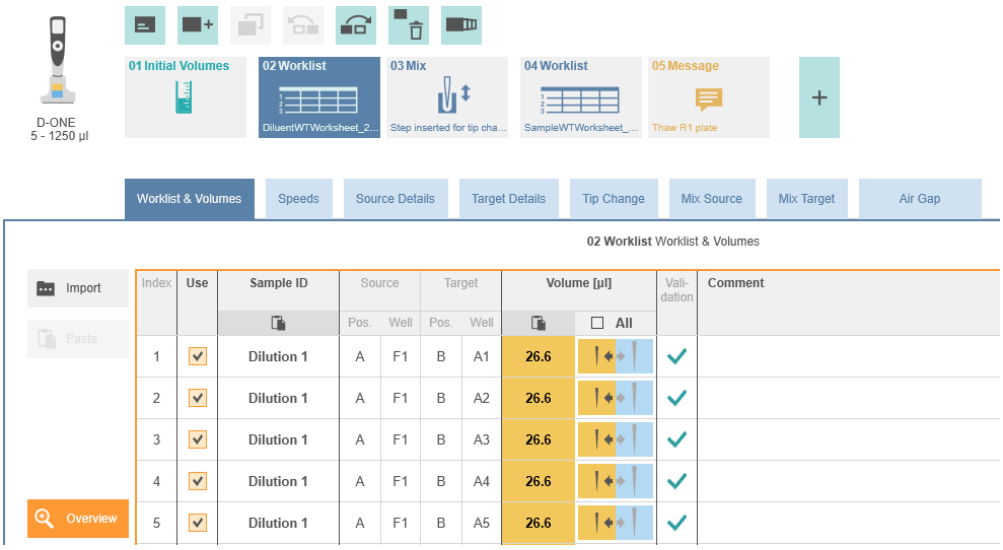


Figure 7: VIALAB worklist generation for diluent volumes using 02 Worklist.

CRITICAL! After uploading the worklist, all pipetting settings will automatically reset to standard default settings. Readjust the pipette settings to the correct worklist parameters. See Appendix A for the complete list of pipette parameters for each worklist.

13. In the "04 Worklist", under the "Worklist and Volumes" tab, upload the "SampleVolume.csv" worklist file generated in Step 10 using the "Import" button (Figure 8).

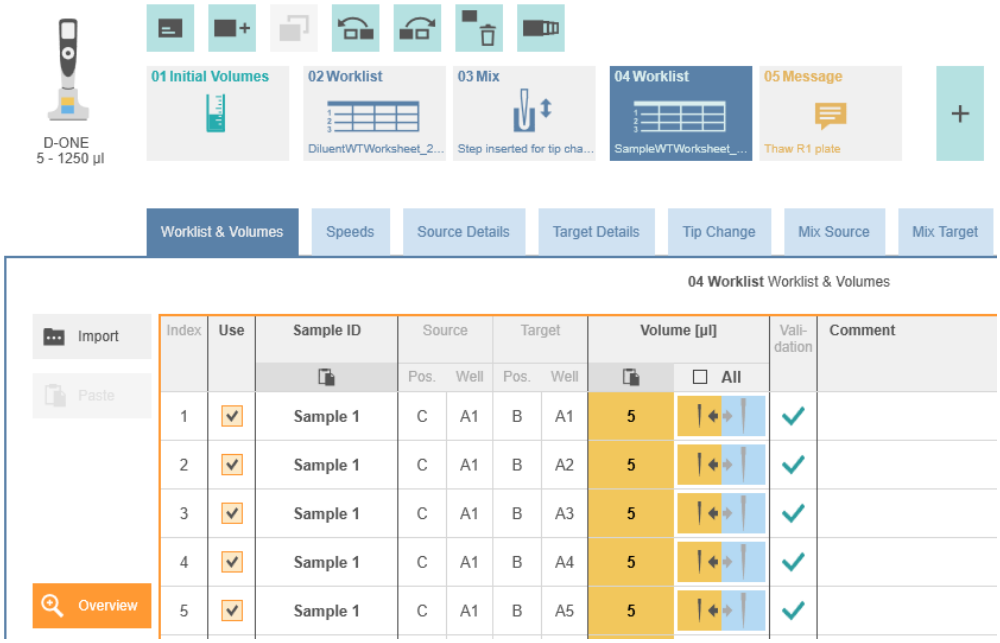


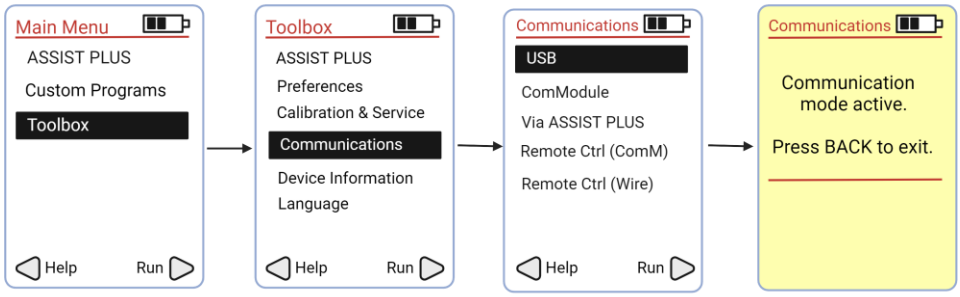
Figure 8: VIALAB worklist generation for sample volumes using 04 Worklist.

CRITICAL! After uploading the worklist, all pipetting settings will automatically reset to standard default settings. Readjust the pipette settings to the correct worklist parameters. See Appendix A for the complete list of pipette parameters for each worklist.

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

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

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

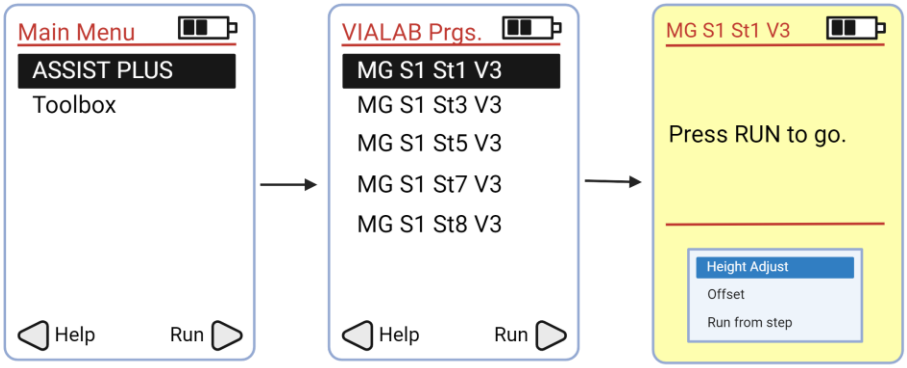


iii. In the VIALAB on your computer, select “Transfer”.

iv. Confirm that the pipette has been recognized by the software. Click "Send to pipette". This will upload the **MG S1 St1 V3** program to the D-ONE Pipette.

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

vi. If done correctly, a program named **MG S1 St1 V3** will be found on your pipette as shown in the diagram below.



15. Place the Barcoding Reagents in an ice bucket.

16. Remove both Thermochromic PCR Cold Blocks with Thermochromic PCR Cold Block Risers from -20°C and thaw at room temperature for **10 minutes**. Ensure that the corner sizes align between the colored Thermochromic PCR Cold Block and the gray Thermochromic PCR Cold Block Riser (Figure 9).

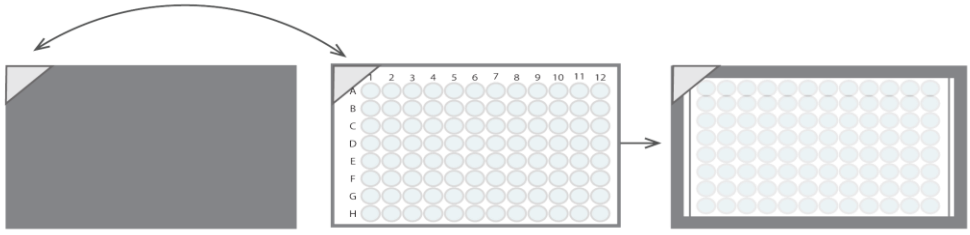
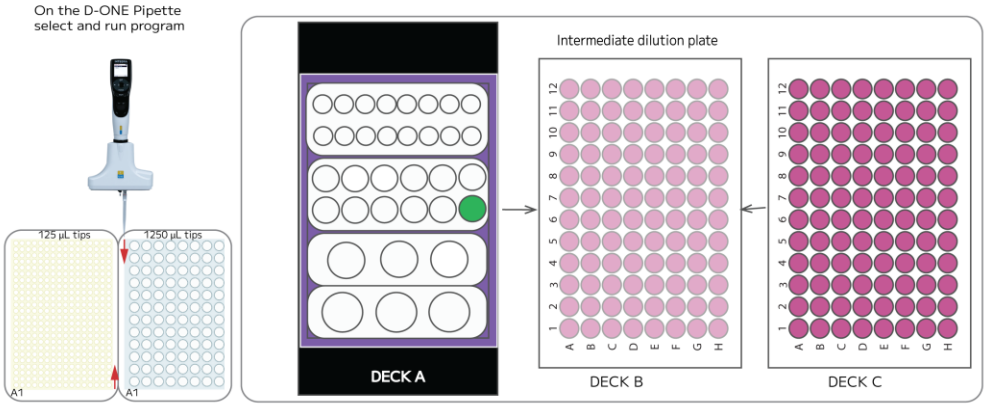


Figure 9: Corners are aligned between the Thermochromic PCR Cold Block and the Thermochromic PCR Cold Block Riser.

17. Set up the 3 Position Universal Deck according to the deck configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 1 24 mm Labware Pedestal	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser
CONSUMABLES		Semi skirted plate (empty)	Semi skirted plate (with sample)
REAGENTS	Sample Dilution Buffer		Sample Suspension Mix

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

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.

19. Remove the reagent caps, then select and run the program **MG S1 St1 V3.**

Section 1.2. Load and Pool Round 1

The program loads the normalized cells from Section 1.1 on Deck B into the Round 1 Plate on Deck C. After Barcoding Round 1 incubation, move the Round 1 plate onto Deck B.

The program then pools all the samples in the Round 1 Plate into rows A and E.

To load the sample(s):

1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse-Provided	2	Pull the Freezer Block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	2	
Round 1 Plate	-20°C Reagents (Parse Reagents)	1	Place directly on ice.

2. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Replace it with VIAFLO 12-Ch 5-125 µL Pipette and corresponding Tip Deck.

Note: Before removing the D-ONE Pipetting Module 1-Ch, 5-1250 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove tip box lid prior to start the program.

3. **When prompted**, thaw the ▭ Round 1 Plate using the thermocycling program below.

THAW ROUND 1 PLATE		
Run Time	Lid Temperature	Sample Volume
10 min	70°C	26 µL
Step	Time	Temperature
1	10 min	25°C
2	Hold	4°C

4. Remove Thermochromic PCR Cold Block from -20°C freezer and thaw it at room temperature for the duration of the thermocycling program.

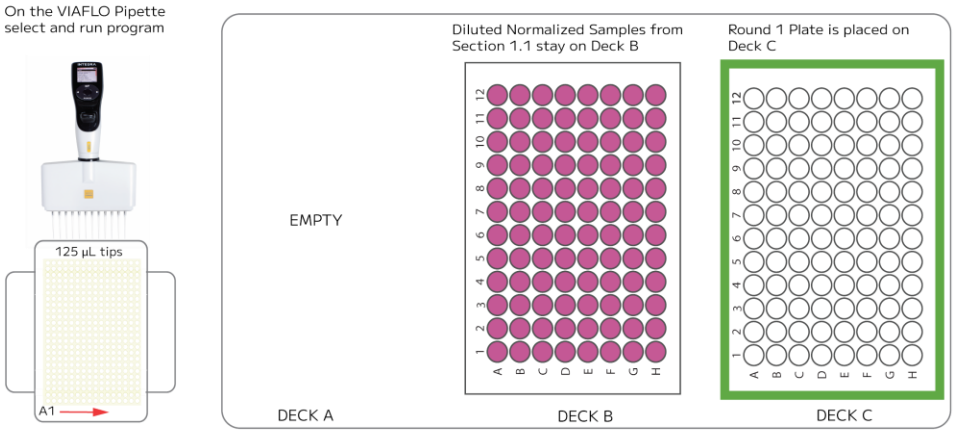
5. Gently remove the ▭ Round 1 Plate from the thermocycler and centrifuge for **1 minute** at 100 x g at 4°C.

6. Remove the ▭ Round 1 Plate from the centrifuge, place in a Thermochromic PCR Cold Block, remove the plate seal.

7. Replace the Thermochromic PCR Cold Block on Deck C with the new Thermochromic PCR Cold Block thawed during step 4. Place this old Thermochromic PCR Cold Block back in the -20°C freezer for later use.

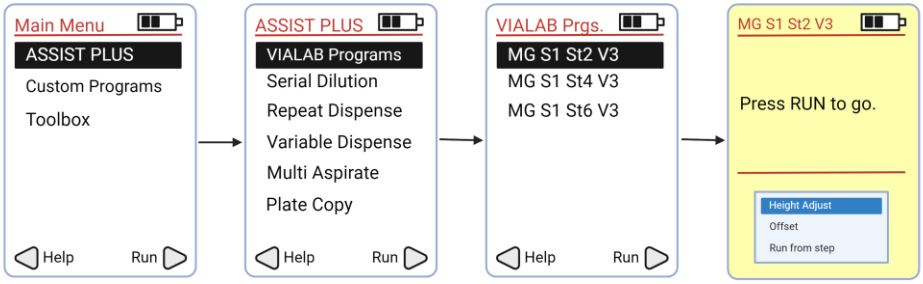
8. **When prompted**, place the ▭ Round 1 Plate in the Thermochromic PCR Cold Block located on Deck C. Ensure A1 is oriented towards the bottom left corner. Deck should correspond to the diagram below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE		Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser
CONSUMABLES			
REAGENTS		● Sample Suspension Mix	▭ Round 1 Plate

9. On the VIAFLO Pipette 12-Ch, select and run the program **MG S1 St2 V3** following the diagram below.



10. **When prompted**, seal the ▭ Round 1 Plate from Deck C using a new plate seal. This is best achieved while the plate is secured in a PCR plate rack and on a flat surface.

11. Place the ▭ Round 1 Plate into a thermocycler and run the following program.

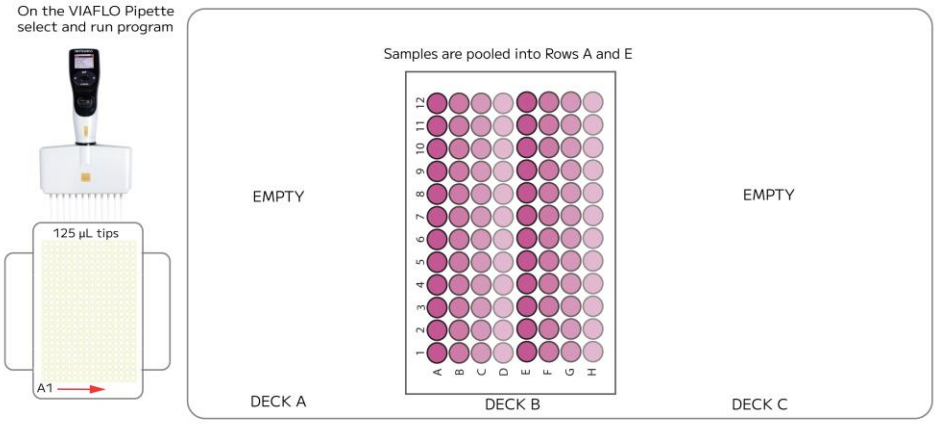
BARCODING ROUND 1			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 µL	
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	Hold

12. Discard the used semi-skirted plate on Deck B. Freeze the Thermochromic PCR Cold Block on Deck B in a -20°C freezer.

13. Ten minutes before Barcoding Round 1 thermocycling program is over, take the Thermochromic PCR Cold Block out of the freezer and place it on Deck B.

14. **When prompted**, once Barcoding Round 1 thermocycling program is over, place ▭Round 1 Plate in Thermochromic PCR Cold Block located on Deck B with A1 oriented towards the bottom left corner. Deck layout should correspond to the configuration below.

Deck Configuration



15. Remove the plate seal.

16. Press "Run" on the pipette to continue the program.

17. At the conclusion of the run, we recommend removing the Thermochromic PCR Cold Block with the Thermochromic PCR Cold Block Riser from the deck and storing them in the freezer.

Section 1.3. Round 2 Ligation Preparation

The program pools all the samples from the plate on Deck B into a 10 mL tube on Deck C. After centrifugation, removes supernatant and resuspends the cells in ○Resuspension Buffer. Mixes ● Round 2 Ligation Enzyme and ○Round 2 Ligation Buffer with the resuspended cells. The cells are then put on the left basin (A1) 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	N/A	
Thermochromic PCR Cold Block	Parse-Provided	1	Pull the freezer block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	1	
Parse Cold Block 1	Parse-Provided	1	Keep on ice when not in use.
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	N/A	
10 mL transport tube	Consumables	1	
Dual Reservoir Adapter (ANSI/SLAS footprint)	INTEGRA Components	N/A	
25 mL Basin Reservoir Liners	INTEGRA-Provided	2	
▭ Round 2 Plate	-20°C Reagents (Parse Reagents)	1	Place directly on ice.
● Round 2 Ligation Enzyme	-20°C Reagents (Parse Reagents)	1.5 mL tube	Place directly on ice. Briefly centrifuge before use.
○ Round 2 Ligation Buffer	-20°C Reagents (Parse Reagents)	5 mL tube	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Resuspension Buffer	-20°C Reagents (Parse Reagents)	5 mL tube	
● Spin Additive	4°C Reagents (Parse Reagents)	1.5 mL tube	Briefly centrifuge. Keep at room temperature.

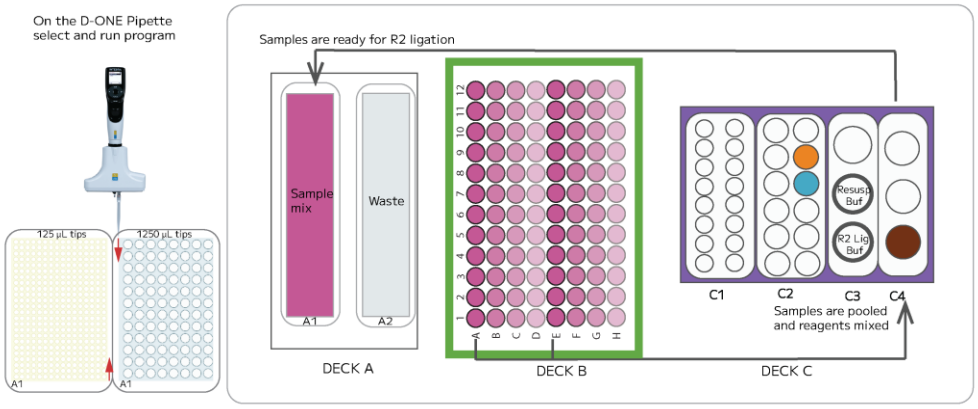
2. Place the Tube Block 1 on Deck C.

3. Place the Dual Reservoir Adapter (INTEGRA logo oriented to the front) on Deck A lined with two new 25 mL basin reservoir liners.

4. Configure the deck layout as follows:

Note: Centrifuge reagents before loading on Deck C.

Deck Configuration



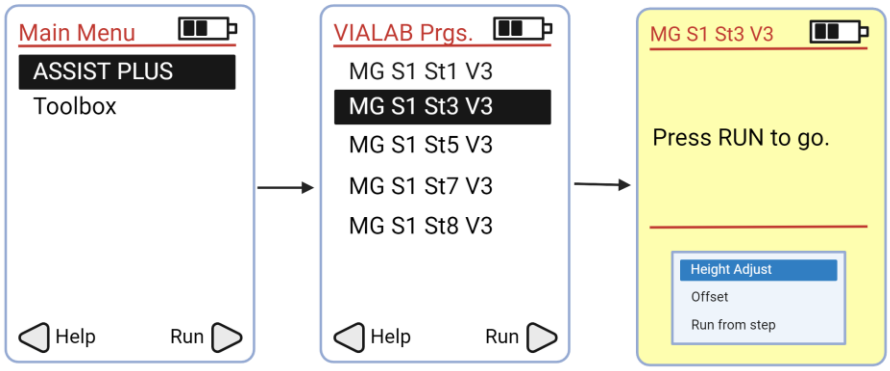
	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	Parse Cold Block 1
CONSUMABLES	25 mL basin reservoir liners		● 10 mL transport tube
REAGENTS	● Sample Suspension Mix	▭ Round 1 Plate	● Spin Additive ● Round 2 Ligation Enzyme ○ Resuspension Buffer ○ Round 2 Ligation Buffer

5. Remove VIAFLO Pipette 12-Ch, 5-125 µL and corresponding Tip Deck. Replace it with the D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck.

Note: Before removing the VIAFLO Pipette 12-Ch, 5-125 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove tip box lid prior to start the program.

6. Remove the reagent caps, then run the program **MG S1 St3 V3** following the diagram below.



7. **When prompted**, cap and invert the 10 mL transport tube containing the pooled cells. Centrifuge the sample in a swinging bucket centrifuge cooled to 4°C for **10 minutes** at 200 x g.

8. During the 10 minutes spin, **when prompted**, thaw the ▭ Round 2 Plate using the program below for later use. Proceed to the next step while the program is still running.

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

9. Remove a new Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser from -20°C freezer and thaw for **10 minutes** during centrifugation for later use.

10. Once centrifugation is complete, **when prompted**, remove the cap from the 10 mL transport tube and place it back in its original position within the Parse Cold Block 1 located on Deck C4. Immediately proceed to the next step.

11. Press “Run” to continue.

12. Clear the deck from the Thermochromic PCR Cold Block, the Thermochromic PCR Cold Block Riser and store them in the freezer. Remove the Parse Cold Block 1 and place it on ice.

Section 1.4. Round 2 Ligation

The program transfers Cell Suspension Mix from the left reservoir (A1) on Deck A to ▭Round 2 Plate on Deck B. Following Round 2 Incubation, the program will load Round 2 Stop Buffer in the right reservoir (A2) into each well. After Round 2 Stop incubation, it pools all samples together in the left reservoir (A1) on Deck A.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	N/A	
Tip Deck for VIAFLO Pipette 12-Ch, 5-125 µL	INTEGRA Components	N/A	
Thermochromic PCR Cold Block	Parse-Provided	1	Pull the Freezer Block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	1	
INTEGRA Dual 25mL Basin Reservoir Adapter	INTEGRA-Provided	N/A	
25 mL Basin Reservoir Liners	INTEGRA-Provided	2	
● Round 2 Stop Buffer	-20°C Reagents (Parse Reagents)	2 mL tube	Thaw at room temperature then store on ice. Mix by vortexing.

2. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Replace it with VIAFLO 12-Ch 5-125 µL and corresponding 125 µL Tip Deck.

Note: Before removing the D-ONE Pipetting Module 1-Ch, 5-1250 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove tip box lid prior to start the program.

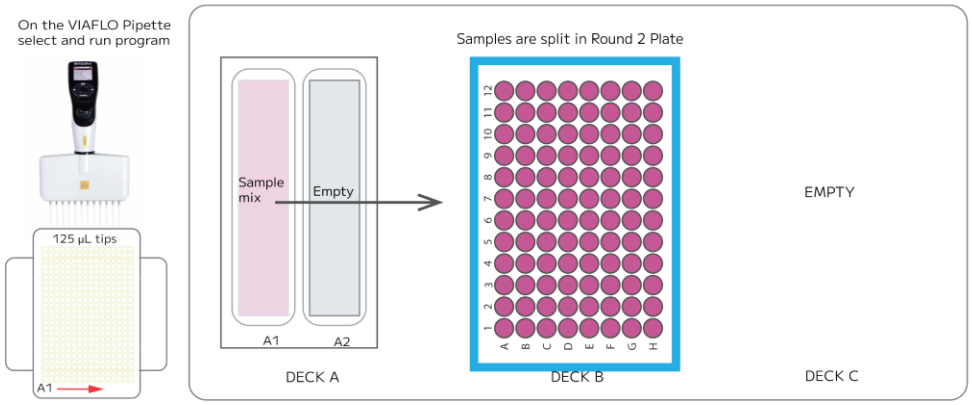
3. Remove the ▭ Round 2 Plate from the thermocycler and centrifuge for **1 minute** at 100 x g at 4°C.

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

5. Place the Thermochromic PCR Cold Block thawed during Section 1.3 step 5 on Deck B.

6. Place the ▭ Round 2 Plate on Deck B in the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser with A1 oriented towards the bottom left corner and remove the seal. The deck should correspond to the configuration below.

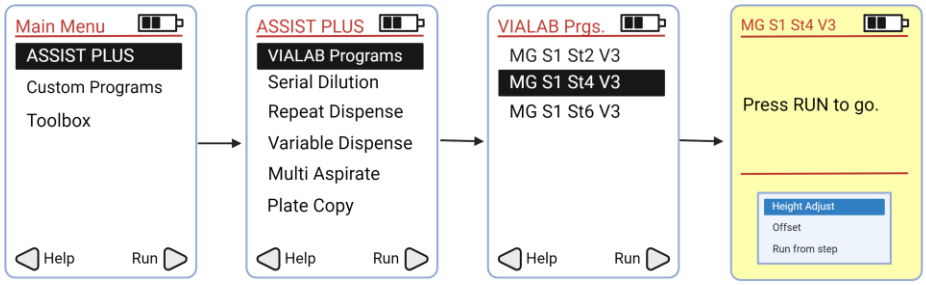
Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	
CONSUMABLES	25 mL Reservoir liners		
REAGENTS	● Sample Suspension Mix	▭ Round 2 Plate	

7.

8. Select and run the program **MG S1 St4 V3** following the diagram below.



8. **When prompted**, reseal the ▭ Round 2 Plate with an adhesive seal, place it into a thermocycler, and run the following program. Upon completion, proceed immediately to the next step.

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

9. Remove the ▭ Round 2 Plate from the thermocycler and place in a PCR plate rack.

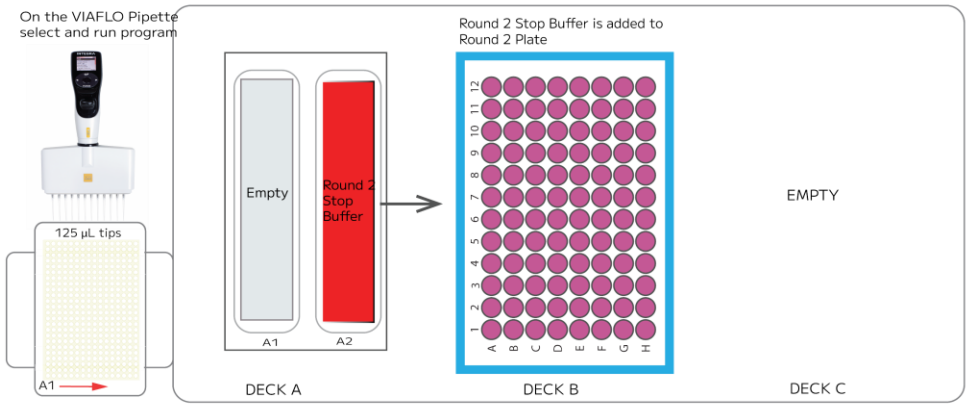
10. Remove the plate seal and place the ▭ Round 2 Plate back on Deck B with A1 oriented towards the lower left corner.

11. **When prompted**, replace the 25 mL basin reservoir liners with a new 25 mL basin reservoir liner.

Note: The right basin liner contains general waste and can be flushed down the sink. Dispose basin liners in biohazard waste.

12. Briefly vortex (2-3 seconds) and centrifuge the Parse ● Round 2 Stop Buffer. **When prompted**, using a P1000 pipette, add the total volume (~1.4 mL) to the right basin (A2) on Deck A.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	
CONSUMABLES	25 mL reservoir liners		
REAGENTS	● Round 2 Stop Buffer	▭ Round 2 Plate	

CRITICAL! When adding the ● Round 2 Stop Buffer to the reservoir ensure the volume is evenly distributed for optimal pipetting.

13. Remove the reagent caps, then press "Run" to continue.

14. **When prompted**, reseal the ▭ Round 2 Plate with an adhesive seal, and incubate the ▭ Round 2 Plate in a thermocycler using the following protocol. Upon completion, proceed immediately to the next step.

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

15. Place the ▭ Round 2 Plate within the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser located on Deck B with A1 oriented towards the bottom left.

16. Remove the seal and press "Run".

17. At the conclusion of the run, we recommend removing the Thermochromic PCR Cold Block with the Thermochromic PCR Cold Block Riser from the deck and storing them in the freezer.

Section 1.5. Round 3 Ligation Preparation

The pooled cell suspension in the left reservoir (A1) on Deck A is strained into the 10 mL transport tube on Deck C4. The program adds ● Round 3 Ligation Enzyme into the cells and mixes. It then transfers the cell suspension mix to the top reservoir (A1) 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Parse Cold Block 1	Parse-Provided	1	Keep on ice when not in use.
Dual Reservoir Adapter (ANSI/SLAS footprint)	INTEGRA Component	N/A	
ASSIST PLUS Slanted Plate Holder	INTEGRA Component	N/A	
10 mL transport tube	Consumables	1	
25 mL Basin Reservoir Liners	INTEGRA-Provided	2	
40 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	
▭ Round 3 Plate	-20°C Reagents (Parse Reagents)	1	Place directly on ice.
● Round 3 Ligation Enzyme	-20°C Reagents (Parse Reagents)	1.5 mL tube	Place directly on ice. Briefly centrifuge before use.

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

Note: Ensure that the lowest side is positioned adjacent to Deck A.

3. Place the Parse Cold Block 1 on Deck C.

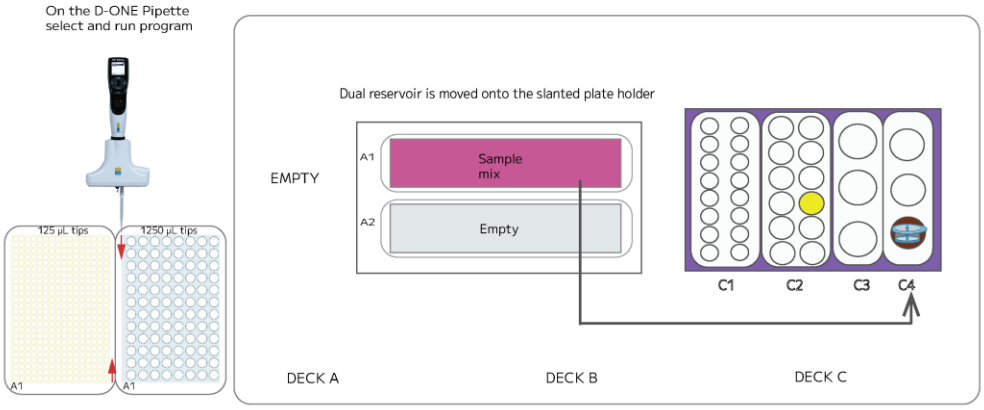
4. Briefly centrifuge and insert the ● Round 3 Ligation Enzyme tube in the appropriate location in the Parse Cold Block 1.

5. Place a 10 mL transport tube with the 40 µm cell strainer within its respective location in the Parse Cold Block 1 located in the Reagent Block in C4 position. Deck layout should correspond to the Deck Configuration below.

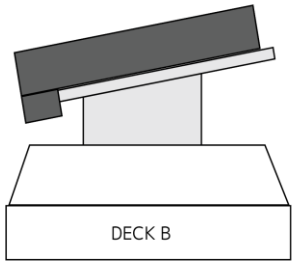
Deck Configuration

Note: When moving the dual reservoir adapter onto the slanted plate holder on Deck B, ensure the INTEGRA logo is oriented towards the front left.
Note: Use extra care when moving the cell suspension to avoid spills.

Deck Configuration



Slanted Plate Holder (10°) front view



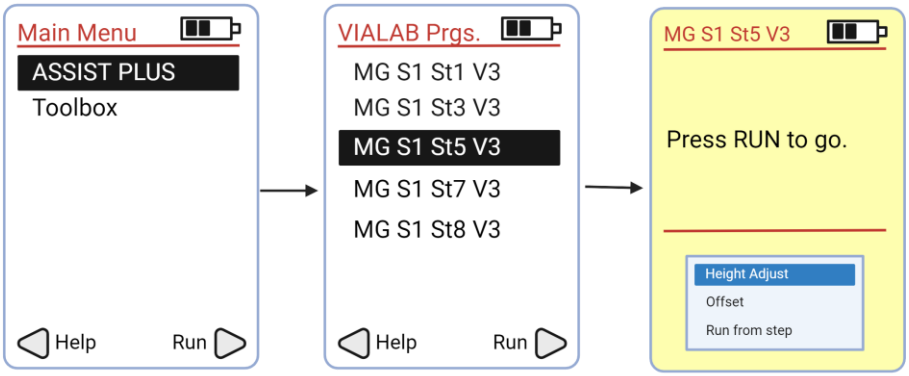
	DECK A	DECK B	DECK C
HARDWARE		Slanted Plate Holder (10°) Dual Reservoir Adapter	Parse Cold Block 1
CONSUMABLES		25 mL reservoir liners	● 10 mL transport tube with cell strainer
REAGENTS		● Sample Suspension Mix	● Round 3 Ligation Enzyme

6. Remove VIAFLO Pipette 12-Ch, 5-125 µL and corresponding tip deck. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck.

Note: Before removing the VIAFLO Pipette 12-Ch, 5-125 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.

7. Remove the reagent caps, then select and run the program **MG S1 St5 V3** following the diagram below.



8. Proceed to the next step while the program is still running.

9. Place the ▭ Round 3 Plate into a second thermocycler and run the program below. Proceed to the next step while the program is still running.

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

10. Take a new Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser from -20°C freezer and thaw for **10 minutes** during Round 3 Plate Thaw.

11. **When prompted**, remove the 40 µm cell strainer.

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

12. **When prompted**, replace the basin reservoirs containing the supernatant with new 25 mL basin reservoirs and press "Run" to continue the protocol.

13. At the conclusion of the run, remove Parse Cold Block 1 from the Deck C and place it on ice.

Section 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 Ligation 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	1	Pull the freezer block with stabilizer from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	1	
INTEGRA Dual 25mL Basin Reservoir Adapter	INTEGRA Component	1	
10 mL transport tube	Consumables	1	
25 mL Basin Reservoir Liners	INTEGRA-Provided	2	
40 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA-Provided	1	

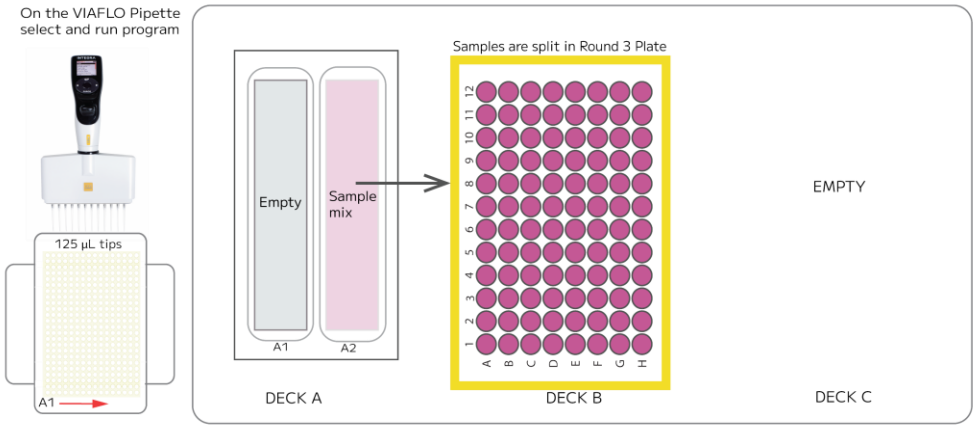
ITEM	SOURCE	QTY	HANDLING AND STORAGE
1250 µL Tip Rack	INTEGRA-Provided	1	
○ Round 3 Stop Buffer	-20°C Reagents (Parse Reagents)	5 mL tube	Thaw at room temperature then store on ice. Mix by vortexing.

2. Place Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser on Deck B.

3. Remove the ▭ Round 3 Plate from the thermocycler and centrifuge for **1 minute** at 100 x g at 4°C.

4. **When prompted**, place the ▭ Round 3 Plate within the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser located on Deck B with A1 oriented towards the bottom left corner, and remove the seal. Deck layout should correspond to the configuration below.

Deck Configuration



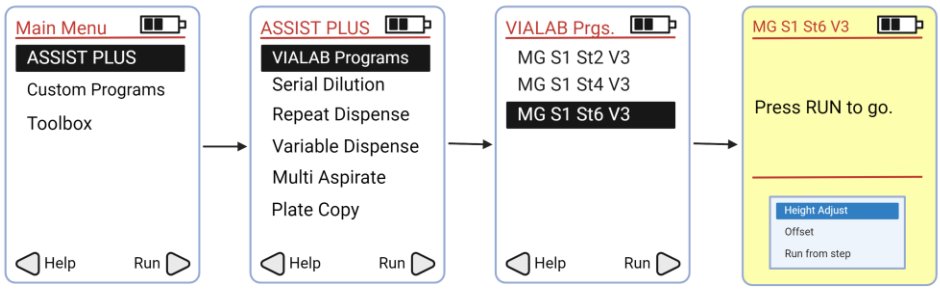
	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	
CONSUMABLES	25 mL Reservoir Liner		
REAGENTS	● Sample Suspension Mix	▭ Round 3 Plate	

5. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding tip deck. Replace it with VIAFLO Pipette 12-Ch, 5-125 µL and corresponding Tip Deck.

Note: Before removing the D-ONE Pipetting Module 1-Ch, 5-1250 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.

6. Once the pipette and tip deck with tips are loaded on deck, select and run the program **MG S1 St6 V3** following the diagram below.



7. **When prompted**, reseal the ▭ Round 3 Plate with an adhesive seal and incubate the ▭ Round 3 Plate in a thermocycler with the following protocol.

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

8. **When prompted**, place the ▭ Round 3 Plate on Deck B.

9. **When prompted,** replace the 25 mL basin reservoir liners with new 25 mL basin reservoir liners. Dispose used basin liners in biohazard waste.

10. Press "Run" on Pipette.

11. **When prompted,** follow the prompts to add all the ○Round 3 Stop Buffer to the A2 basin ensuring that the volume added into the reservoir basin is evenly distributed.

12. Press "Run" to continue.

13. At the conclusion of the run, we recommend removing the Thermochromic PCR Cold Block with the Thermochromic PCR Cold Block Riser from the deck and storing them in the freezer.

Section 1.7. Pre-Lysis

The pooled cell suspension on Deck B is strained into the 10 mL transport tube on Deck C4. The ● Spin Additive is then added into the cells and centrifuged. Supernatant is removed, the cells are resuspended in ○ Pre Lyse 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Components	N/A	
Parse Cold Block 1	Parse-Provided	1	Keep on ice when not in use.
Dual 25mL Basin Reservoir Adapter	INTEGRA Components	N/A	
10 mL transport tube	Consumables	1	
25 mL basin reservoir liners	INTEGRA-Provided	2	
40 µm cell strainer	Consumables	1	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Spin Additive	4°C Reagents (Parse Reagents)	1.5 mL tube	Keep at room temperature.
○ Pre-Lysis Wash Buffer	-20°C Reagents (Parse Reagents)	5 mL tube	Thaw at room temperature then store on ice. Mix by pipetting 3x.

2. Add slanted plate holder on Deck B and incorporate a 10 degree tilt (lowest side adjacent to Deck A).

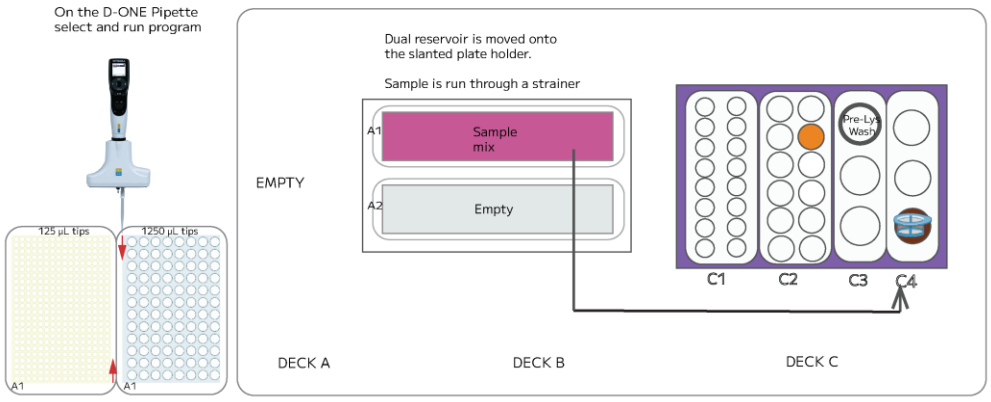
3. Place the Parse Cold Block 1 on Deck C.

4. Place the reagent tubes in their respective orientation found in the deck configuration.

5. Put the 10 mL transport tube with the cell strainer in the C4 position of the Parse Cold Block 1.

6. Put the new 1.5 mL tube in the C2 position of the Parse Cold Block 1. Deck layout should correspond to the configuration below.

Deck Configuration



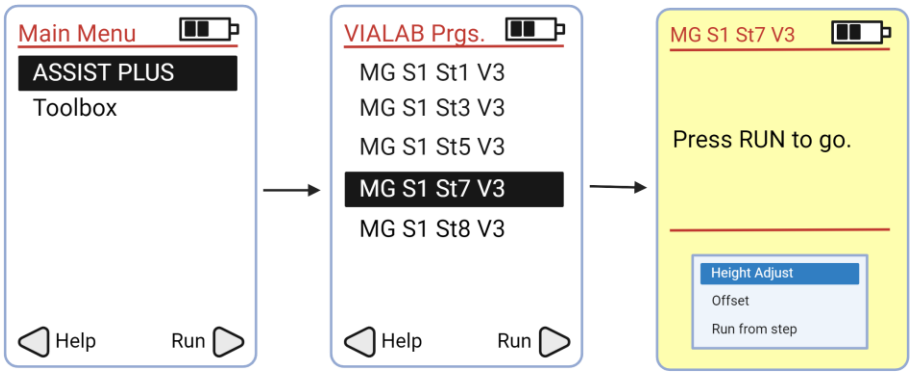
	DECK A	DECK B	DECK C
HARDWARE		Slanted Plate Holder (10°) Dual Reservoir Adapter	Parse Cold Block
CONSUMABLE		25 mL reservoir liner	● 10 mL transport tube and cell strainer
REAGENTS		● Sample Suspension Mix	● Spin Additive ○ Pre Lyse Wash Buffer

7. Remove the VIAFLO Pipette 12-Ch, 5-125 µL Pipette and corresponding Tip Deck. Attach the D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck.

Note: Before removing the VIAFLO Pipette 12-Ch, 5-125 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place.

8. Remove reagent caps, select and run the program **MG S1 St7 V3** following the diagram below.



9. **When prompted**, remove the cell strainer.

10. **When prompted**, cap and invert the 10 mL transport tube and centrifuge the pooled cells in a swinging bucket centrifuge cooled to 4°C for **10 minutes** at 200 x g.

11. **When prompted** after the centrifugation is complete, remove the cap from the 10 mL transport tube and place it back in its original position within the Parse Cold Block 1 located on Deck C4.

12. Press "Run" to continue.

13. **When prompted**, centrifuge the 10 mL transport tube in a swinging bucket centrifuge cooled to 4°C for **10 minutes** at 200 x g for a second spin.

14. Press "Run" to continue.

15. **When prompted** after the centrifugation is complete, remove the cap from the 10 mL transport tube and place it back in its original position within the Parse Cold Block 1 located on Deck C4.

16. Press "Run" to continue.

17. While minimizing time on ice, count the number of cells in the sample from the 10 mL transport tube on Deck C4 with a hemocytometer or alternative cell counting device. Record the cell count.

18. Clear the deck from the Dual 25 mL Basin Reservoir Adapter.

Section 1.8. Lysis and Sublibrary Generation

The program requires a dilution of the sample to 2500 cells per µL with a volume of 420 µL. The program will create sixteen lysates with 62,500 cells 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:

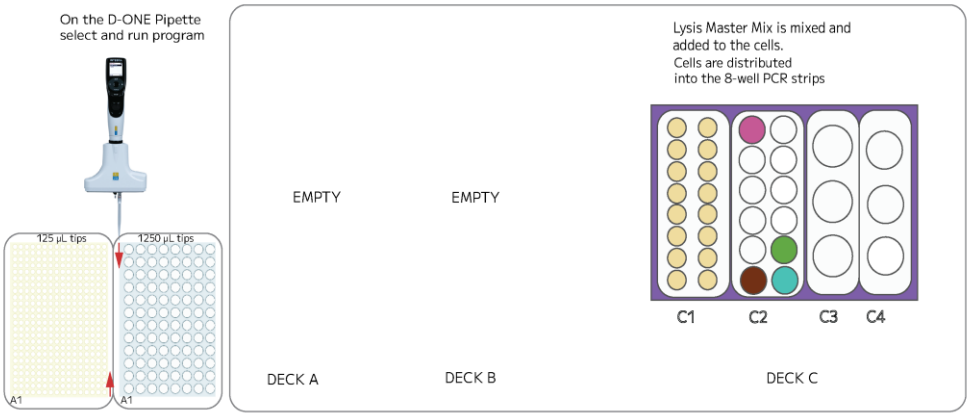
ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block 1	Parse-Provided	1	Keep on ice when not in use.
8 PCR strip tubes	Consumables	2	
1.5 mL tube	Consumables	2	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	
● Pre-Lysis Dilution Buffer	-20°C Reagents (Parse Reagents)	2 mL tube	Thaw at room temperature then store on ice. Mix by pipetting 3x.
● Lysis Buffer	4°C Reagents (Parse Reagents)	1.5 mL tube	Place directly on ice. Briefly centrifuge before use.
● Lysis Enzyme	-20°C Reagents (Parse Reagents)	1.5 mL tube	Place in a 37°C water bath until use.

2. Dilute the cells to a concentration of 2500 cells per µL for a total volume of **420 µL** using the ● Pre-Lysis Dilution Buffer in a 1.5 mL transport tube. Store this dilution on ice until ready to use.

3. Place the PCR strip tubes on Deck C1.

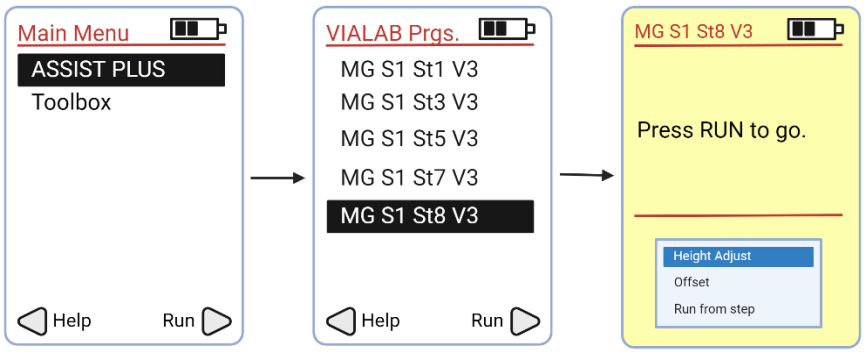
4. Place the Lysis Buffer, Lysis Enzyme, empty 1.5 mL transport tube, and diluted sample from Step 2 on Deck C2. Deck should correspond to the Deck Configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE			Parse Cold Block 1
CONSUMABLES			● 16-count PCR Tube ● 1.5 mL Transport Tube
REAGENTS			● 250 µL Sample Suspension Mix suspension ● Lysis Buffer ● Lysis Enzyme

5. Remove the reagent caps, ensure that all strip tubes are open, select and run the program **MG S1 St8 V3** following the diagram below.



6. Vortex the 0.2 mL tube(s) for **10 seconds**. Briefly centrifuge.

7. Place the tube(s) into a thermocycler and run the following program.

CELL 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

8. If continuing to Section 2 without freezing the sample, proceed to **Section 2: cDNA Capture and Amplification** in the Evercode TCR Mega User Guide while the program is still running.

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

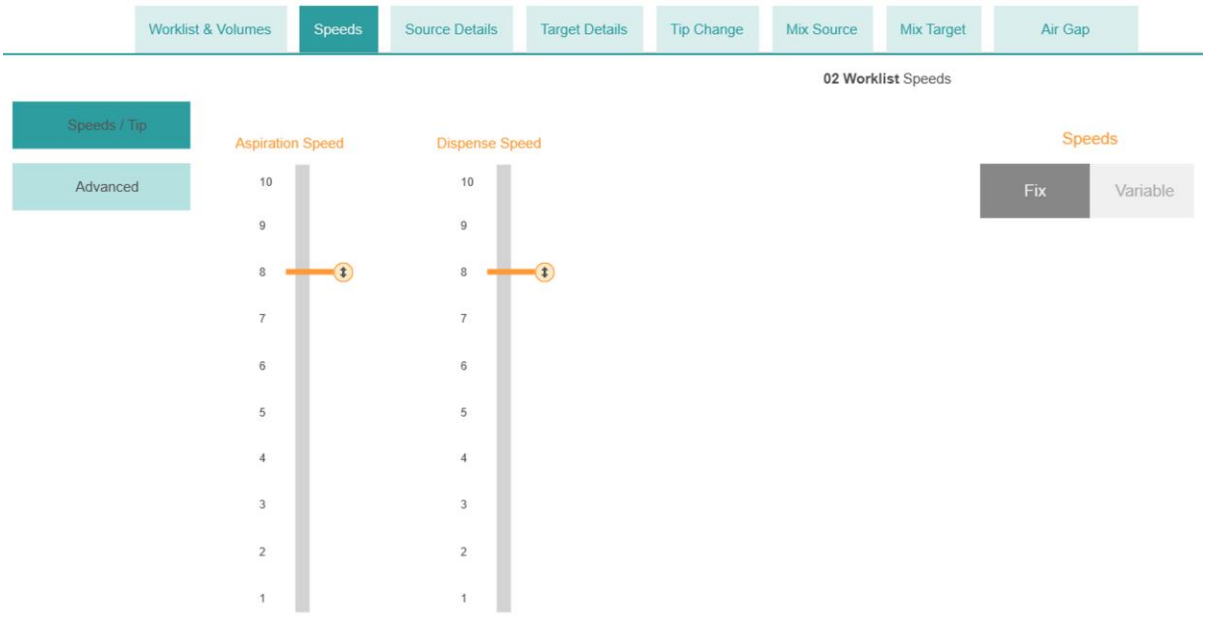
Appendices

Appendix A: Worklist Settings

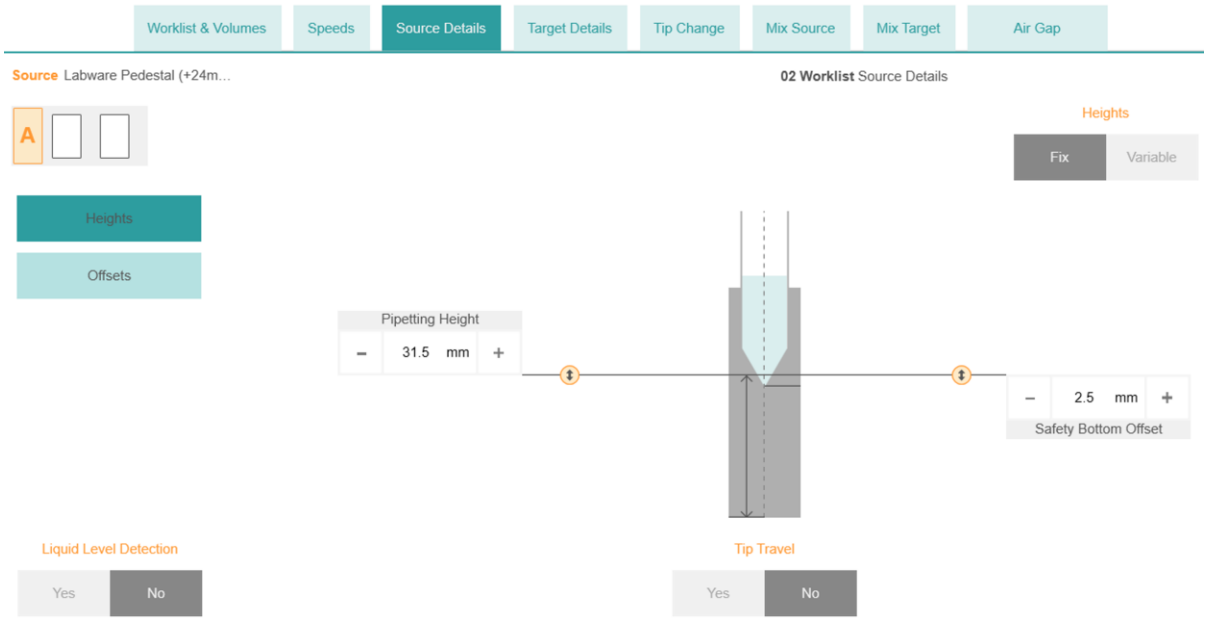
The worklist settings are shown below. Ensure that the settings in both the diluent addition and sample addition worklist steps match the settings shown below. Anything not shown can be left at its default settings.

Diluent Addition

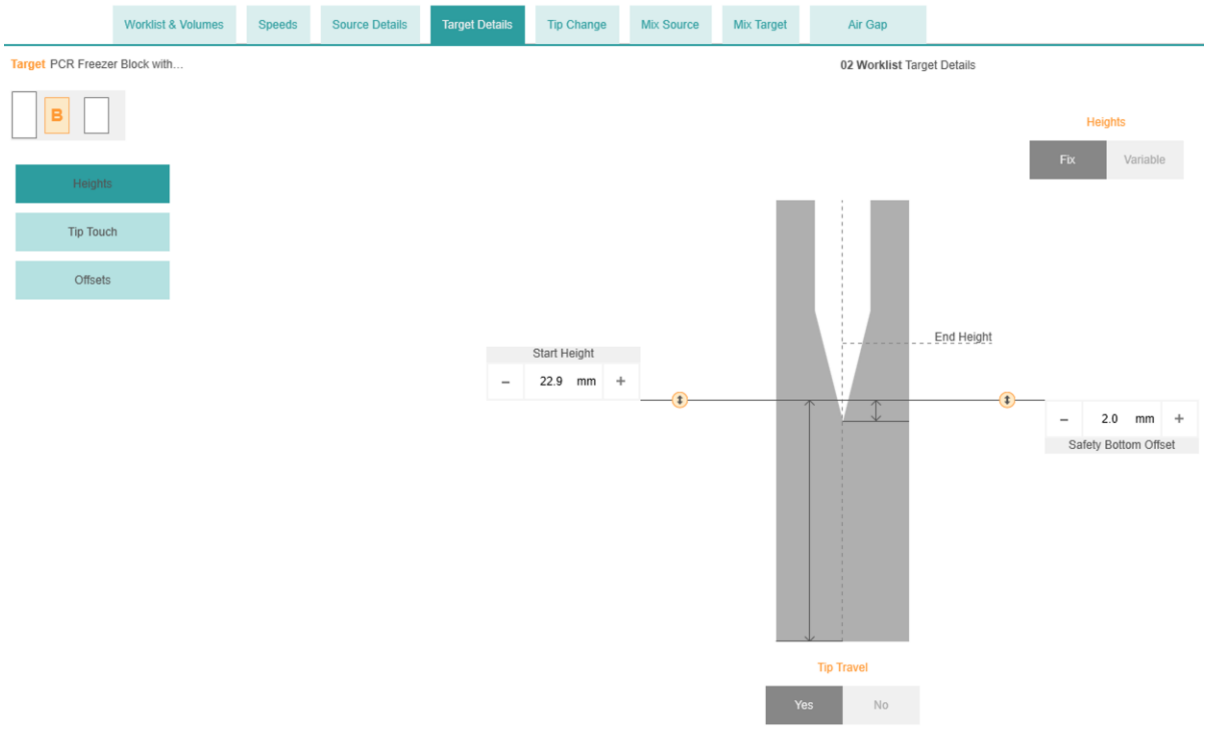
Dilution 1



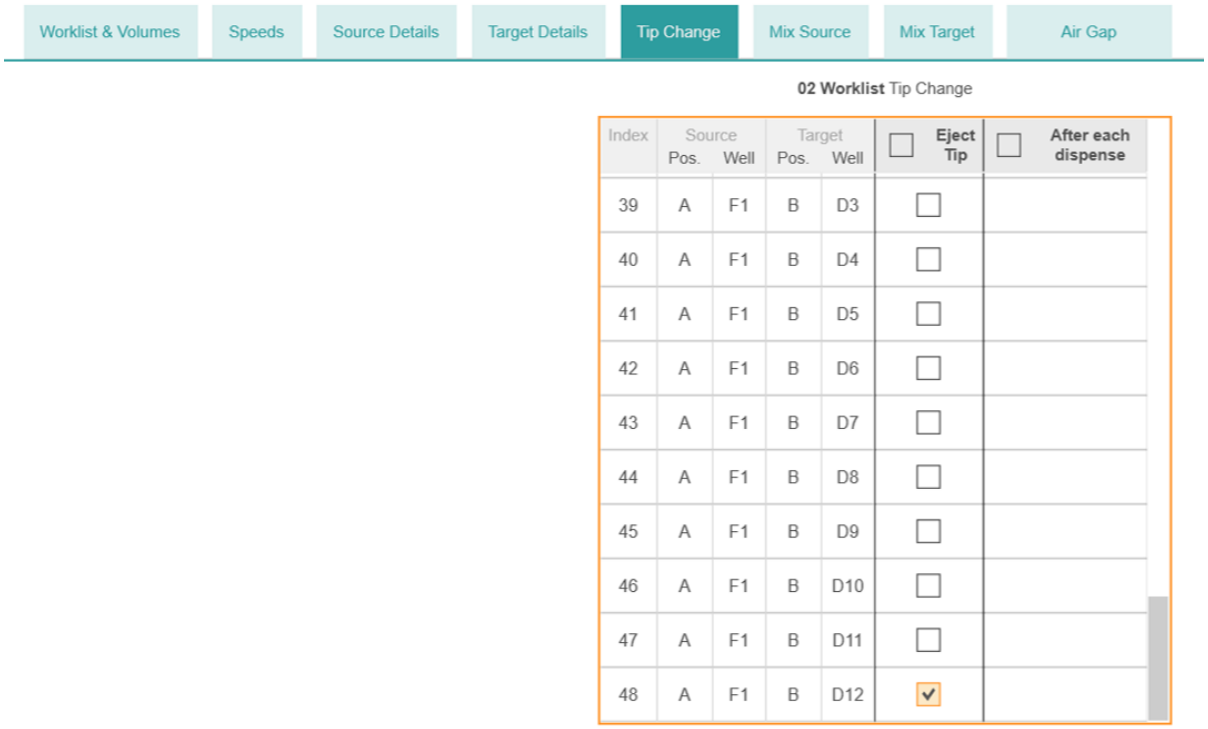
Dilution 2



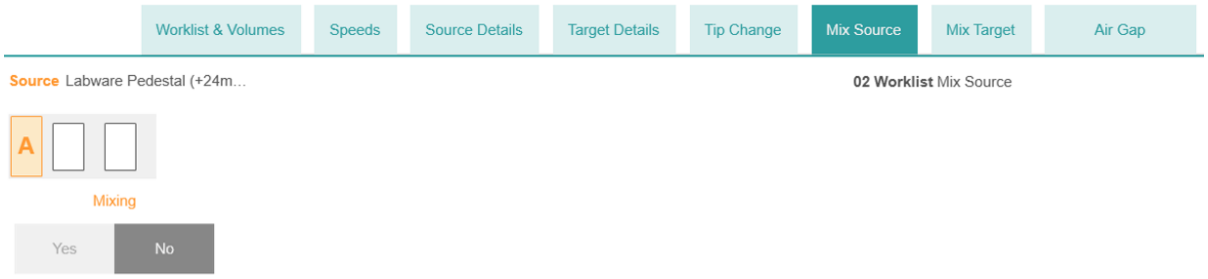
Dilution 3



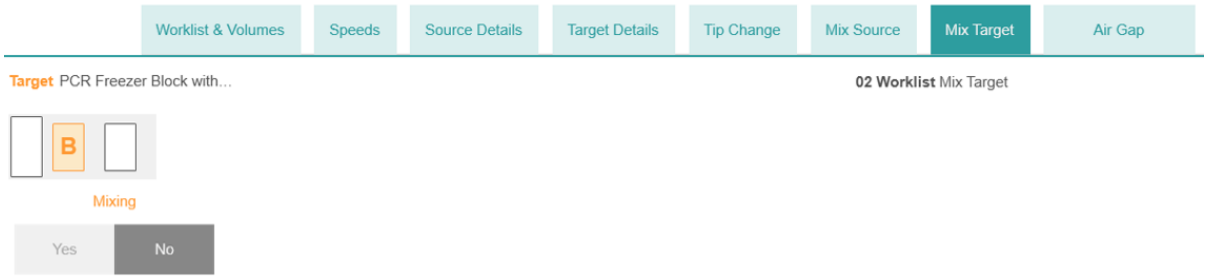
Dilution 4



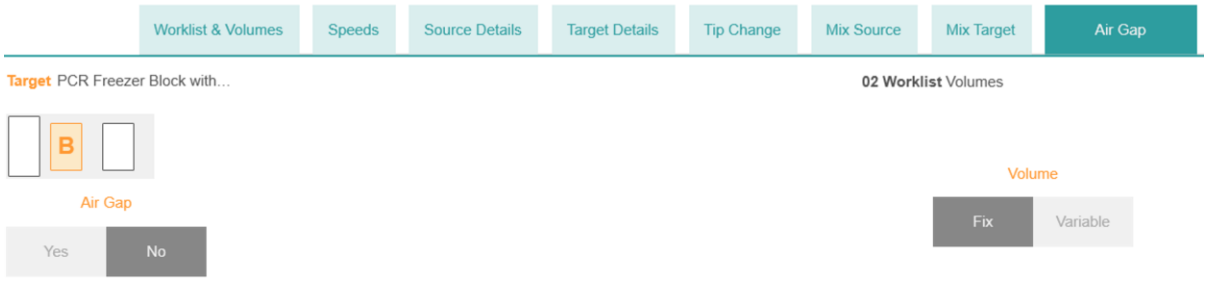
Dilution 5



Dilution 6

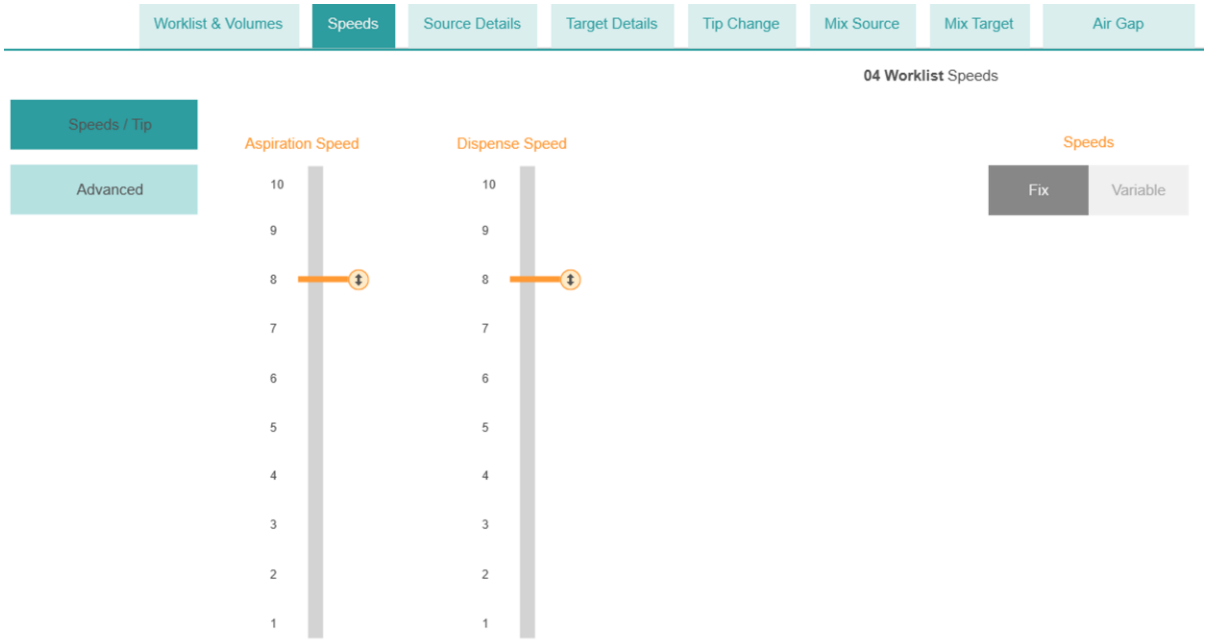


Dilution 7

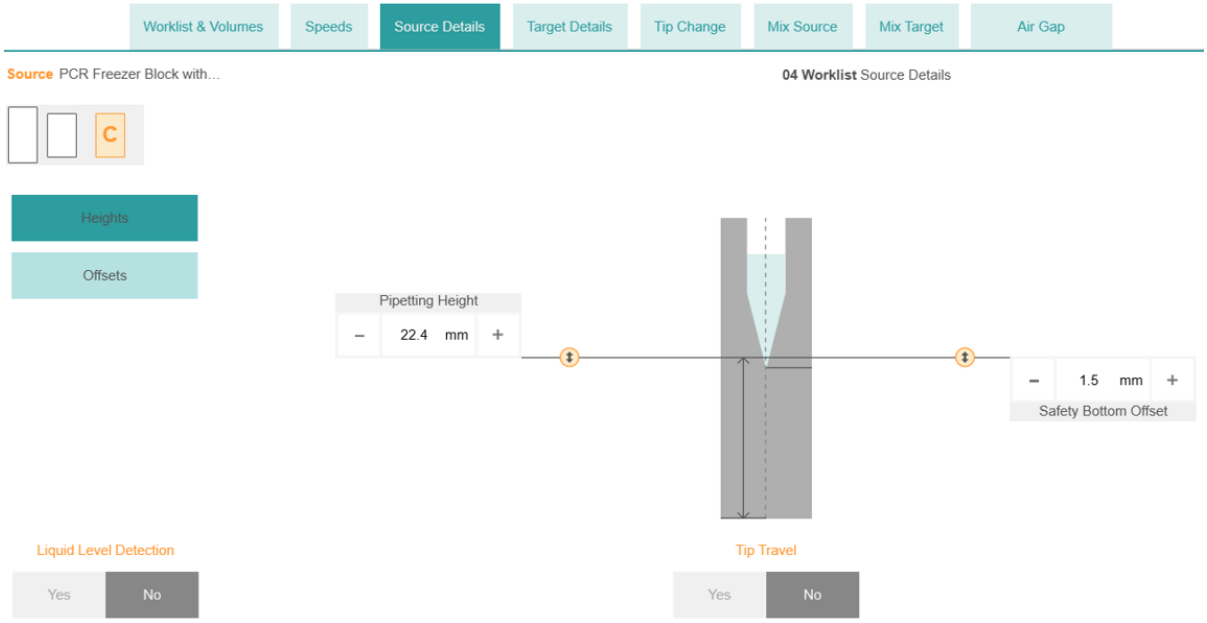


Sample Addition

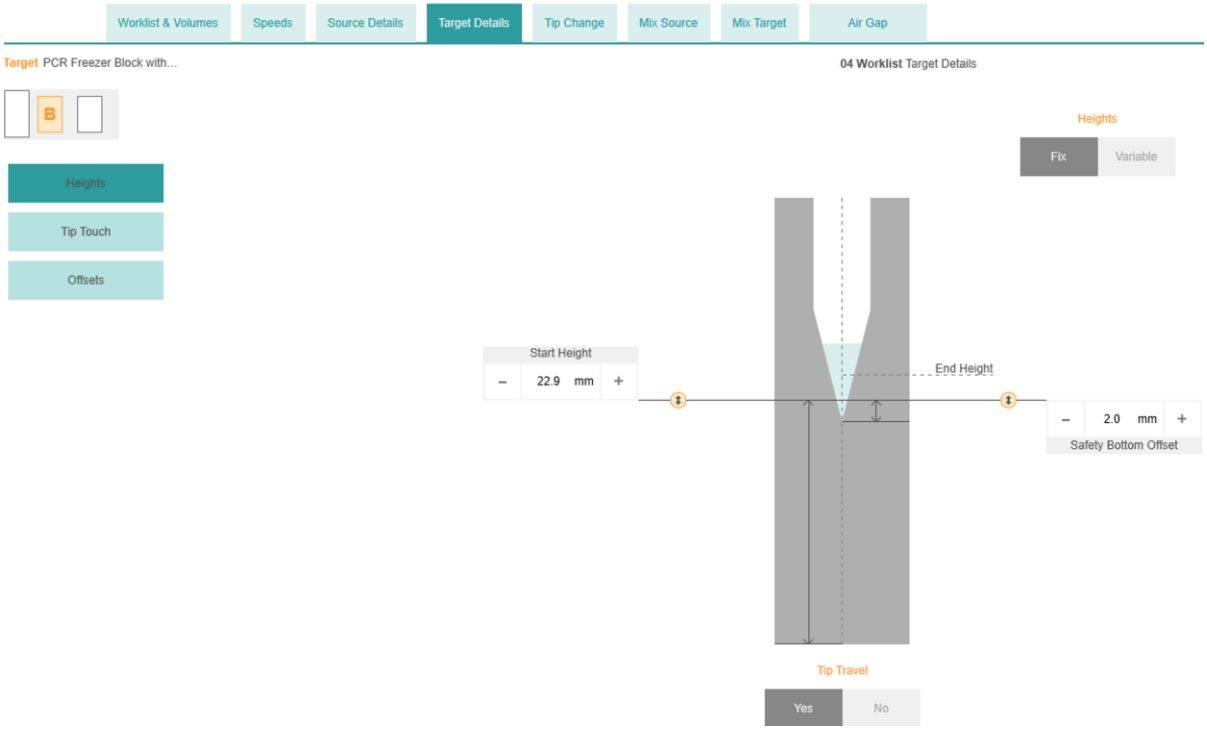
Sample 1



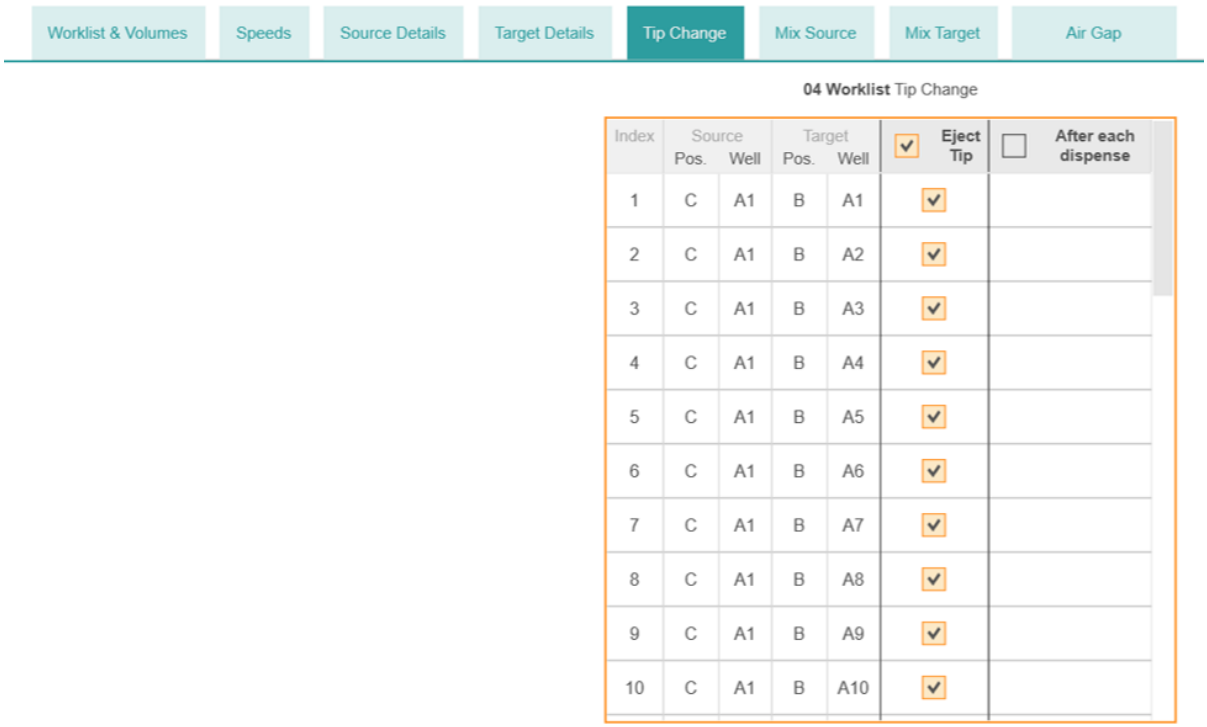
Sample 2



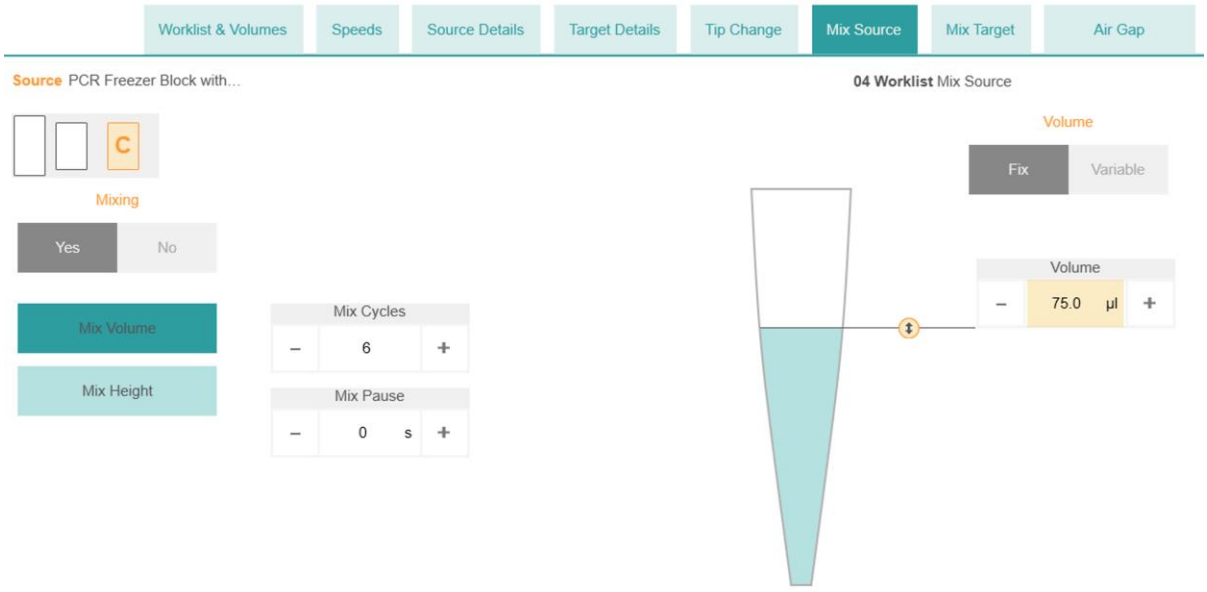
Sample 3



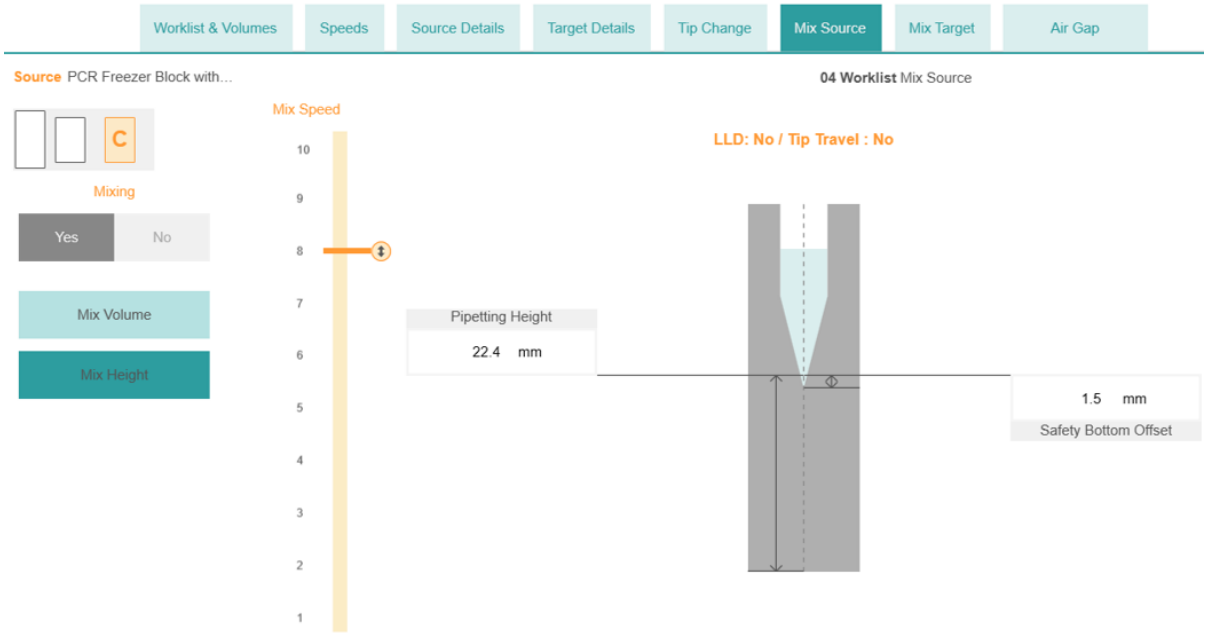
Sample 4



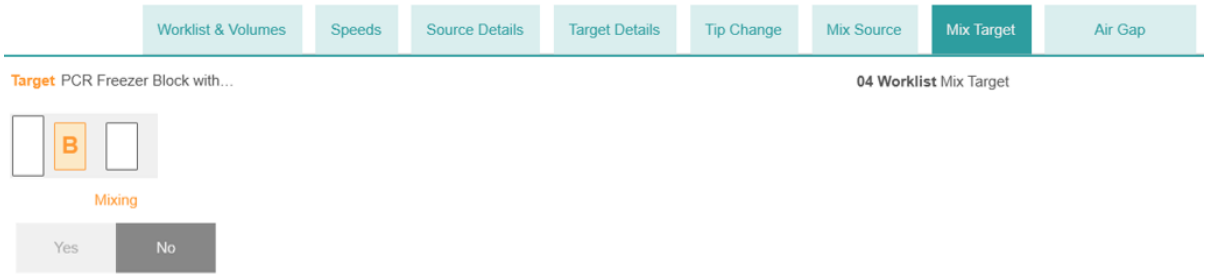
Sample 5



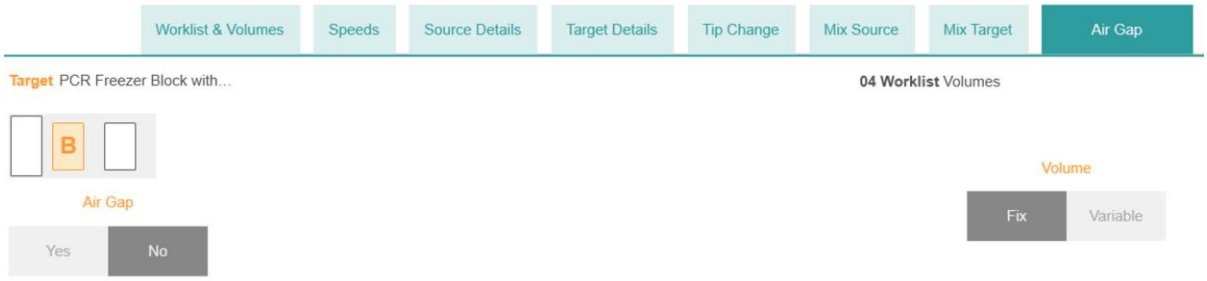
Sample 6



Sample 7



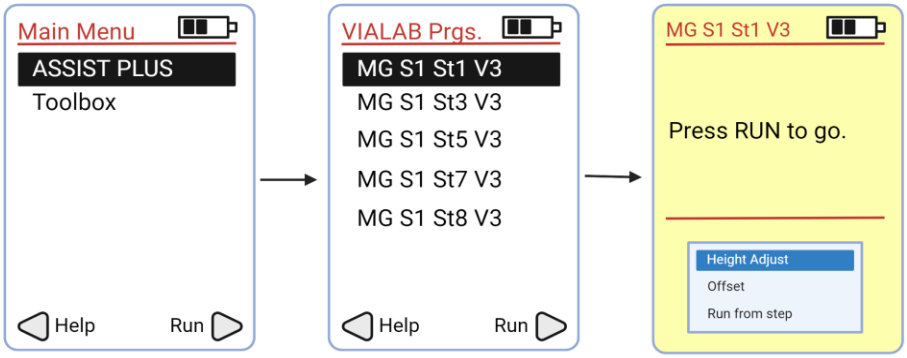
Sample 8



Appendix B: Pipetting Programs

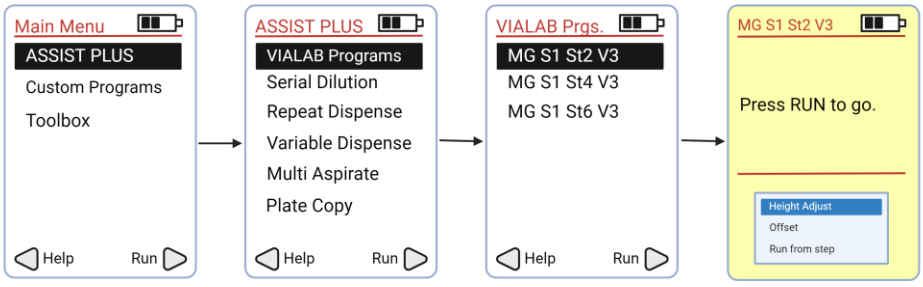
Section 1.1. Sample Normalization

MG S1 St1 V3



STEPS	ACTION
1	Initial Volumes
2	Diluent Worklist
3	Discard Tip
4	Sample Worklist
5	"Thaw R1 Plate" message

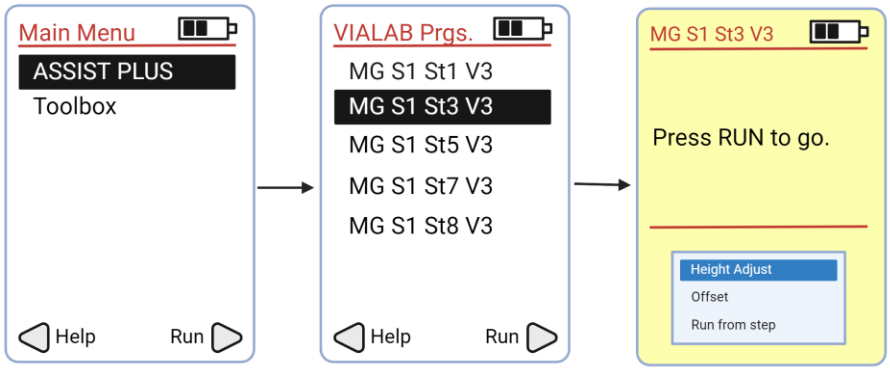
Section 1.2. Round 1 Plate Loading and Pooling
MG S1 St2 V3



STEPS	ACTION
1	Initial Volumes

STEPS	ACTION
2	“Change Plate on Deck C to R1 plate” message
3	Transfer Diluted Fixed Sample to Round 1 Barcoding Plate
4	“Seal and incubate for Round 1 RT” message
5	“Plate R1 on Deck B” message
6	Volume Change
7-8	Pool Cells to Intermediate Wells

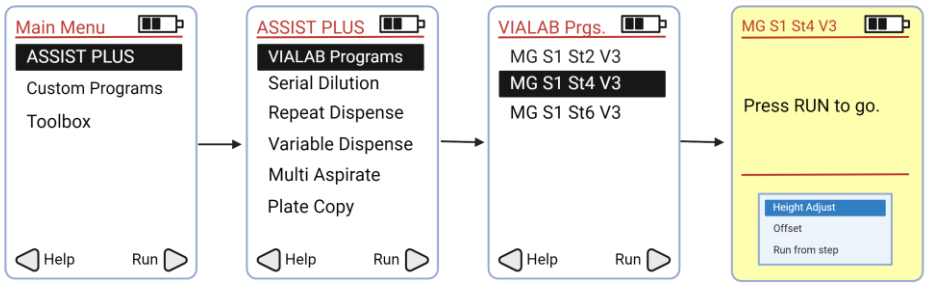
Section 1.3. Round 2 Ligation Preparation
MG S1 St3 V3



STEPS	ACTION
1	Initial Volumes
2	Pool row A into 10 mL Tube with 1000 µL tips
3	Pool row E into 10 mL Tube with 1000 µL tips
4	Pool rows into 10 mL Tube with 125 µL tips
5	Add Spin Additive
6	“Invert Tube and Spin for 10 min” message
7	“Thaw R2 Plate” message
8	“Return tube to Deck C” message

STEPS	ACTION
9	Volume Change
10-16	Remove Supernatant
17-18	Resuspend Cell Pellet with Resuspension Buffer
19	Add R2 Ligation Enzyme to Buffer
20	Mix Cells in Ligation Mix
21	Volume Change
22	Transfer Cells to Ligation Mix
23	Mix Cells in Ligation Mix
24-27	Transfer Ligation Mix to Basin
28-30	Mix Sample in Basin

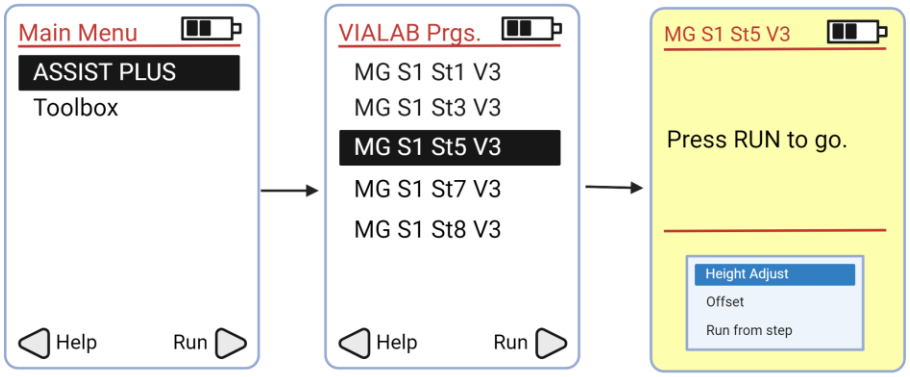
Section 1.4. Round 2 Ligation
MG S1 St4 V3



STEPS	ACTION
1	Initial Volumes
2-4	Mix samples in basins
5-12	Load Sample into Round 2 Plate
13	“Seal and incubate for R2 Ligation” message

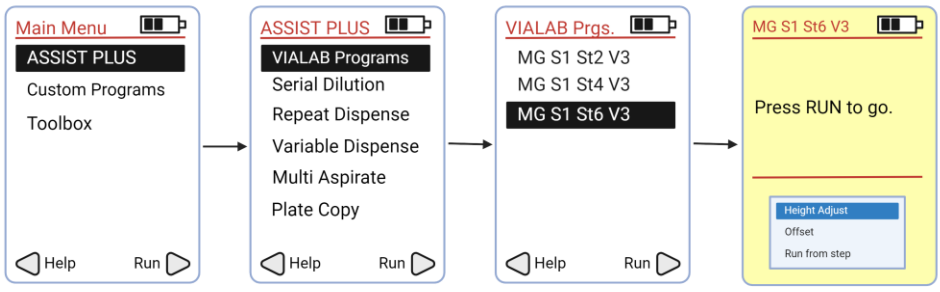
STEPS	ACTION
14	"Replace both basin liners" message
15	"Reload R2 plate on Deck B" message
16	"Add R2 Stop to right basin" message
17	Volume Change
18	Add Stop
19	"Seal and incubate for Round 2 Stop" message
20	"Thaw R3 plate" message
21	"Replace right basin liner" message
22	"Reload R2 Plate on Deck B" message
23	Pool R2 Plate to basin

Section 1.5. Round 3 Ligation Preparation
MG S1 St5 V3



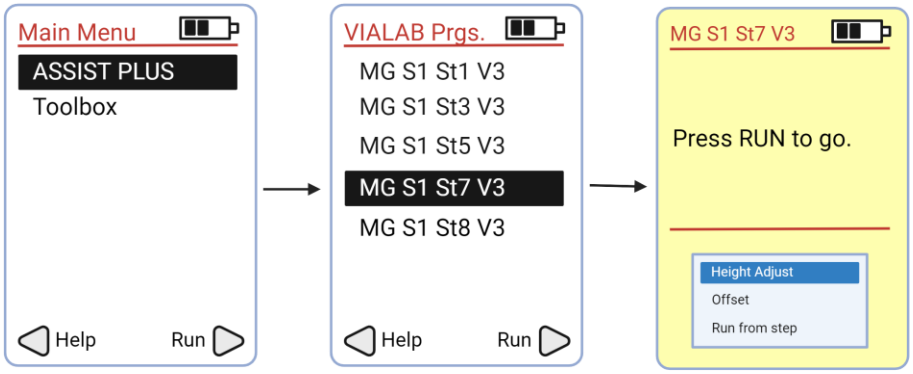
STEPS	ACTION
1	Initial Volumes
2	"Move basin to slanted holder" message
3	"Insert cell strainer in 10 mL tube" message
4	Wash basin mix
5	Volume change
6-10	Strain cells
11	"Move Basin Holder to Deck A" message
12	"Remove cell strainer" message
13	Volume change
14	Add R3 Ligation Enzyme
15	Mix Ligation Enzyme with sample
16	Volume change
17-21	Transfer cells to right basin

Section 1.6. Round 3 Ligation
MG S1 St6 V3



STEPS	ACTION
1	Initial Volumes
2-4	Mix sample in basin
5-12	Add sample to Round 3 Plate
13	"Seal and incubate for R3 Ligation" message
14	"Change both basin liners" message
15	"Reload R3 Plate on Deck B" message
16	"Add R3 Stop to right basin" message
17	Volume change
18	Add R3 Stop to plate
19	Pool R3 Plate

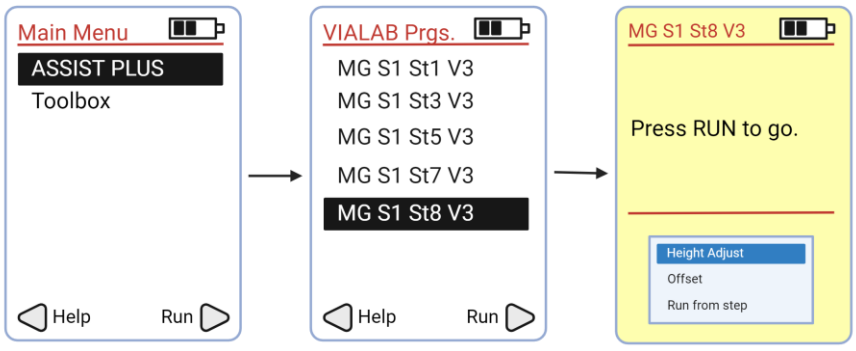
Section 1.7. Pre-Lysis
MG S1 St7 V3



STEPS	ACTION
1	Initial Volumes
2	“Move Basin Holder to Deck B” message
3	"Insert cell strainer in 10 mL tube" message
4	Wash basin

STEPS	ACTION
5	Volume change
6-12	Strain cells
13	“Move Basin Holder to Deck A” message
14	"Remove cell strainer" message
15	Add Spin Additive
16	“Invert and spin for 10 min” message
17	"Return 10 mL tube to Deck C" message
18	Volume change
19-23	Remove supernatant
24-25	Resuspend Pre-Lyse
26	"Spin for 10 min" message
27	“Return 10 mL Tube to Deck C” message
28	Volume change
29-33	Remove supernatant
34	"Count cells/nuclei" message

Section 1.8. Lysis and Sublibrary Generation
MG S1 St8 V3



STEPS	ACTION
1	Initial Volumes
2-5	Aliquot 25 µL of sample
6	Lysis Mastermix
7	Mix Lysis Mastermix
8	Add Lysis Mastermix to sample
9	“Vortex and centrifuge samples” message

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	May 2024
1.1	Updated configuration decks	June 2024
1.2	Updated box configurations	August 2024

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