User Manual

Version 1.1 – UMWT3300INT

Evercode™ WT v3 with INTEGRA ASSIST PLUS

For use with

ECWT3300

INTEGRA ASSIST PLUS

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Legal Notices

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Overview

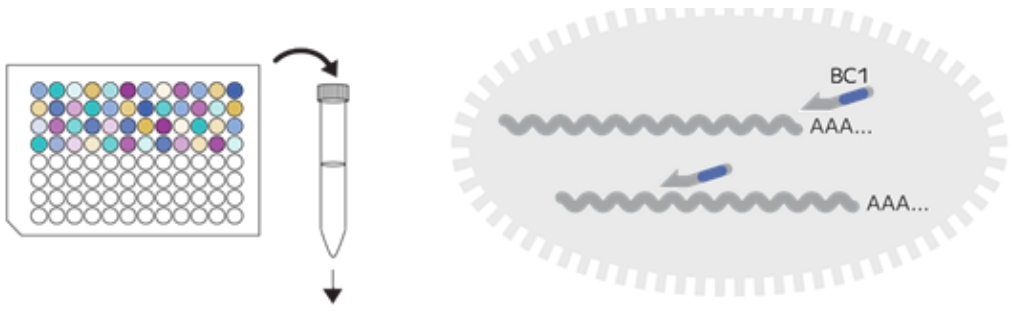
Workflow

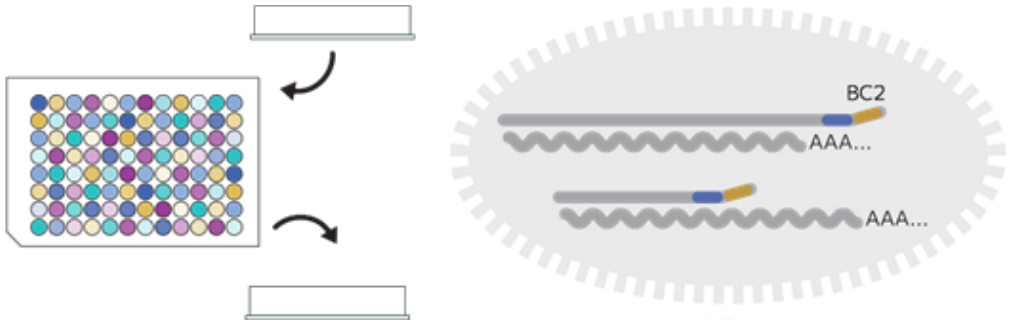
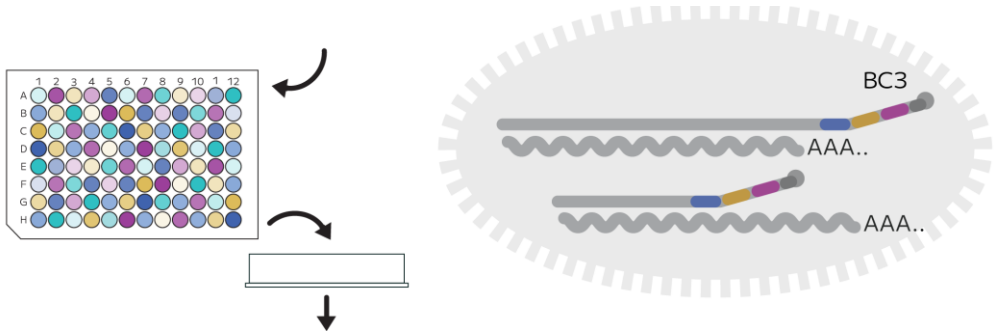
The Evercode combinatorial barcoding workflow is now compatible with the INTEGRA ASSIST PLUS to enable large-scale single cell RNA-Seq experiments through a robust, semi-automated process.

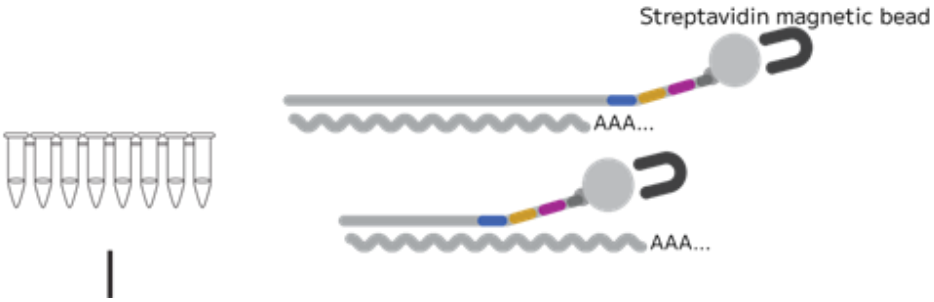
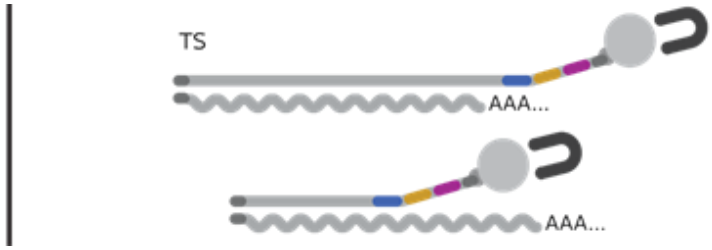
The Evercode WT v3 kit can profile up to 100,000 cells across up to 48 different biological samples or experimental conditions. Evercode Fixation kits first fix and permeabilize cells/nuclei so they act as individual reaction compartments. Through four rounds of barcoding, the transcriptome of each fixed 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 100,000 cells/nuclei while avoiding doublets. After sequencing, the Parse Biosciences Analysis Pipeline assigns reads with the same four barcode combinations to a single cell/nuclei.

By integrating the Evercode 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/nuclei into the 48 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/nuclei are then pooled and resuspended.

	Subsections 1.4 - 1.5: Round 2 Barcoding
2	 Pooled cells/nuclei 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/nuclei are then pooled and resuspended.
	Subsections 1.6-1.7: Round 3 Barcoding
3	 Pooled cells/nuclei 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/nuclei are then pooled and suspended.
	Subsection 1.8: Lysis and Sublibrary Generation
4	 Pooled cells/nuclei are split across 8 sublibraries and lysed.

	Sections 2.1-2.2: cDNA Capture
1	 Biotinylated cDNA is captured via streptavidin beads.
	Sections 2.3-2.7 Template Switch and cDNA Amplification
2	 **cDNA Template Switch:** A template switch reaction adds an adapter to the 3′ end of the cDNA.
3	 **cDNA Amplification:** The cDNA is amplified with primers specific to the template switch adapter and Illumina Truseq Read 2.

	Sections 3.1-3.3: Fragmentation and End Repair
1	 DNA is fragmented, ends are repaired, and A-tailed.
	Section 3.4-3.5 Adapter Ligation
2	 An Illumina Truseq Read 1 Adapter is ligated to the 5' end of the DNA.
	Section 3.6-3.7: Round 4 Barcoding
3	 The sequencing library is amplified, adding P5/P7 Adapters and a fourth barcode via the UDI – WT Plate.

This recommended protocol is intended to be followed when performing the Evercode assays on the INTEGRA ASSIST PLUS liquid handler. The protocol replaces "Section 1. *In Situ* Cell/Nuclei Barcoding", "Section 2. cDNA Purification and Amplification", and "Section 3. Sequencing Library Preparation" of the standard Evercode WT v3 User Guide.

Important Guidelines

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

Comprehensive guidance on optimizing the complete standard Evercode WT v3 workflow is provided in the Evercode WT v3 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 or nuclei previously fixed with an Evercode Cell Fixation v3 or Evercode Nuclei Fixation v3 kit. It is also compatible with samples fixed with Evercode Cell Fixation v2 or Evercode Nuclei Fixation v2 kits.

- 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.

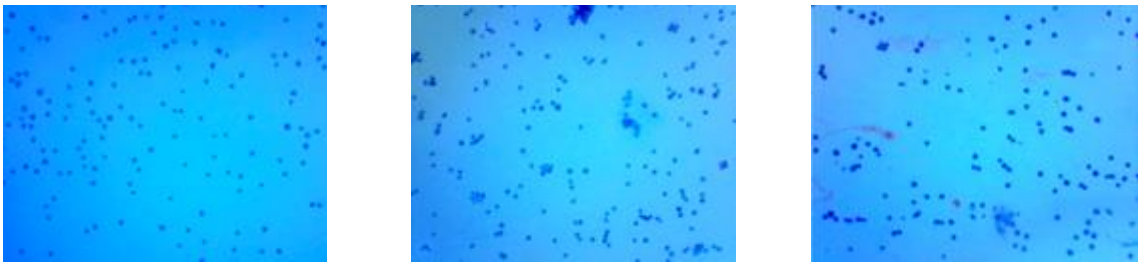
- Once fixed samples have been thawed, they should not be refrozen.

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.

- 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/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 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/nuclei loss.

Sample Loading Table

- Ensure that the Parse Evercode WT 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 Evercode WT INTEGRA 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/nuclei.

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

Plate Sealing

- 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

- Dilute the sample with the Pre-Lysis Dilution Buffer to the desired concentration. If the expected sample concentrations are too high, additional Sample Dilution Buffer in the Dilution Accessory Box should be purchased before starting the barcoding workflow.

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 precheck has been confirmed prior to running the INTEGRA + Parse workflow. This can be done by running the Evercode WT with INTEGRA ASSIST PLUS Precheck Scripts available on the Customer Support Suite.

- Ensure that the .csv worklists generated via the Parse Evercode WT INTEGRA Sample Loading Table "CombinedWTWorksheetYYYYMMDD.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.

- 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.

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 WT v3 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.

-20°C Reagents Store -20°C, PN WT100

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	WT101	Green semi-skirted 96 well plate	1
	Round 2 Plate	WT102	Blue semi-skirted 96 well plate	1
	Round 3 Plate	WT103	Yellow semi-skirted 96 well plate	1
	Resuspension Buffer	WT104	5 mL tube	1
	Sample Dilution Buffer	WT105	2 mL tube	1
	Round 2 Ligation Buffer	WT106	5 mL tube	1
	Round 2 Ligation Enzyme	WT107	1.5 mL tube	1
	Round 2 Stop Buffer	WT108	2 mL tube	1
	Round 3 Stop Buffer	WT109	5 mL tube	1
	Pre-Lysis Wash Buffer	WT110	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Round 3 Ligation Enzyme	WT111	1.5 mL tube	1
	Pre-Lysis Dilution Buffer	WT112	2 mL tube	1
	Lysis Enzyme	WT113	1.5 mL tube	1
	Bead Wash Buffer	WT114	5 mL tube	1
	Wash Buffer 1	WT115	5 mL tube	1
	Wash Buffer 2	WT116	5 mL tube	1
	Capture Enhancer	WT117	1.5 mL tube	1
	Binding Buffer	WT118	1.5 mL tube	1
	Wash Buffer 3	WT119	5 mL tube	1
	Template Switch Buffer	WT120	1.5 mL tube	1
	Template Switch Enzyme	WT121	1.5 mL tube	1
	Template Switch Primer	WT122	1.5 mL tube	1
	cDNA Amp Mix	WT123	1.5 mL tube	1
	cDNA Amp Primers	WT124	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Buffer	WT125	1.5.mL tube	1
	Fragm/End Prep Enzymes	WT126	1.5 mL tube	1
	Ligation Adapter	WT127	1.5 mL tube	1
	Adapter Ligation Buffer	WT128	1.5 mL tube	1
	Adapter Ligation Enzyme	WT129	1.5 mL tube	1
	Library Amp Mix	WT130	1.5 mL tube	1

4°C Reagents. Store 4°C, PN WT200

LABEL	ITEM	PN	FORMAT	QTY
	Spin Additive	WT201	1.5 mL tube	1
	Lysis Buffer	WT202	1.5 mL tube	1
	Streptavidin Beads	WT203	1.5 mL tube	1

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

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

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	NTAC1107	1

INTEGRA Components

The following are required INTEGRA ASSIST PLUS components needed to run the Evercode assay on the INTEGRA ASSIST PLUS.

ITEM	ITEM TYPE	PN	QTY
Pipette Communication Module for VIAFLO / VOYAGER Pipettes	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
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 Magnetic Module	Adapter	4906	1

Consumables

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

ITEM	SUPPLIER	PN	QTY
25 mL Basin Reservoir Liner	INTEGRA-Provided	4316	6
Sterilized 40 µm Mini Cell Strainer	DiagnoCine	FNK-HT-AMS-14002	2
10 mL Transport Tube	GlobeScientific	6102S	3
1.5 mL Microtube	Genesee Scientific	21-257	6
PCR Strip Tubes	USA Scientific	1402-4700	8
2 mL Microtubes	Genesee Scientific	21-255	4
8 Row Polystyrene Reservoir	INTEGRA-Provided	6373	2
Semi-skirted 96 well plates	Eppendorf	E951020362	5

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
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 & In Situ Barcoding

1.1. Sample Normalization

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

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/nuclei 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:

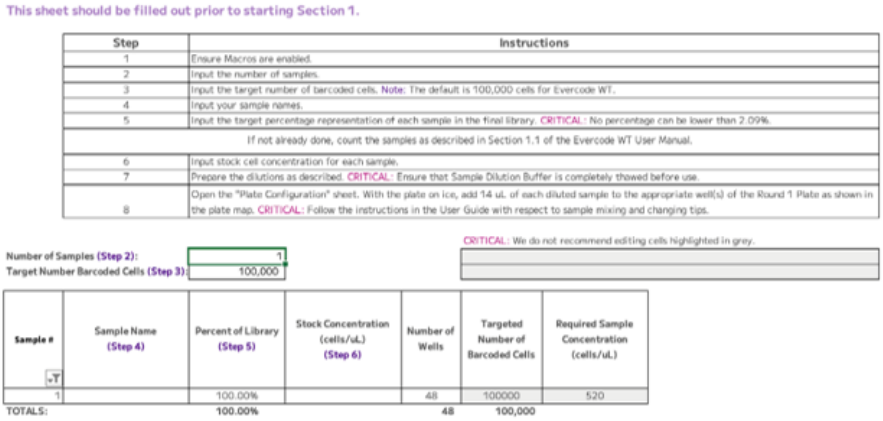
ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	Pull the Freezer Block with riser from the -20°C freezer and leave them at room temperature for 10 minutes prior to use.
Thermochromic PCR Cold Block Riser	Parse-Provided	N/A	
Parse Cold Block	Parse-Provided	N/A	Keep on ice when not in use.
Semi-Skirted 96 Well PCR Plate	Consumables	N/A	
● Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
▭ Round 1 Plate	-20°C Reagents	1	Place directly on ice.

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

Parse Biosciences Customer Support Suite. Customer log-in is required to access the Sample Loading Table.

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 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): 100,000

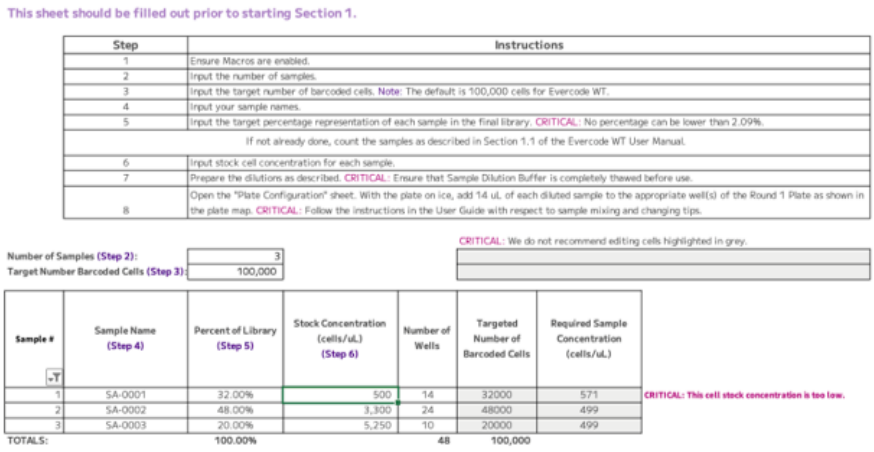
Sample #	Sample Name (Step 4)	Percent of Library (Step 5)	Stock Concentration (cells/uL) (Step 6)	Number of Wells	Targeted Number of Barcoded Cells	Required Sample Concentration (cells/uL)
1		100.00%		48	100000	520
TOTALS:		100.00%		48	100,000	

Figure 1: Evercode WT Sample Loading Table.

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



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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): 3
Target Number Barcoded Cells (Step 3): 100,000

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	SA-0001	32.00%	500	14	32000	571	CRITICAL: This cell stock concentration is too low.
2	SA-0002	48.00%	3,300	24	48000	499	
3	SA-0003	20.00%	5,250	10	20000	499	
TOTALS:		100.00%		48	100,000		

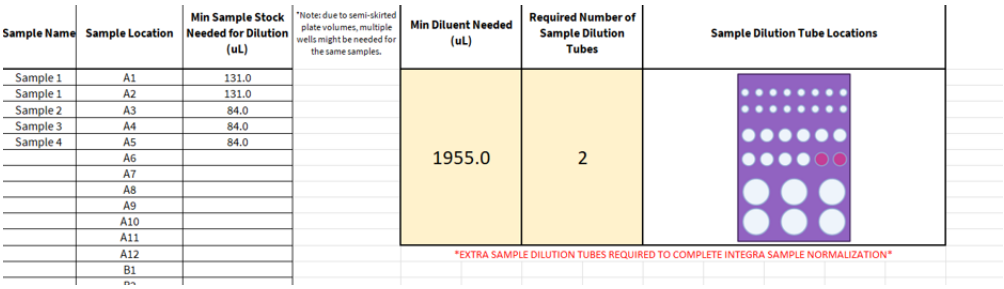
Figure 2: Example error message, noting that the sample stock concentration is too low.

6. Navigate to the "INTEGRA Loading Table" tab and check that the Minimum Diluent Needed (µL) does not exceed 1,800 µL. If the "Required Number of Sample Dilution Tubes" is greater than 1, an additional Sample Dilution Buffer is provided in the Dilution Accessory Box (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 and Sample Location A2 with a minimum volume of 131 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)
Sample 1	A1	131.0
Sample 1	A2	131.0
Sample 2	A3	84.0
Sample 3	A4	84.0
Sample 4	A5	84.0
	A6	
	A7	
	A8	
	A9	
	A10	
	A11	
	A12	
	B1	

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

Min Diluent Needed (uL)	Required Number of Sample Dilution Tubes	Sample Dilution Tube Locations
1955.0	2	

EXTRA SAMPLE DILUTION TUBES REQUIRED TO COMPLETE INTEGRA SAMPLE NORMALIZATION

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

9. 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 "CombinedWTWorksheet.csv") for later use (Figure 4).

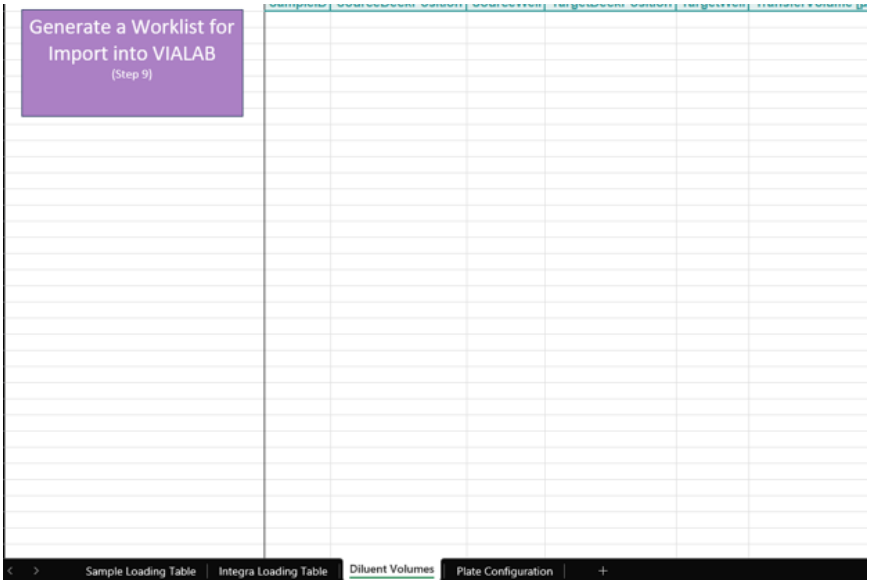


Figure 4: Diluent Buffer Volumes tab for generating a VIALAB worklist.

10. Navigate to the "Plate Configuration" tab to visualize the final sample location and orientation within the 96 well plate format (Figure 5).

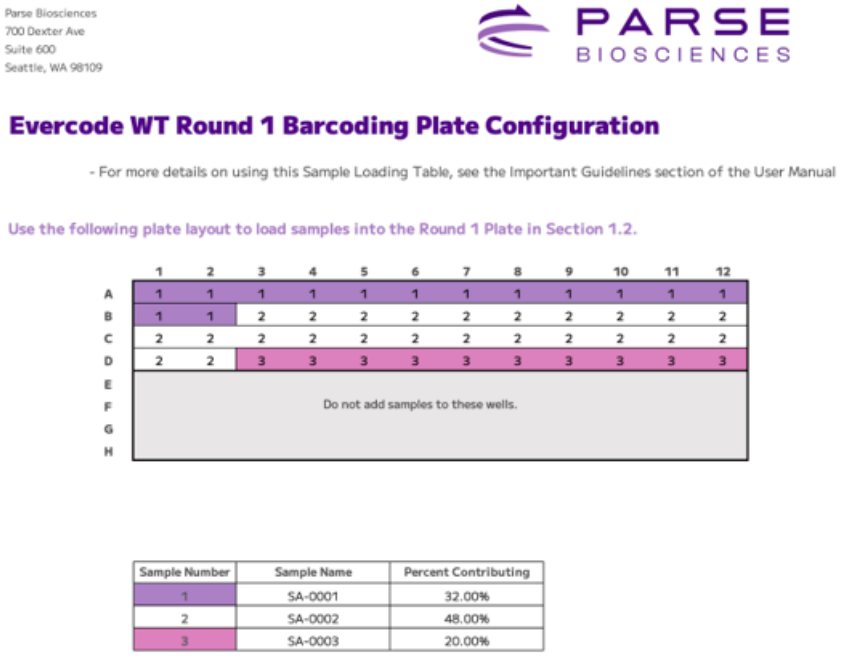
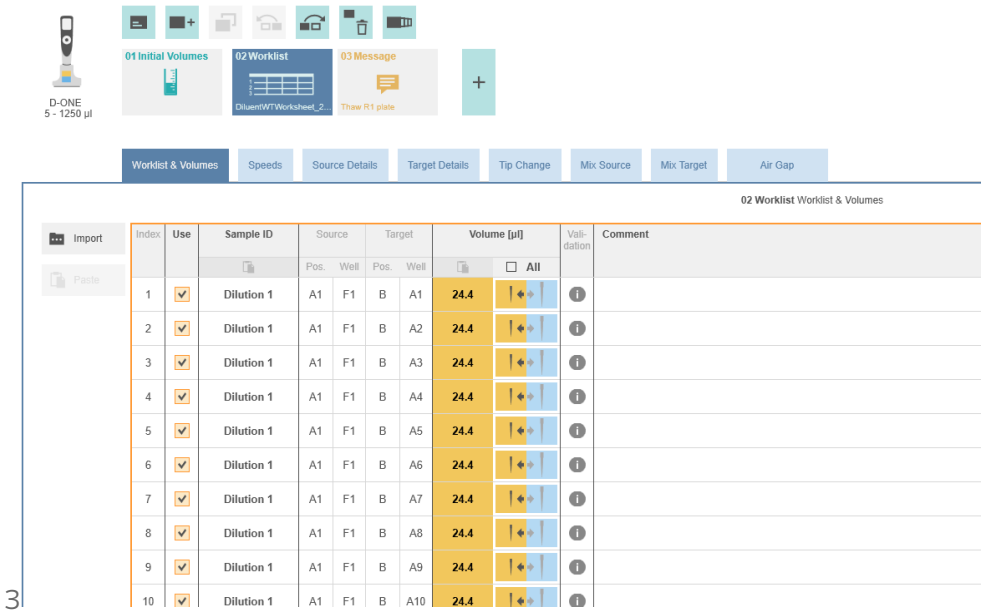


Figure 5: Plate Configuration tab visualizes the sample locations and orientations.

11. Open the VIALAB program **WT S1 St1 DONE V3** and navigate to the "Method" section. In the "02 Worklist", under the "Worklist and Volumes" tab, upload the "CombinedWTWorksheet.csv" worklist file generated in Step 9 using the "Import" button (Figure 6).



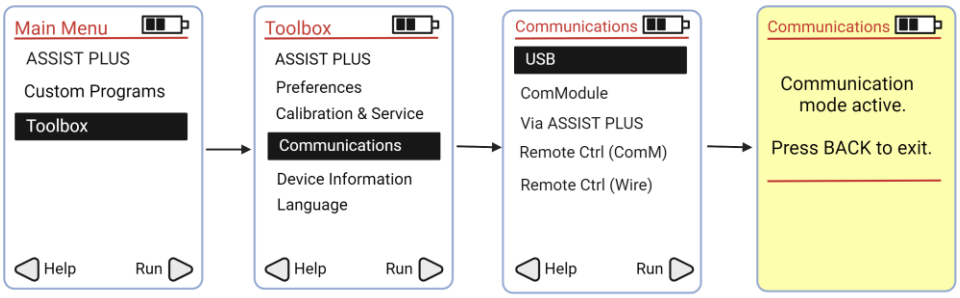
3

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

12. 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:

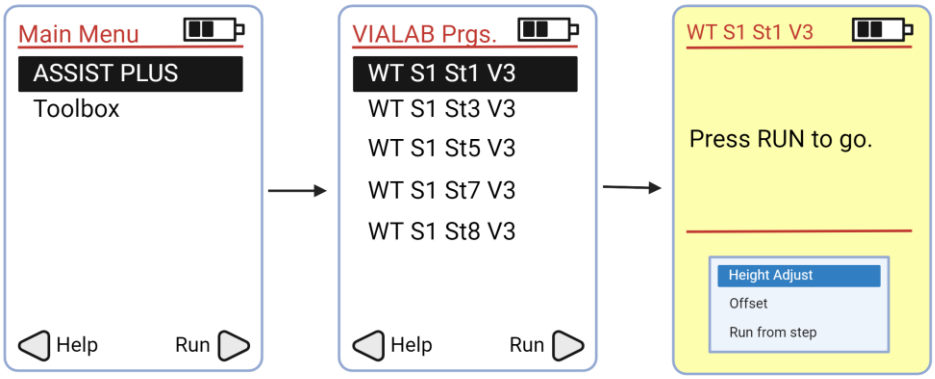


    c. In the VIALAB on your computer, select "Transfer".

    d. Confirm that the pipette has been recognized by the software. Click "Send to pipette". This will upload the **WT S1 St1 V3** 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 **WT S1 St1 V3** will be found on your pipette as shown in the diagram below.



14. Place the Barcoding Reagents in an ice bucket.

15. 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 7).

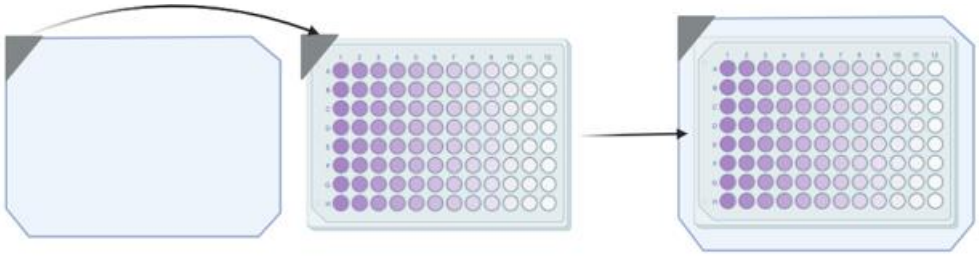
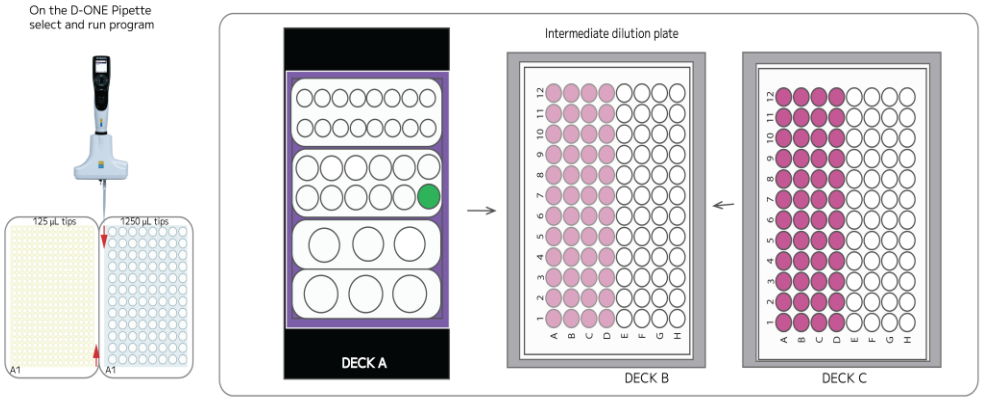


Figure 7: Corners are aligned between the Thermochromic PCR Cold Block and the Thermochromic PCR Cold Block Riser.

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

Note: Refer to the 'Integra Loading Table' tab of the Parse Biosciences Evercode WT INTEGRA Sample Loading Table. Place the dilution tubes according to the locations highlighted in pink under 'Sample Dilution Tube Locations' on the deck configuration (Figure 3)

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 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		● Samples

17. 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.

18. Remove the reagent caps, then select and run the program **WT S1 St1 V3.**

19. **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

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

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

1.2. Load and Pool Round 1

The program loads the normalized cells/nuclei 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 row A.

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	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	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	N/A	

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 the tip box lid prior to starting the program.

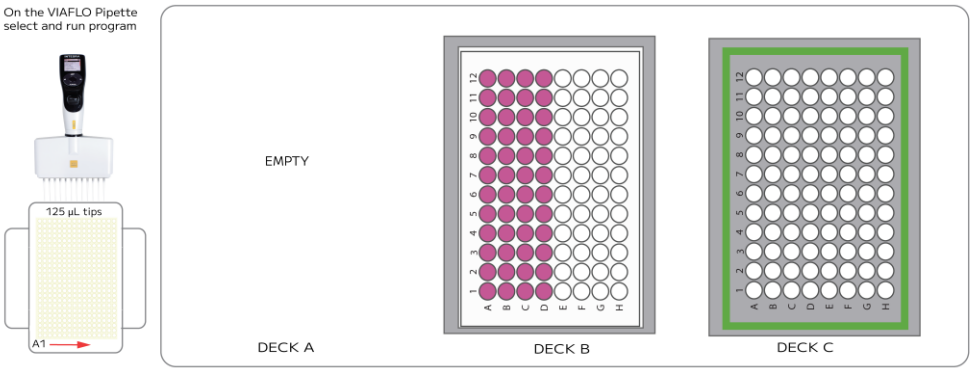
3. Remove all items from Deck A.

4. Remove the Round 1 Plate from the centrifuge, place on a stable surface and remove the plate seal.

5. Replace the Thermochromic PCR Cold Block on Deck C with the new Thermochromic PCR Cold Block thawed in Section 1.1.20. Place this old Thermochromic PCR Cold Block back in the -20°C freezer for later use.

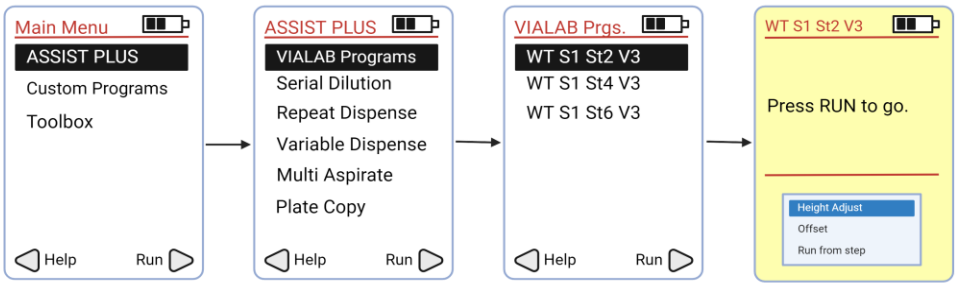
6. **When prompted**, place the ▭ Round 1 Plate in the Thermochromic PCR Cold Block located on Deck C. Ensure A1 is oriented towards the bottom left corner. Deck should correspond to the diagram below.

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		● Samples	▭ Round 1 Plate

9. On the VIAFLO Pipette 12-Ch, select and run the program **WT 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.

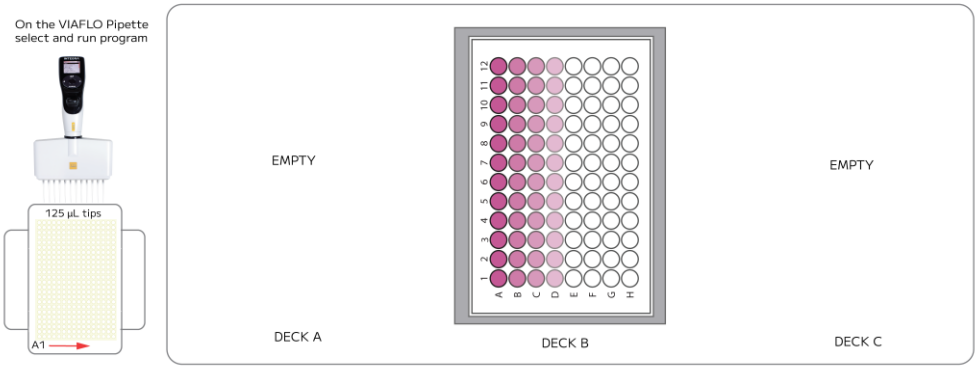
BARCODING ROUND 1			
Run Time	Lid Temperature	Sample Volume	
40 min	70°C	40 µL	
Step	Time	Temperature	Cycles
1	10 min	50°C	1
2	12 s	8°C	3
3	45 s	15°C	
4	45 s	20°C	
5	30 s	30°C	
6	2 min	42°C	
7	3 min	50°C	
8	5 min	50°C	1
9	Hold	4°C	Hold

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

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	N/A	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	N/A	
Parse Cold Block	Parse-Provided	N/A	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	INTEGRA Components	N/A	
25 mL Basin Reservoir Liners	INTEGRA-Provided	2	
▭ Round 2 Plate	-20°C Reagents	1	Place directly on ice.
● 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	
● Spin Additive	4°C Reagents	1	Briefly centrifuge. Keep at room temperature.

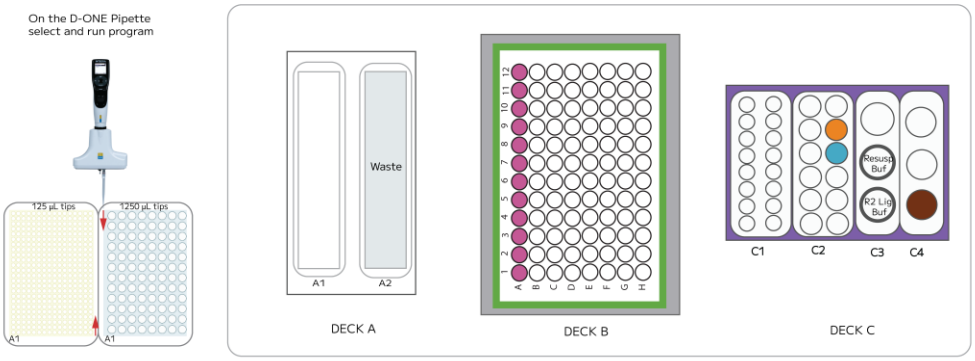
2. Place the Parse Cold Block on Deck C.

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



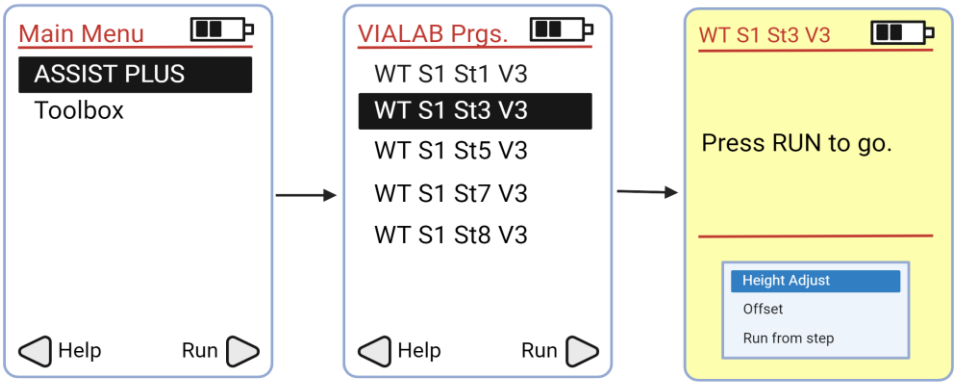
	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	Parse Cold Block
CONSUMABLES	25 mL basin reservoir liners		● 10 mL transport tube
REAGENTS	● Samples	▭ Round 1 Plate	● Spin Additive ● Round 2 Ligation Enzyme ○ Resuspension Buffer ○ Round 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 the tip box lid prior to starting the program.

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



7. **When prompted**, cap and invert 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 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 Decks B and C. Discard the right basin lines and Deck A. Place the Parse Cold Block on ice.

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	N/A	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	N/A	
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	1	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 starting the program.

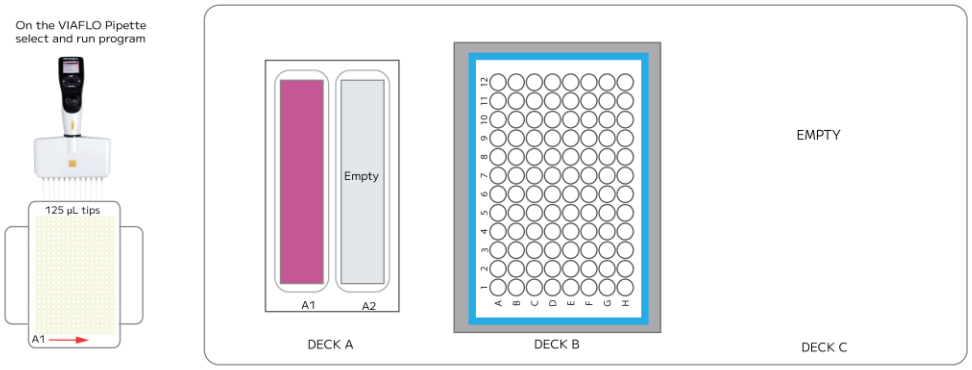
3. Remove the ▭ Round 2 Plate from the thermocycler and centrifuge for **1 minute** at 100 x g at 4°C.

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 1.3.9 on Deck B.

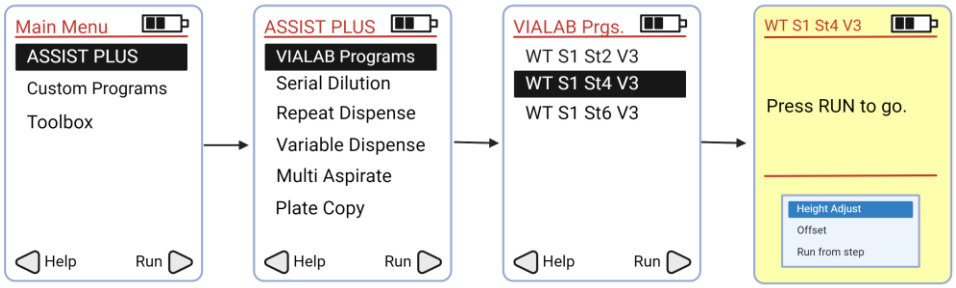
6. Place the ▭ Round 2 Plate on Deck B in the Thermochromic PCR Cold Block with Thermochromic PCR Cold Block Riser with A1 oriented towards the bottom left corner and remove the seal. The deck should correspond to the configuration below.

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	● Samples	▭ Round 2 Plate	

7. Select and run the program **WT 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

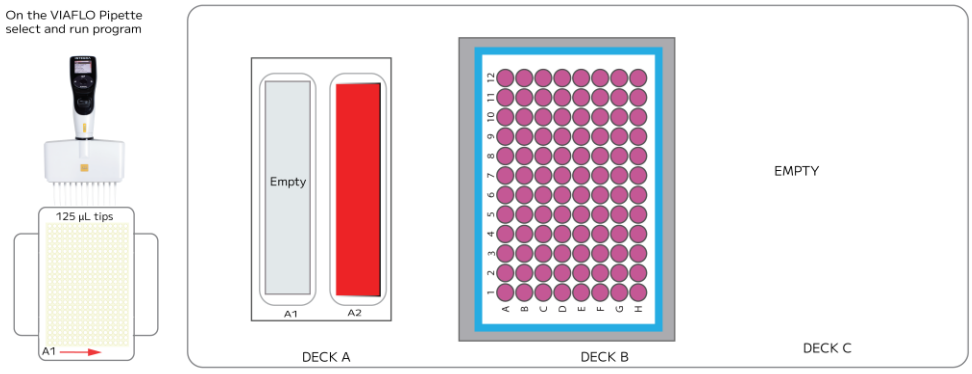
9. Remove the Round 2 Plate from the thermocycler and place in a PCR plate rack.

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 liner on the right with a new 25 mL basin reservoir liner.

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. **When prompted**, replace the right basin liner (A2) on Deck A.

18. 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.

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	
Parse Cold Block	Parse-Provided	N/A	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	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
1250 µL Tip Rack	INTEGRA-Provided	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. 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.

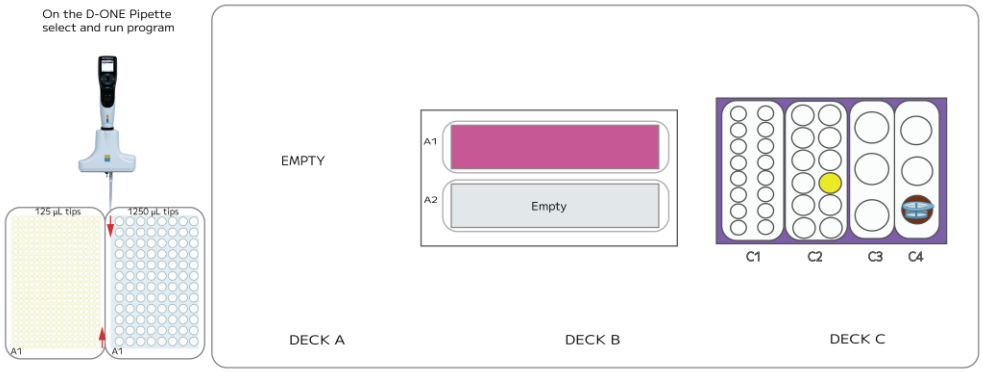
4. Briefly centrifuge and insert the ● Round 3 Ligation Enzyme tube in the appropriate location in the Parse Cold Block.

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.

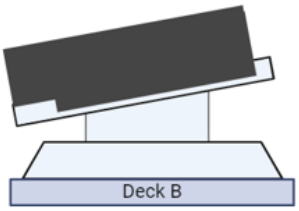
Deck Configuration

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.



Slanted Plate Holder (10°) front view



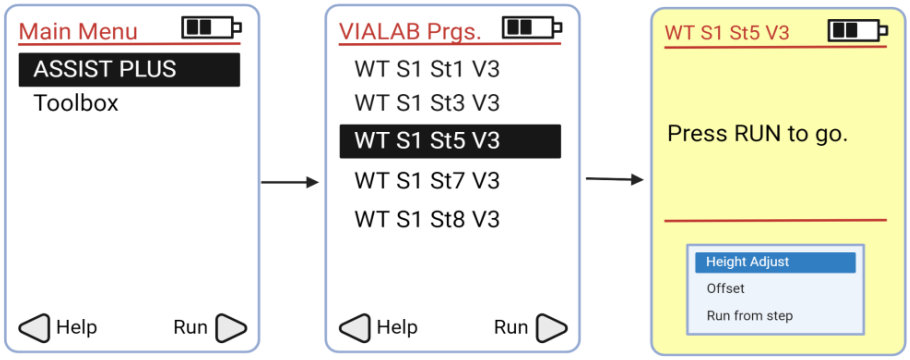
	DECK A	DECK B	DECK C
HARDWARE		Slanted Plate Holder **(10°)** Dual Reservoir Adapter	Parse Cold Block
CONSUMABLES		25 mL basin reservoir liners	10 mL transport tube with cell strainer
REAGENTS		Samples	Round 3 Ligation Enzyme

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 **WT S1 St5 V3** following the diagram below.



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

9. **When prompted**, 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**, move the dual reservoir holder from the slanted plate holder back to Deck A.

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

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

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

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 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	
INTEGRA Dual 25 mL Basin Reservoir Adapter	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 Stop Buffer	-20°C Reagents (Parse Reagents)	1	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.

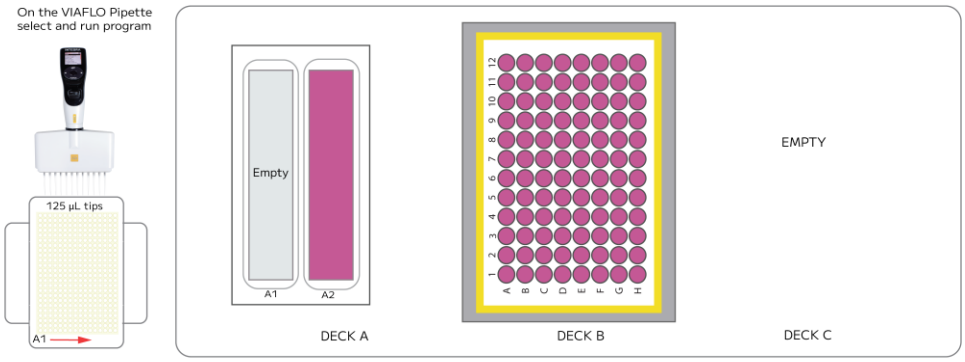
3. Replace the left reservoir.

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

5. 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



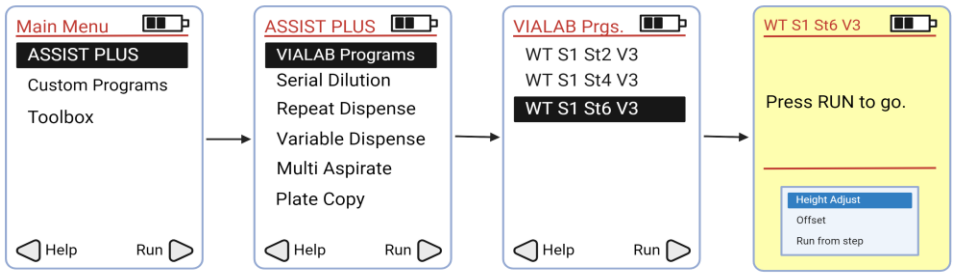
	DECK A	DECK B	DECK C
HARDWARE	Dual Reservoir Adapter	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	
CONSUMABLES	25 mL Reservoir Liner		
REAGENTS	● Samples	▭ Round 3 Plate	

6. 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.

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



8. **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

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

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.

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	Parse-Provided	N/A	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	
● 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.

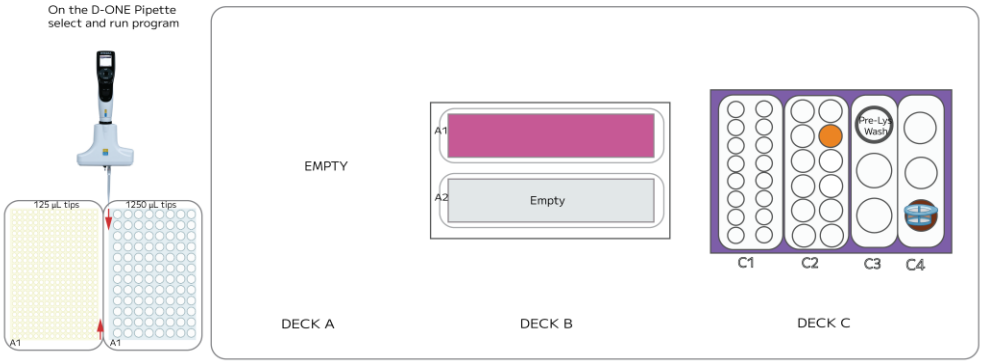
2. Add slanted plate holder on Deck B and incorporate a **10 degree** tilt (lowest side adjacent to Deck A).

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. Deck layout should correspond to the configuration below.

Deck Configuration



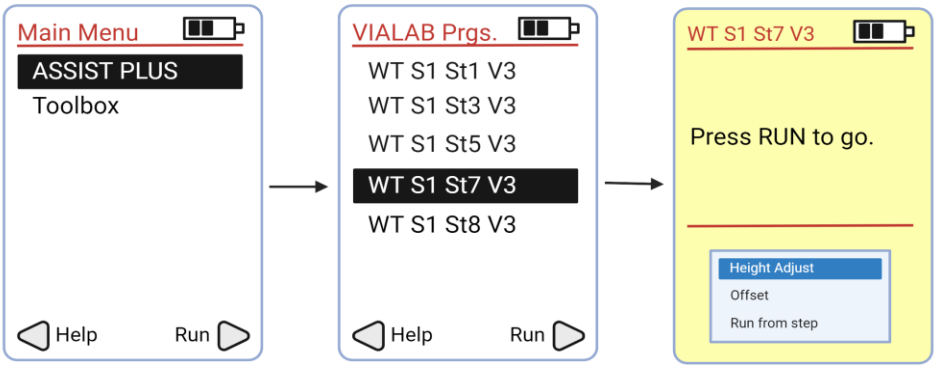
	DECK A	DECK B	DECK C
HARDWARE		Slanted Plate Holder (**10°**) Dual Reservoir Adapter	Parse Cold Block
CONSUMABLE		25 mL reservoir liner	● 10 mL transport tube and cell strainer
REAGENTS		● Samples	● Spin Additive ○ Pre Lyse Wash Buffer

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 **WT S1 St7 V3** following the diagram below.



9. **When prompted**, move the dual reservoir holder back to Deck A.

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

11. **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.

12. **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.

13. Press "Run" on Pipette.

14. **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.

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, mix and count the number of cells/nuclei in the sample from the 10 mL transport tube on Deck C4 with a hemocytometer or alternative cell counting device. Record the cell/nuclei count.

18. Clear the deck.

1.8. Lysis and Sublibrary Generation

The program requires a dilution of the sample to 500 cells/nuclei per µL with a volume of 250 µL. The program will create eight lysates with 12,500 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	1	Keep on ice when not in use.
8 PCR strip tubes	Consumables	1	
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	1	Thaw at room temperature then store on ice. Mix by pipetting 3x.
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.

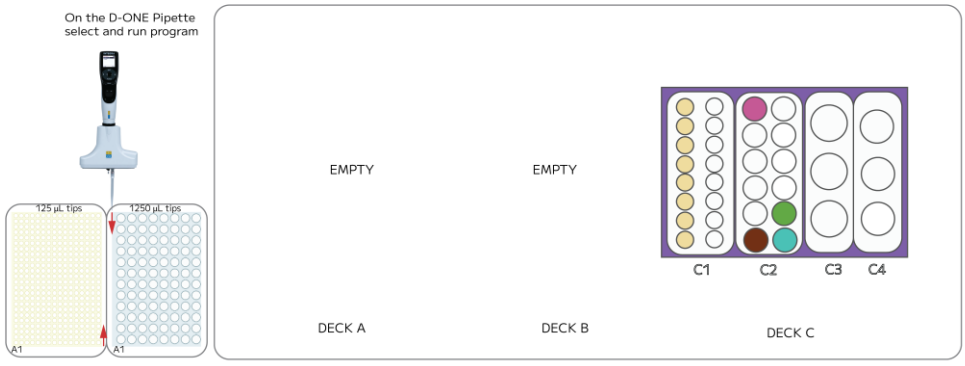
2. Dilute the cells to a concentration of 500 cells/nuclei per µL for a total volume of **250 µL** using the Pre-Lysis Dilution Buffer in a 1.5 mL transport tube. Store this dilution on ice until ready to use.

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/nuclei per µL will create lysates with 7,500 cells.)

3. Place a new PCR strip tube on Deck C1.

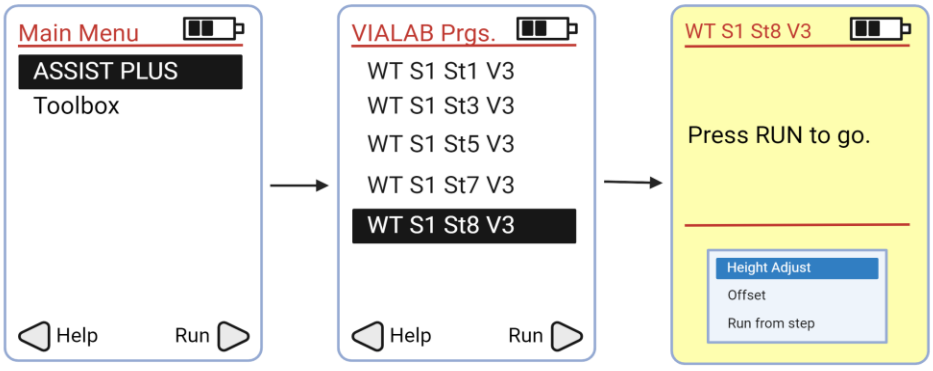
4. Place the Lysis Buffer, Lysis Enzyme, empty 1.5 mL transport tube, and diluted sample from Section 1.8.2 on Deck C2. Deck should correspond to the configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE			Parse Cold Block
CONSUMABLES			8-count PCR Tube 1.5 mL Transport Tube
REAGENTS			Samples Lysis Buffer Lysis Enzyme

5. Remove the reagent caps, ensure that all strip tubes are open, select and run the program **WT 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/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

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

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

The deck is set up with the reagents needed to wash the beads and mix the samples with the beads.

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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	N/A	Keep on ice when not in use.
HEATMAG	INTEGRA Component	N/A	
8 Row Reservoir	INTEGRA-Provided	N/A	Individually wrapped consumable
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
PCR strip tubes	Consumables	3	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	
● Streptavidin Beads	4°C Reagents	1	Keep at room temperature.
● Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
● Capture Enhancer	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
○ Bead Wash Buffer	-20°C Reagents	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Wash Buffer 1	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Wash Buffer 2	-20°C Reagents	1	
○ Wash Buffer 3	-20°C Reagents	1	

3. Place the 24 mm Labware Pedestal and the INTEGRA 8 Row Reservoir Plastic Base on Deck A.
4. Place the 8 Row Reservoir on 24 mm Labware Pedestal on deck A. Ensure that Row 1 is oriented on the left.
5. Place the Parse Cold Block on Deck B.
6. Place the HEATMAG (INTEGRA logo oriented to the front) on Deck C. Ensure HEATMAG power is on for the entire duration of the process.
7. Dispense **750 µL** of SPRI beads in a 2 mL screw cap tube to room temperature.
8. Attach D-ONE Pipetting Module 1-Ch, 5-1250 µL and the corresponding Tip Deck.

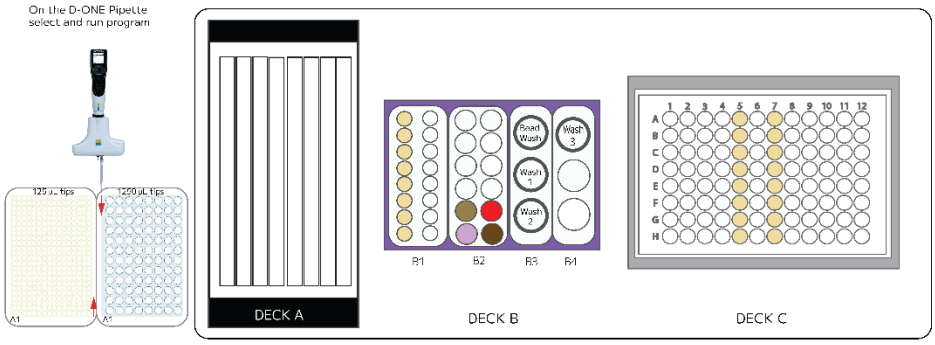
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

9. On Deck B, in the Parse Cold Block, place a clean PCR strip tube in position B1 (left).
10. Briefly centrifuge and add the following reagents to their respective positions on the Parse Cold Block:
    a. B2: **750 µL** ● SPRI beads, ● Streptavidin Beads, ● Binding Buffer, ● Capture Enhancer.
    b. B3: ○ Bead Wash Buffer, ○ Wash Buffer 1, ○ Wash Buffer 2.
    c. B4: ○ Wash Buffer 3.

Note: Thoroughly mix the ● Streptavidin Beads and SPRI Beads before placing them on the deck.

11. Place 2 clean PCR strip tubes with the caps facing to the right in columns 5 and 7 on Deck C. Deck layout should correspond to the configuration below.

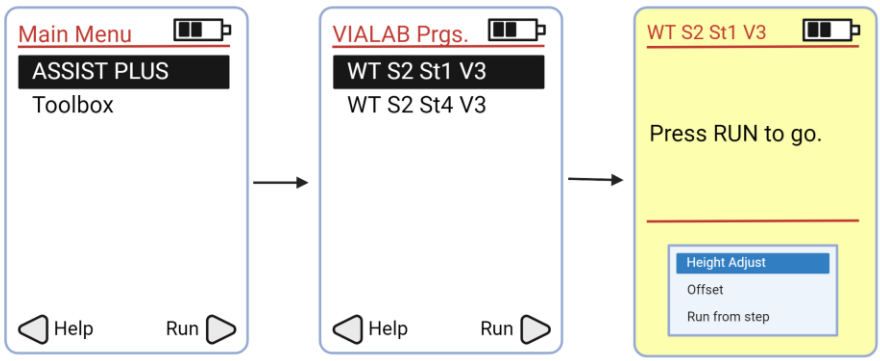
Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir	PCR strip tube	PCR strip tubes
REAGENTS		SPRI Beads Capture Enhancer Binding Buffer Streptavidin Beads Bead Wash Buffer Wash Buffer 1 Wash Buffer 2 Wash Buffer 3	

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

13. On the D-ONE Pipette select and run the program **WT S2 St1 V3** following the diagram below.



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

15. If previously frozen and **when prompted**, incubate the lysates in a water bath or 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: Ensure there is no precipitate before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

16. When the lysates are finished thawing, briefly centrifuge and store at room temperature.

17. **When prompted**, load the thawed lysates from Section 1 in column 1 of the HEATMAG on Deck C. Press "Run" to continue.

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.

18. **When prompted**, cap and store the PCR strip tubes on Deck B1 at room temperature until after cDNA cleanup. Label these tubes as SPRI beads.

19. After the program has completed, verify the following:

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

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

    c. All wash buffers have been transferred to the 8 Row Reservoir.

20. Remove and discard empty tubes on Deck B.

21. Proceed immediately to Section 2.2.

2.2. cDNA Capture

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

To capture the cDNA:

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

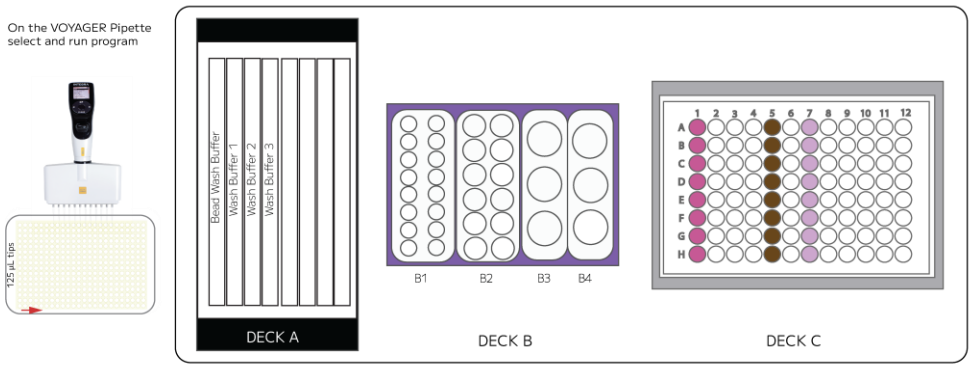
ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
125 µL Tip Rack	INTEGRA-Provided	1	

2. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Replace it with VOYAGER Pipetting Module 8-Ch 5-125 µL and corresponding Tip Deck. Deck layout should correspond to the configuration below.

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.

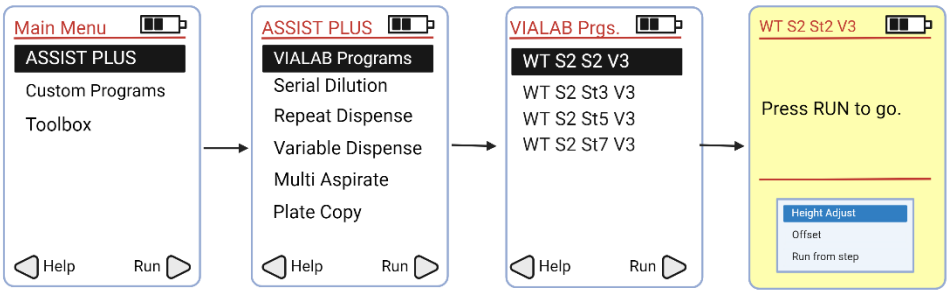
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir		
REAGENTS	○ Bead Wash Buffer ○ Wash Buffer 1 ○ Wash Buffer 2 ○ Wash Buffer 3		● Samples ● Streptavidin Beads ● Binding Buffer

3. Select and run the program **WT S2 St2 V3** following the diagram below.



4. After the program has completed, cap the sample strip tubes on Deck C column 1. Ensure the caps are secured tightly.

5. Cover the 8 Row Reservoir to avoid contamination.

5. Place the sample strip tubes from Deck C column 1 onto a vortex mixer and vortex on 100% power for **1 minute.**

6. **When prompted**, vortex at 20% power (~800-1000 RPM) for **30 minutes** at room temperature. Press "Run" to continue the program.

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.

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

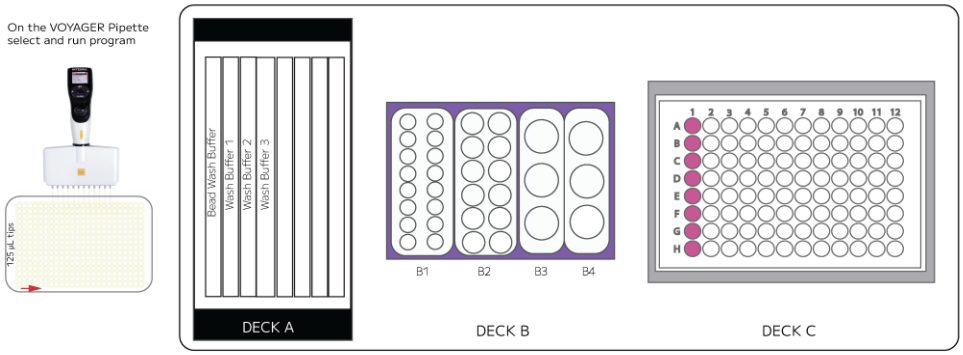
2.3. Binder Beads Wash

Barcoded cDNA samples are washed to remove cellular debris.

1. Briefly centrifuge the barcoded cDNA sample tubes.
2. Place the sample tubes back on the HEATMAG on Deck C, column 1.

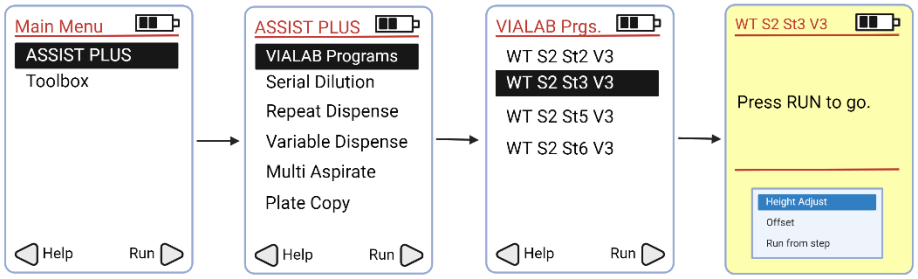
Note: Ensure the PCR strip tube caps are facing the same direction. Push the PCR strip tube caps wide open to avoid interference with the pipette's tips.

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



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir		
REAGENTS	○ Bead Wash Buffer ○ Wash Buffer 1 ○ Wash Buffer 2 ○ Wash Buffer 3		● Samples

4. Select and run the program **WT S2 St3 V3** following the diagram below.



5. When the program is completed, 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	1	
24 mm Labware Pedestal	INTEGRA Component	N/A	
HEATMAG	INTEGRA Component	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
8 Row Reservoir	INTEGRA-Provided	1	Individually wrapped consumable
PCR strip tubes	Consumables	2	
2 mL tubes	Consumables	2	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	
○ Wash Buffer 3	-20°C Reagents	1	Thaw and store at room temperature. Mix by inverting 3x.
● 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.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● 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. In the Parse Cold Block on Deck B, place the following using the deck configuration below:

    a. Two clean PCR strip tubes on Deck B1.

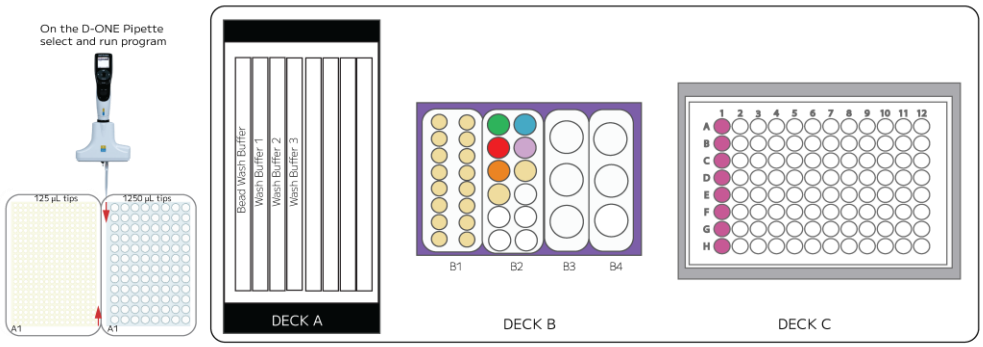
    b. ● Template Switch Buffer, ● Template Switch Primer, ● Template Switch Enzyme, ● cDNA Amp Mix, ● cDNA Amp Primers, and 2 clean 2 mL tubes on Deck B2.

3. Remove the VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Deck layout should correspond to the configuration below.

Note: Before removing the VOYAGER 8-Ch 5-125 µL Pipette, ensure it is disconnected by pressing the back button until the main menu is displayed.

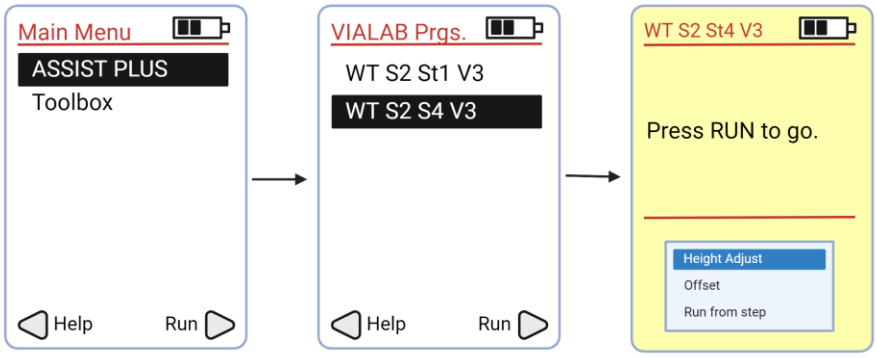
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir	● Clean PCR strip tubes ● Clean 2 mL tubes	
REAGENTS	○ Bead Wash Buffer ○ Wash Buffer 1 ○ Wash Buffer 2 ○ Wash Buffer 3	● Template Switch Buffer ● Template Switch Primer ● Template Switch Enzyme ● cDNA Amp Mix ● cDNA Amp Primers	● Samples

4. Remove the reagent caps, select and run the program **WT S2 St4 V3** following the diagram below.



5. **When prompted**, cap, label, and store the cDNA Amplification Master Mix dispensed in the right PCR strip tube on Deck B1 on ice. Verify the volume is even.

6. Press "Run" to continue.

7. After the program has completed, verify that the volume of the Template Switch Master Mix in the left PCR strip tube on Deck B1 is even.

8. Remove and discard used 1.5 mL and 2 mL reagent tubes on Deck B2.

9. Proceed immediately to Section 2.5.

2.5. Template Switch and cDNA Amplification

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

To perform template switch:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER 8-Ch 5-125 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
HEATMAG	INTEGRA Component	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
8 Row Reservoir	INTEGRA-Provided	1	
125 µL Tip Rack	INTEGRA-Provided	1	

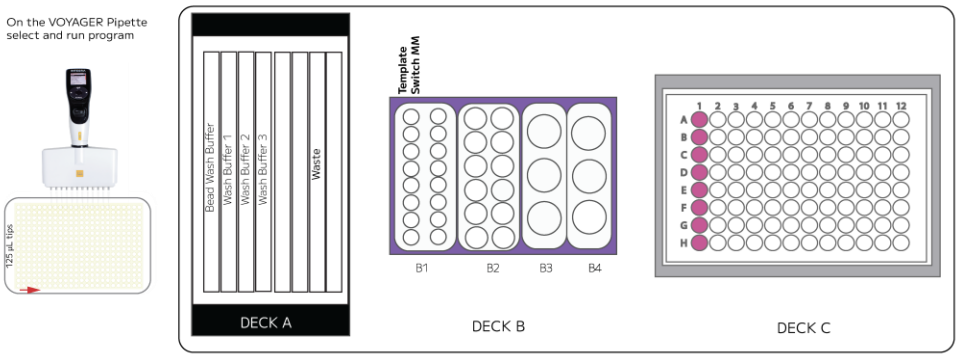
2. Remove the D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Replace it with VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck.

Note: Before removing the D-ONE Pipetting Module 1-Ch, 5-1250 µL Pipette, 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 the tip box lid prior to starting the program.

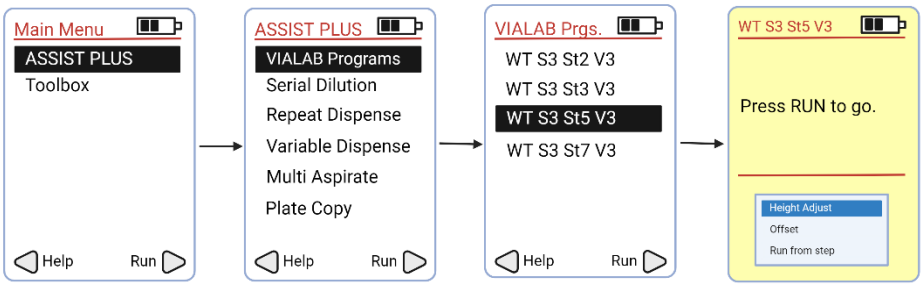
3. Ensure the samples on Deck C, column 1 with the PCR strip tube caps open. Deck layout should correspond to the Deck Configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir		
REAGENTS	○ Bead Wash Buffer ○ Wash Buffer 1 ○ Wash Buffer 2 ○ Wash Buffer 3	Template Switch Master Mix	● Samples

5. On the VOYAGER Pipette select and run the program **WT S2 St5 V3** following the diagram below.



Note: The addition of the Template Switch Master Mix is followed by **30 minutes** incubation. We recommend covering the 8 Row Reservoir during this time to reduce contamination.

6. **When prompted**, incubate the cDNA samples on Deck C, column 1 at room temperature for **30 minutes**. Ensure the PCR strip tube caps are closed. Add a cover to the reagent reservoir on Deck A during the 30 minute incubation.

7. After the 30 minute incubation, press "Run" to continue the program.

8. **When prompted**, reload the samples on the HEATMAG on Deck C, column 1 with the strip cap tubes open. 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.

9. **When prompted**, remove the samples from the Deck C, column 1 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

10. Once the thermocycling program has completed, remove the cover from the 8 Row Reservoir on Deck A and remove the samples from the thermocycler. Press "Run" to continue the program.

11. **When prompted**, place the samples on Deck C column 1. Press "Run" to continue the program.

12. **When prompted**, place the cDNA Amplification Master Mix from section 2.3.5 back into the right B1 lane on Deck B. Press "Run" to continue the program.

13. **When prompted**, remove the samples from Deck C column 1 and place them into a thermocycler. Remove and discard used strip tubes on Deck B. Press "Run" to complete the program.

14. 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

NUMBER OF PCR CYCLES			
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

13. Run the following program on the thermocycler.

Note: We recommend covering the 8 Row Reservoir during this time to reduce contamination.

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.

STOP 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.

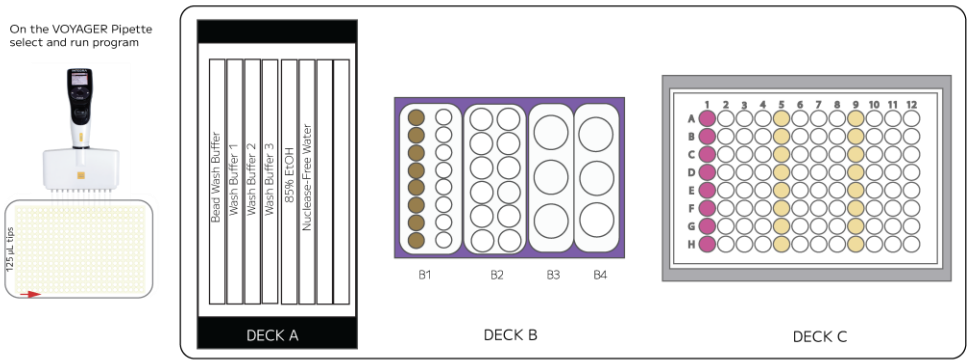
To purify the cDNA:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER 8-Ch 5-125 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
HEATMAG	INTEGRA Component	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
8 Row Reservoir	INTEGRA-Provided	1	
125 µL Tip Rack	INTEGRA-Provided	1	
PCR strip tubes	Consumables	2	

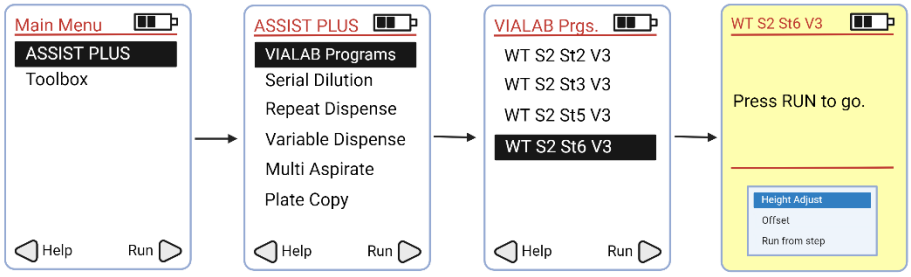
Note: Ensure the SPRI beads have been equilibrated to room temperature for at least 30 minutes.

2. With a P1000 pipette, add:
    a. **5 mL** Ethanol in lane 5 of the 8 Row Reservoir on Deck A
    b. **3 mL** nuclease free water in lane 6 of the 8 Row Reservoir on Deck A.
3. Once the thermocycler program is complete, place the sample strip tube in column 1 on the HEATMAG on Deck C.
4. Place 2 clean PCR strip tubes in columns 5, 9 on Deck C. The deck layout should correspond to the Deck Configuration below.



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Parse Cold Block	HEATMAG
CONSUMABLES	8 Row Reservoir		Clean strip tubes
REAGENTS	Bead Wash Buffer Wash Buffer 1 Wash Buffer 2 Wash Buffer 3 Ethanol Nuclease free water	SPRI Beads	Samples

5. Select and run program **WT S2 St6 V3** following the diagram below. The final cDNA libraries are in column 9.



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 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.

To quantify the cDNA:

1. Measure the concentration of each tube of purified cDNA from Section 2.4 with the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. Record the concentration(s), which will be used in Section 3.

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

Safe stopping point: 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.

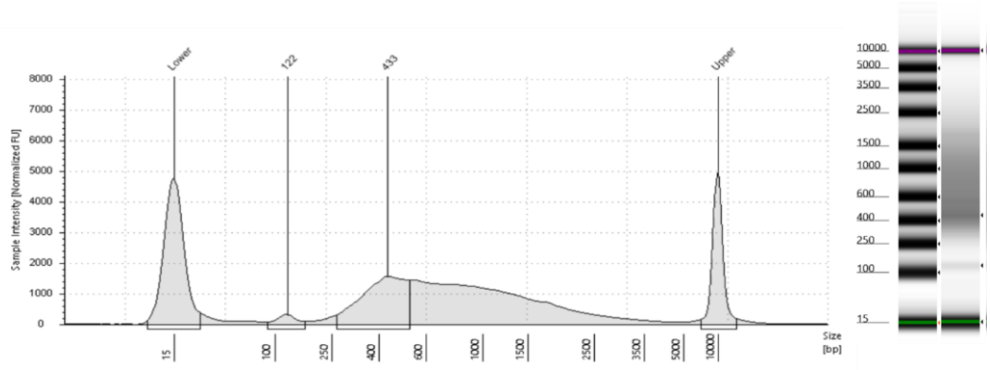


Figure 8: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a Tapestation.

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 is 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

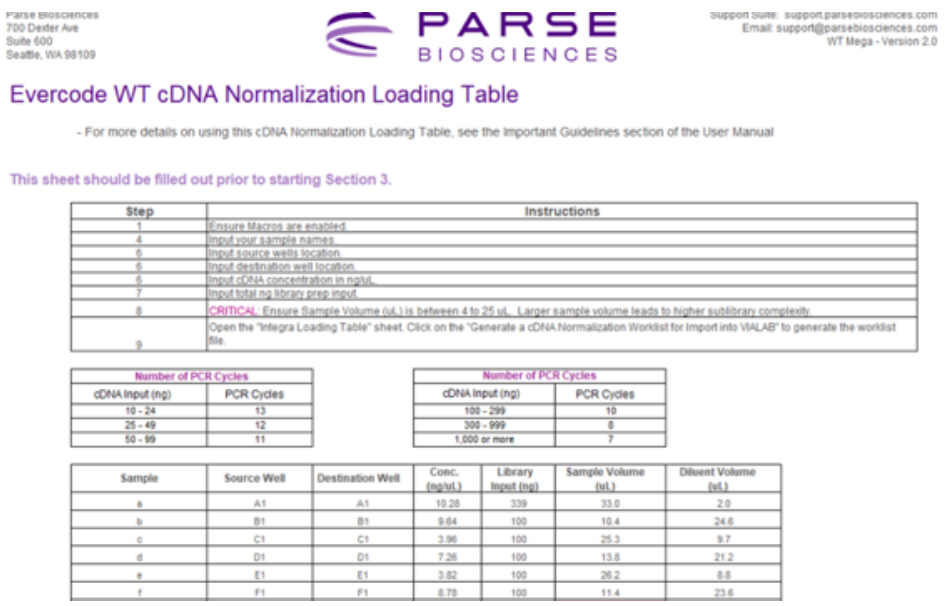
3.0. cDNA Normalization

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 the sample dilution buffer on Deck B2 to normalize amplified cDNA from tube strip(s) on Deck B1.

To normalize amplified cDNA:

1. If frozen, thaw the amplified cDNA and store it on ice.
2. Download the Parse Biosciences Evercode Mega/WT Integra Normalization v1.0.xlsm. The most current version can be found on the Parse Biosciences Customer Support Suite.
3. Obtain recorded cDNA concentrations from Section 2.7.
4. 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

PARSE BIOSCIENCES

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.
5	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

Figure 9: Evercode WT Mega cDNA normalization loading table.

3. Navigate to the "INTEGRA Loading Table" tab and click on "Generate a cDNA Normalization Workflist 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

Generate a cDNA Normalization Worklist for Import

Figure 10: Generated cDNA normalization worklist.

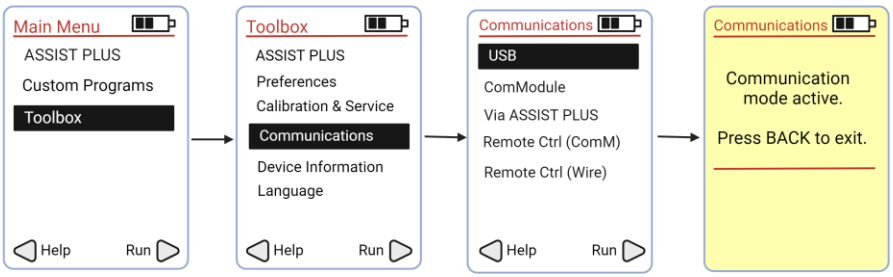
4. Open the Vialab program **WT S3 St0 V3 DONE** and navigate to the "Method" section. In the "02 Worklist", under the "Worklist and Volumes" tab, import the "Section3NormWTWorksheet_xxxxxxx_xxxxxxx.csv" worklist file generated in the previous step. The "Import" button is located in the upper left of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

Figure 11: VIALAB worklist generation for diluent volumes using 02 Worklist. The blue arrow indicates the Import button.

5. 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". This will upload the **WT S3 St0 V3** 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 **WT S3 St0 V3** will be found on your pipette.

6. If present, remove the VOYAGER 8-Ch 5-125 µL and corresponding Tip Deck. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL Pipette and corresponding Tip Deck.

Note: Before removing the VOYAGER 8-Ch 5-125 µL Pipette, ensure it is disconnected by pressing the back button until the main menu is displayed.

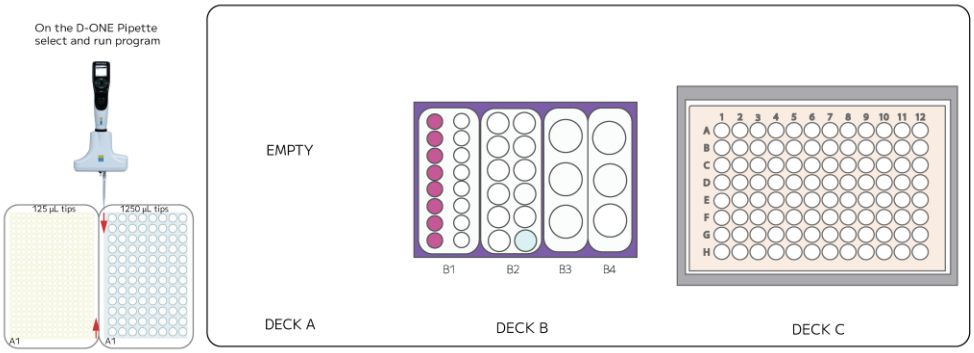
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

7. Briefly vortex and centrifuge the amplified cDNA and place on the Parse Cold block on Deck B1.

8. Fill a 2 mL tube with 1.8 mL nuclease-free water and place it on the Parse Cold Block on Deck B2.

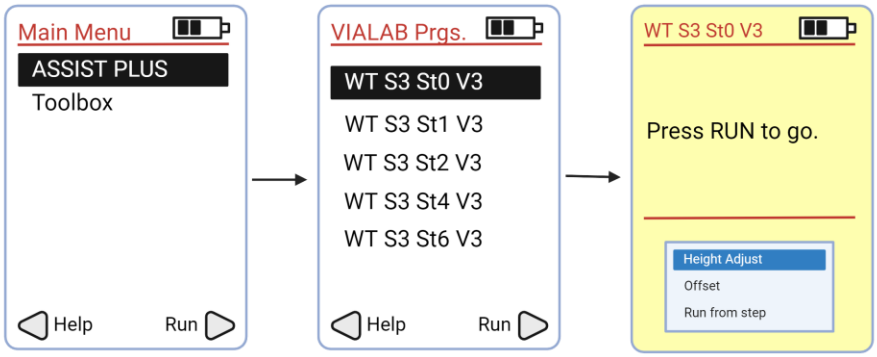
9. Place a clean semi-skirted plate on the HEATMAG on Deck C. The deck layout should correspond to the Deck Configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE		Parse Cold Block	HEATMAG
CONSUMABLES			Semi-skirted 96 well PCR plate
REAGENTS		● Samples ● Nuclease free water	

10. Select and run program **WT S3 St0 V3** following the diagram below.



11. Once the program is complete, the cDNA samples from Deck B1 can be stored at -20°C.

12. 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 when needed. SPRI beads should be kept at room temperature.

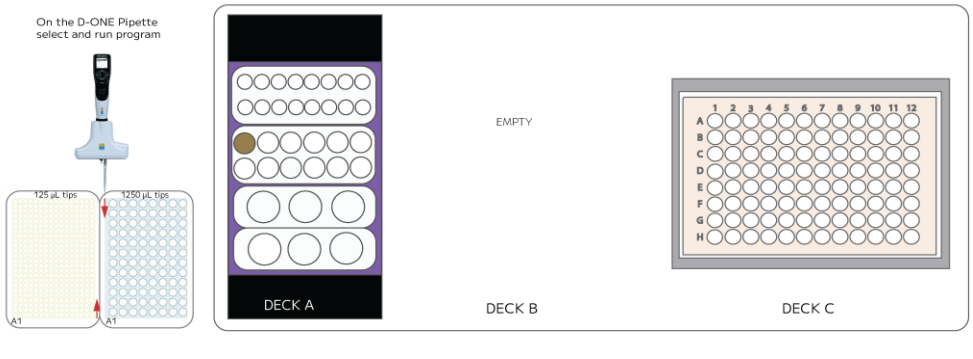
1. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
D-ONE Pipetting Module 1-Ch, 5-1250 µL	INTEGRA Component	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
1.5 mL tube	Consumables	1	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	1	

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● SPRI Beads	Consumables		

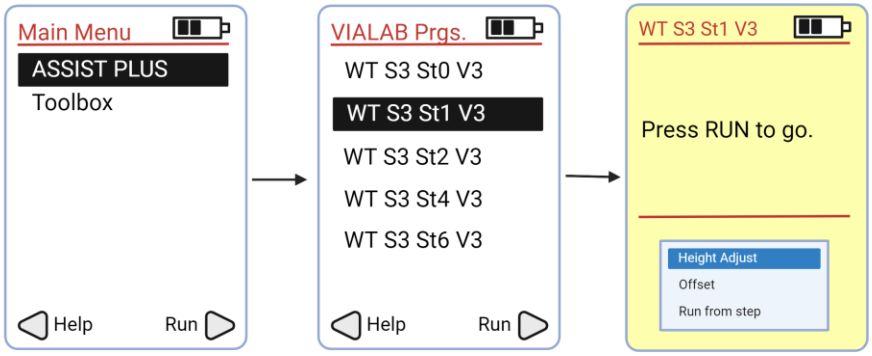
2. If not connected, connect the D-ONE pipette to the ASSIST PLUS and load the D-ONE tip pedestal.
3. Place the Parse Cold Block on the 24 mm Labware pedestal on Deck A.
4. Place a 1.5 mL tube with **1,600 µL** ● SPRI beads in the Parse Cold Block.
5. Place a new semi-skirted 96 well PCR plate on Deck C. The deck layout should correspond to the deck configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 24 mm Labware Pedestal		HEATMAG
CONSUMABLES	1.5 mL screw cap tube		semi-skirted 96 well PCR plate
REAGENTS	● SPRI Beads		

6. Select and run the program **WT S3 St1** following the diagram below.



7. Wait for the method to complete.

8. 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.

9. Remove and store the Parse 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.

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	Parse-Provided	1	
24 mm Labware Pedestal	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	
HEATMAG	INTEGRA Component	1	
Semi-Skirted 96 Well PCR Plate	Consumables	1	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	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 pipette to the ASSIST PLUS and load the D-ONE tip pedestal.

3. Remove both Thermochromic PCR Cold Block with Riser from -20°C and thaw at room temperature for **10 minutes**. Ensure the corner sizes align between the colored Thermochromic PCR Cold Block and the gray Thermochromic PCR Cold Block Riser.

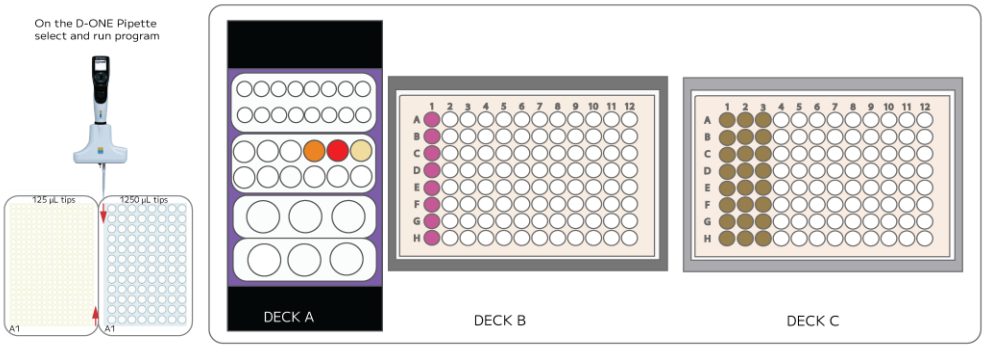
4. Place the cooled Parse Cold Block back on Deck A.

5. Place a new 1.5 mL tube, ● Fragm/End Prep Enzymes, and ● Fragm/End Prep Buffer into the Parse Cold Block.

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

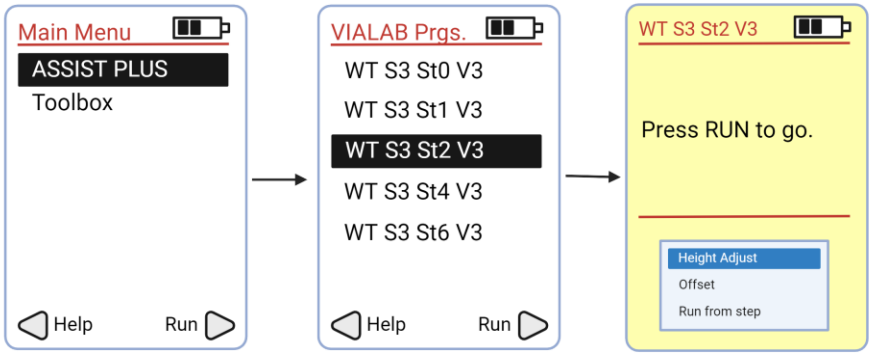
7. Remove cDNA samples from ice and place them in the Thermochromic PCR Cold Block on Deck B, pressing firmly to ensure the plate is fully seated. Deck layout should correspond to the deck configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 24 mm Labware Pedestal	Thermochromic PCR Cold Block (cold) Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	● Clean 1.5 mL tube	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS	● Fragm/End Prep Enzymes ● Fragm/End Prep Buffer	● Samples	● SPRI Beads

8. Remove the reagent caps, then select and run the program **WT S3 St2 V3** following the diagram below.



9. **When prompted**, start the Fragmentation and End Prep program on the thermocycler to pre-chill, ensuring that Step 1 of the cycling program is set to 4°C, so it will be at the correct temperature when needed during Section 3.3.6.

10. Press "Run" to continue.

11. When the run is completed, remove Parse Cold Block from the Deck A and place it on ice.

12. 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. Prepare 5 mL of 85% ethanol with nuclease-free water.

2. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Module 8-Ch, 5-1250 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	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	N/A	
HEATMAG	INTEGRA Component	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
125 µL Tip Rack	INTEGRA-Provided	1	
8 Row Reservoir	INTEGRA-Provided	1	

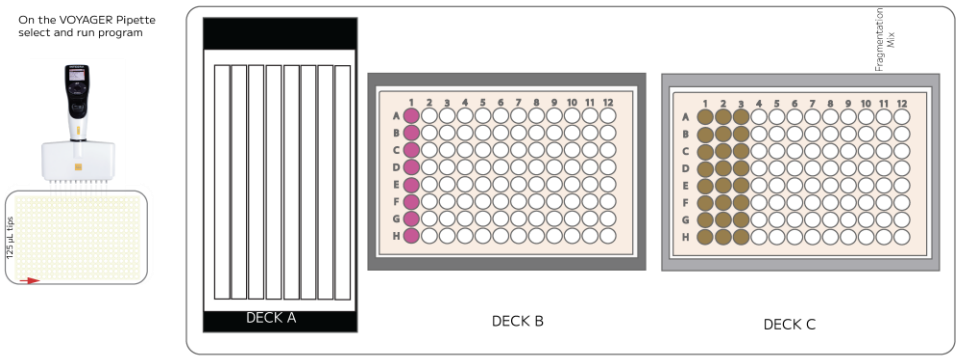
3. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Replace it with VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Deck layout should correspond to the configuration below.

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 the tip box lid prior to starting the program.

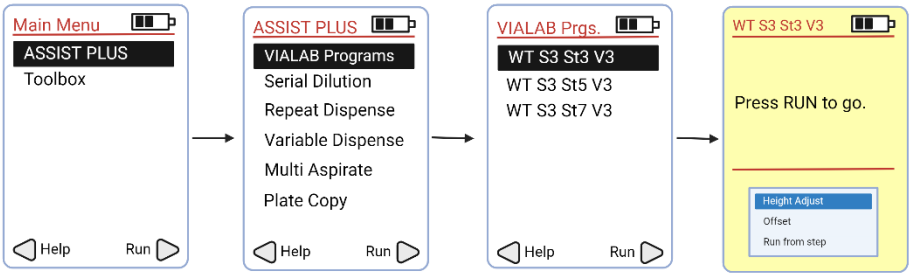
4. Place a new 8 Row Reservoir on Deck A. Deck layout should correspond to the deck configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Reservoir Plastic Base 24 mm Labware Pedestal	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	8 Row Reservoir	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS		● Samples	● SPRI Beads Fragmentation Master Mix

5. Select and run the program **WT S3 St3 V3** following the diagram below.



6. **When prompted**, remove, seal, and load the sublibrary plate on Deck B into the cooled thermocycler from Section 3.2.7. Press "Run" to continue.

7. Ensure the thermocycler is cool prior to use and start the following program.

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

8. While the thermocycler is running, and **when prompted**, replace the frozen Thermochromic PCR Cold Block with Riser with the one fully thawed. Press "Run" to continue.

9. **When prompted**, move the plate on Deck C onto the thawed Thermochromic PCR Cold Block on Deck B. Press "Run" to continue.

10. **When prompted**, with a P1000 pipette, add:

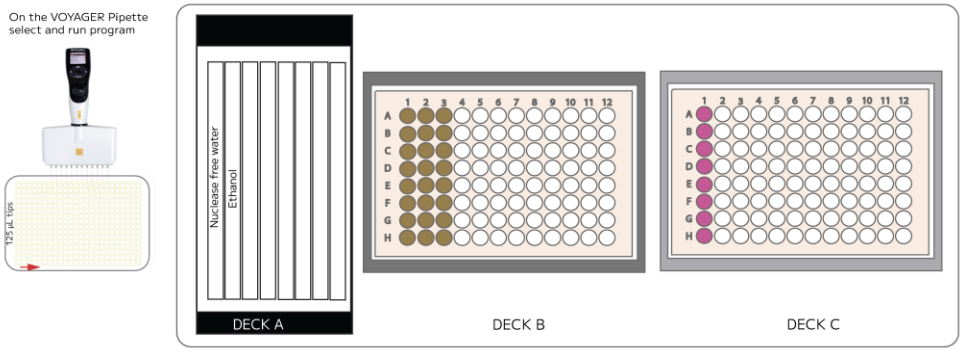
    a. **4 mL** nuclease free water in row 1 of the 8 Row Reservoir on Deck A.

    b. **5 mL** 85% Ethanol in row 2 of the 8 Row Reservoir on Deck A.

11. Press "Run" to continue.

12. When Fragmentation has completed and **when prompted**, load the sublibrary plate onto the HEATMAG on Deck C. Ensure the nuclease free water and the Ethanol are evenly distributed within their rows. Deck layout should correspond to the configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Plastic Adapter 24 mm Labware Pedestal	Thermochromic PCR Cold Block (thawed) Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	8 Row Reservoir	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS	Nuclease free water 85% Ethanol	● SPRI Beads	● Samples

13. Press "Run" to continue the program.

14. When the program is complete, continue to Section 3.4.

STOP 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	1	
24 mm Labware Pedestal	INTEGRA Component	1	
Thermochromic PCR Cold Block	Parse-Provided	1	
Thermochromic PCR Cold Block Riser	Parse-Provided	1	
HEATMAG	INTEGRA Component	1	
125 µL Tip Rack	INTEGRA-Provided	1	
1250 µL Tip Rack	INTEGRA-Provided	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. Remove VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck. Deck layout should correspond to the configuration below.

Note: Before removing the VOYAGER 8-Ch 5-125 µL Pipette, ensure it is disconnected by pressing the back button until the main menu is displayed.

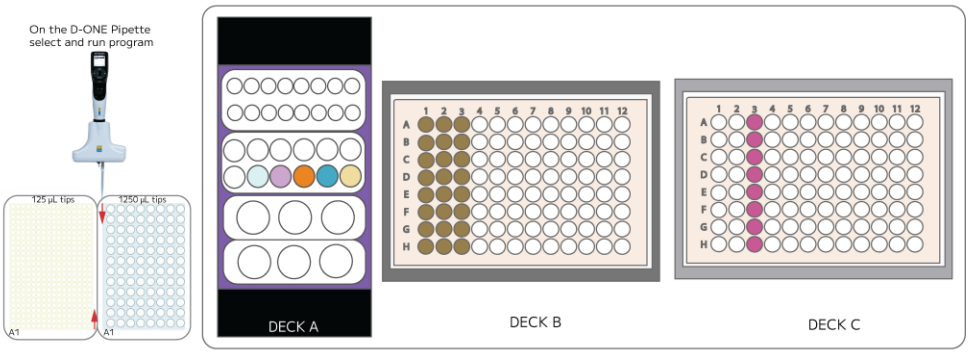
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

3. Cover and remove the reagent reservoir on Deck A. Store at room temperature for later use.

4. Place the Parse Cold Block on Deck A.

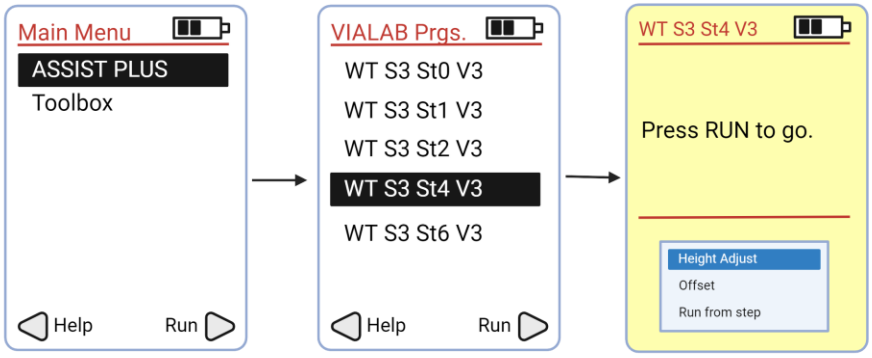
5. Fill a 1.5 mL clean tube with 200 µL of nuclease free water and place in the Parse Cold Block on Deck A. Deck layout should correspond to the deck configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 24 mm Labware Pedestal	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	Clean 1.5 mL tube	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS	Nuclease free water Adapter Ligation Buffer Adapter Ligation Enzyme Ligation Adapter	SPRI Beads	Samples

5. Remove the reagent caps, select and run the program **WT S3 St4 V3** following the diagram below.



6. When the program has completed, 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 thermal cycling program, sublibraries are loaded back onto the ASSIST PLUS and a single-sided SPRI cleanup is carried out.

1. Prepare **5 mL** of 85% ethanol with nuclease free water.

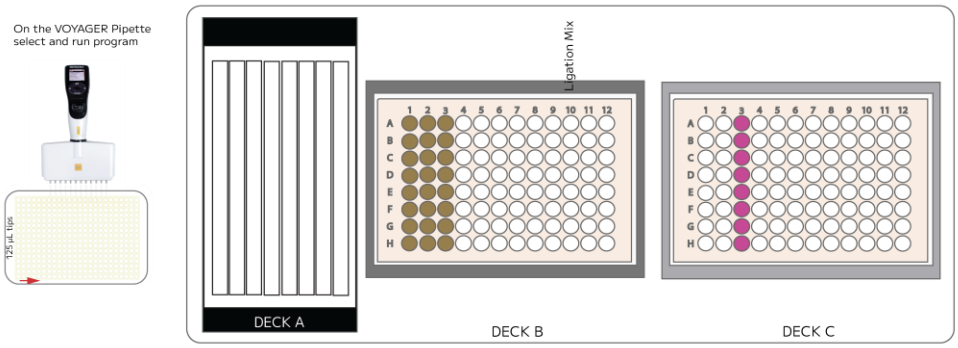
2. Gather the following components and reagents:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch, 5-1250 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	
Thermochromic PCR Cold Block Riser	Parse-Provided	N/A	
HEATMAG	INTEGRA Component	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
125 µL Tip Rack	INTEGRA-Provided	1	
8 Row Reservoir	INTEGRA-Provided	1	

3. Remove Parse Cold Block on Deck A and replace it with the reagent reservoir stored at room temperature from Section 3.4.3. Deck layout should correspond to the configuration below.

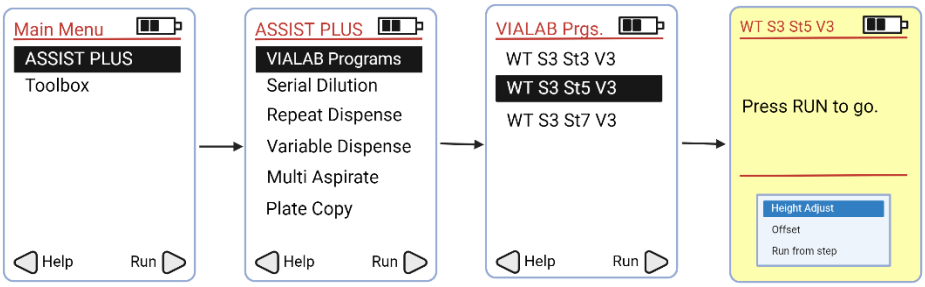
Note: Ensure the reagent reservoir on Deck A is uncovered.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	24 mm Labware Pedestal INTEGRA 8 Row Reservoir Plastic Base	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	8 Row Reservoir	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS		● SPRI Beads	● Samples

4. Navigate to the VIALAB programs on the Voyager Pipetting Module, select and run the program **WT S3 St5 V3** following the diagram below.



5. **When prompted**, seal the sample plate 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

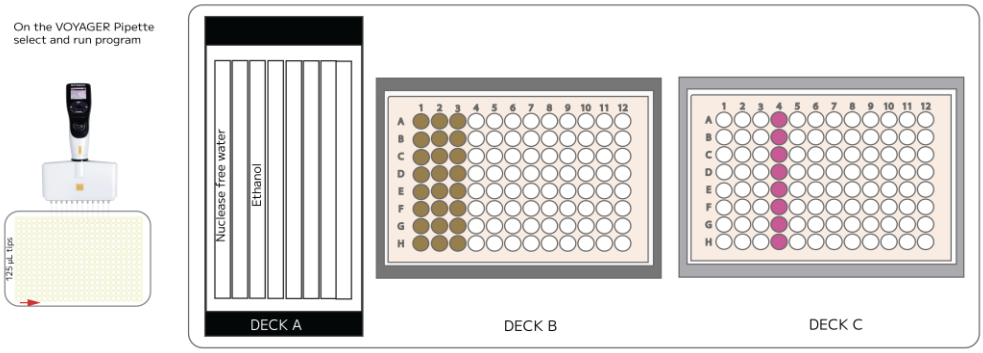
Note: * If the thermocycler's lid can't reach the recommended temperature of 30°C, turn the lid heating off.

6. Press "Run" to continue.

7. **When prompted**, with a P1000 pipette, add **5 mL** 85% Ethanol to row 3 of the 8 Row Reservoir on Deck A. Press "Run" to continue.

8. Upon thermocycling completion and **when prompted**, place the sample plate onto the HEATMAG located on Deck C and remove the seal. Deck layout should correspond to the configuration below. Press "Run" to continue.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	24 mm Labware Pedestal INTEGRA 8 Row Reservoir Plastic Base	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	8 Row Reservoir		Semi-skirted 96 well PCR plate
REAGENTS	Nuclease free water 85% Ethanol	● SPRI Beads	● Samples

8. Upon completion of the program proceed to Section 3.6.

3.6. Barcoding Round 4

Program aliquots out the Library Amp Mix such that it can be easily pipetted into sublibraries using the multichannel. During this program, the user will also 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	N/A	
Tip Deck for D-ONE Pipetting Module	INTEGRA Component	N/A	
Parse Cold Block	Parse-Provided	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	
Thermochromic PCR Cold Block Riser	Parse-Provided	N/A	
HEATMAG	INTEGRA Component	N/A	
125 µL Tip Rack	INTEGRA-Provided	N/A	
1250 µL Tip Rack	INTEGRA-Provided	N/A	
● 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	Place directly on ice. Briefly centrifuge before use.

2. Remove VOYAGER 8-Ch 5-125 µL Pipette and corresponding Tip Deck. Replace it with D-ONE Pipetting Module 1-Ch, 5-1250 µL and corresponding Tip Deck.

Note: Before removing the VOYAGER 8-Ch 5-125 µL, ensure it is disconnected by pressing the back button until the main menu is displayed.

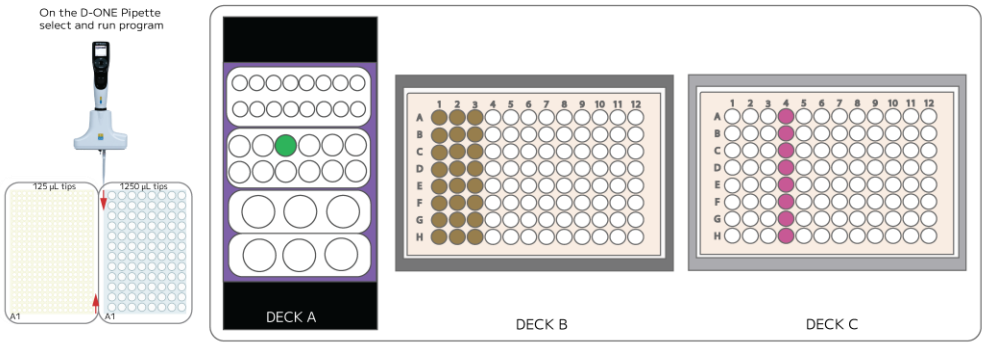
Note: Ensure the Tip Deck and Tip Boxes are securely positioned by pressing them down firmly until they click into place. Remove the tip box lid prior to starting the program.

3. Cover and remove the reagent reservoir on Deck A. Store at room temperature for later use.

4. Place the Parse Cold Block on Deck A.

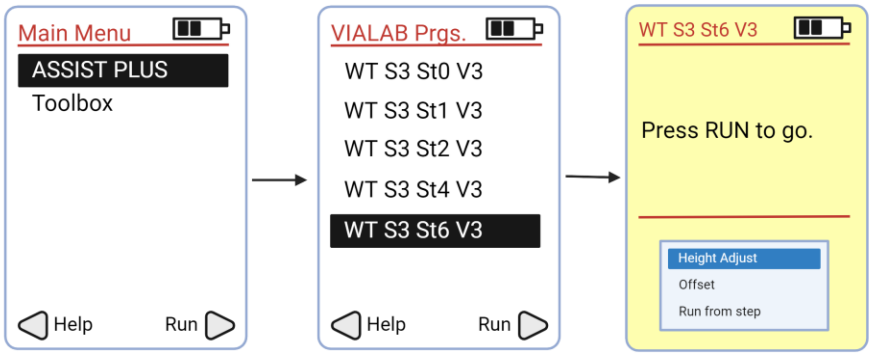
5. Place the ● Library Amp Mix in the Parse Cold Block. Deck layout should correspond to the configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	Parse Cold Block 24 mm Labware Pedestal	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES		semi-skirted 96 well PCR plate	semi-skirted 96 well PCR plate
REAGENTS	● Library Amp Mix	● SPRI Beads	● Samples

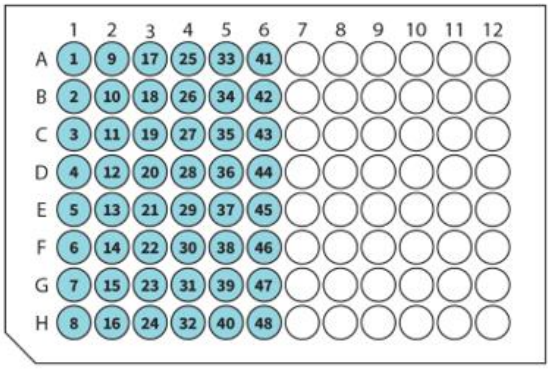
6. Uncap the reagents caps, select and run the program **WT S3 St6 V3** following the diagram below.



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

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

9. 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.



10. With a multichannel P20, pierce the seal of the chosen wells of the UDI Plate - WT.

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

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

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

6. Press "Run" to continue the program.

7. When the program is completed 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 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:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
VOYAGER Pipetting Module 8-Ch 5-125 µL	INTEGRA Component	N/A	
Tip Deck for VOYAGER Pipetting Module	INTEGRA Component	N/A	
24 mm Labware Pedestal	INTEGRA Component	N/A	
Thermochromic PCR Cold Block	Parse-Provided	N/A	
Thermochromic PCR Cold Block Riser	Parse-Provided	N/A	
INTEGRA 8 Row Reservoir Plastic Base	INTEGRA Component	N/A	
HEATMAG	INTEGRA Component	N/A	
125 µL Tip Rack	INTEGRA-Provided	1	
8 Row Reservoir	INTEGRA-Provided	1	

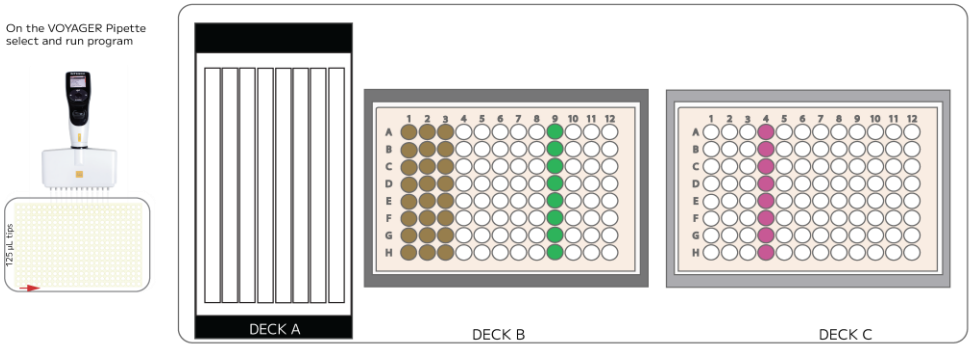
2. Remove D-ONE Pipetting Module 1-Ch, 5-1250 µL Pipette and corresponding Tip Deck. Replace it with VOYAGER 8-Ch 5-125 µL and corresponding Tip Deck. Deck layout should correspond to the configuration below.

Note: Before removing the D-ONE Pipetting Module, 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 the tip box lid prior to starting the program.

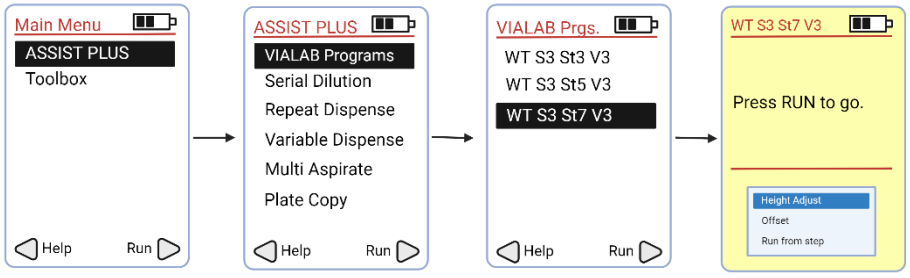
3. Remove Parse Cold Block on Deck A and replace with the reagent reservoir stored at room temperature from Section 3.6.3. Deck layout should correspond to the configuration below.

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	24 mm Labware Pedestal INTEGRA 8 Row Reservoir Plastic Base	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	HEATMAG
CONSUMABLES	8 Row Reservoir	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS		● SPRI Beads ● Library Amp Mix	● Samples

4. Select and run the program **WT S3 St7 V3** following the diagram below.



5. 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

NUMBER OF PCR CYCLES	
25-49	12
50-99	11
100-299	10
300-999	8
1,000 or more	7

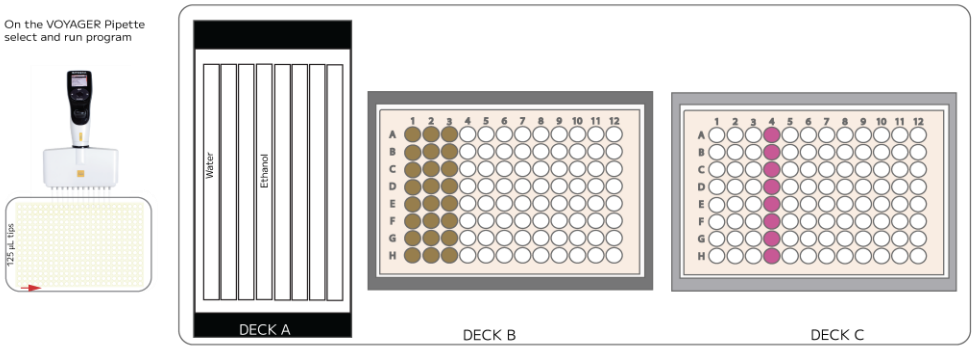
6. **When prompted**, remove the sublibrary plate from the INTEGRA ASSIST PLUS, seal the sample plate on Deck C and place it into a thermocycler. Run the following program.

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

7. When the Indexing PCR thermocycling program is complete, press "Run" to continue.

8. **When prompted**, return the sample plate onto the HEATMAG on Deck C. Deck layout should correspond to the deck configuration below

Deck Configuration



	DECK A	DECK B	DECK C
HARDWARE	INTEGRA 8 Row Plastic Adapter 24 mm Labware Pedestal	Thermochromic PCR Cold Block Thermochromic PCR Cold Block Riser	Heated Magnet
CONSUMABLES	8 Row Reservoir	Semi-skirted 96 well PCR plate	Semi-skirted 96 well PCR plate
REAGENTS	Nuclease free water 85% Ethanol	SPRI Beads	● Samples

9. Press "Run" to continue the program.

10. When the program is complete, the sequencing libraries will be in column 6 on Deck C.

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

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 3.6 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.

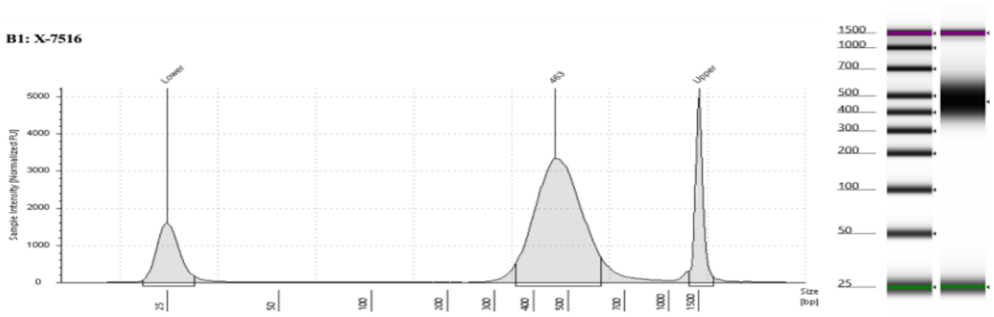


Figure 12: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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 amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.

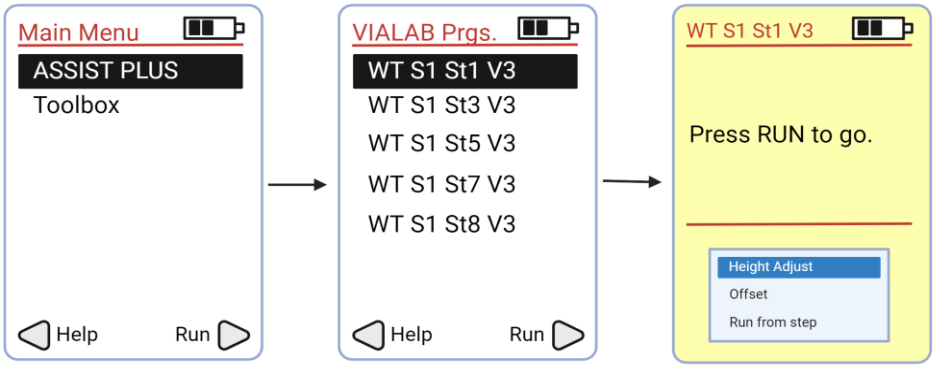
Note: If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 400-500 bp). Do not use this additional peak when estimating amplicon size.

Appendices

Appendix A: Pipetting Programs

Section 1.1. Sample Normalization

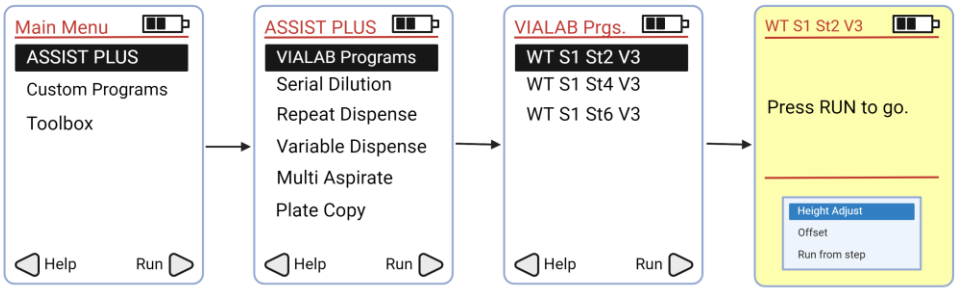
WT S1 St1 V3



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

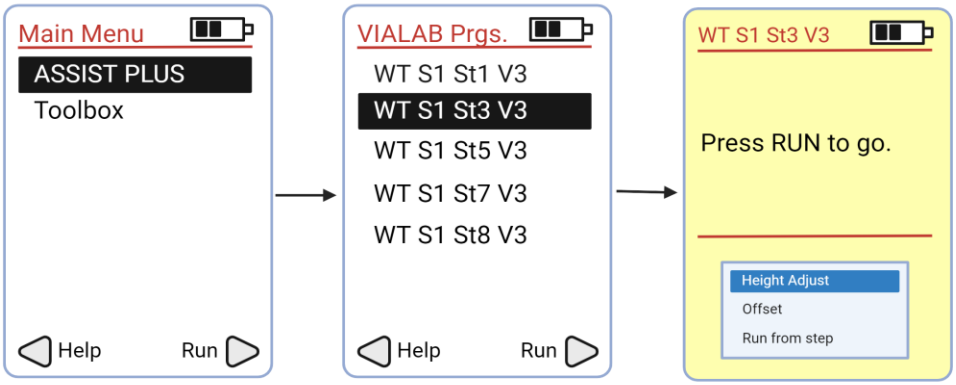
WT S1 St2 V3



STEPS	ACTION
1	Initial Volumes
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	Pool Cells to Intermediate Wells
7-8	Pool Cells to Intermediate Wells

Section 1.3. Round 2 Ligation Preparation

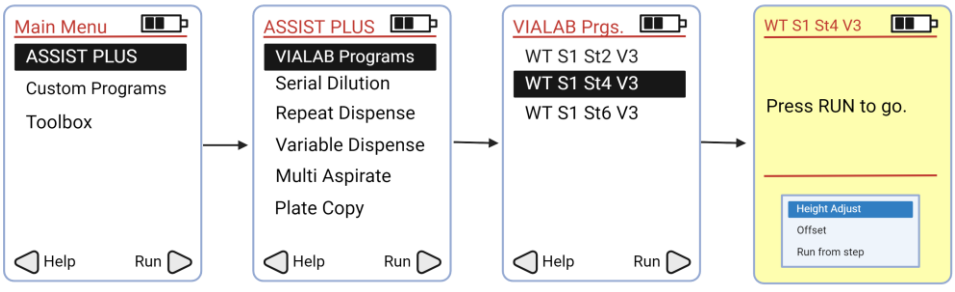
WT S1 St3 V3



STEPS	ACTION
1	Initial Volumes
2	Pool row A into 10 mL Tube with 1000 µL tips
3	Pool row into 10 mL Tube with 125 µL tips
4	Add Spin Additive
5	"Invert Tube and Spin for 10 min" message
6	"Thaw R2 Plate" message
7	"Return tube to Deck C –" message
8-13	Remove Supernatant
14-15	Resuspend Cell Pellet with Resuspension Buffer
16	Add R2 Ligation Enzyme to Buffer
17	Mix Ligation Mix
18	Volume Change
19	Transfer Cells to Ligation Mix
20	Mix Cells in Ligation Mix
21-24	Transfer Ligation Mix to Basin
25-27	Mix Sample in Basin

Section 1.4. Round 2 Ligation

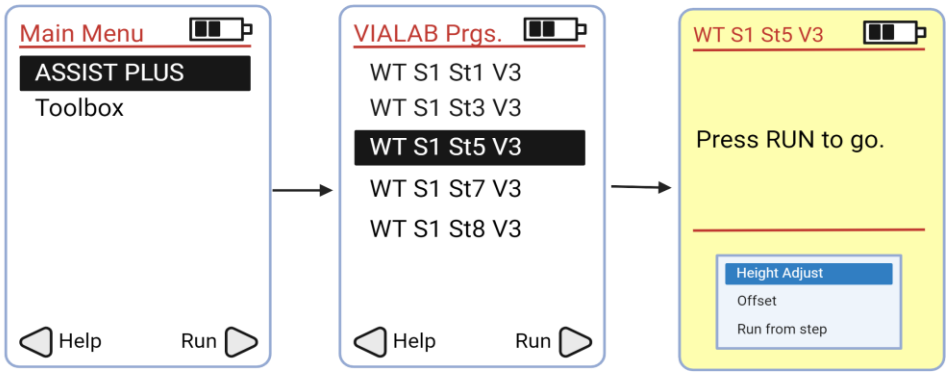
WT S1 St4 V3



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
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

WT S1 St5 V3

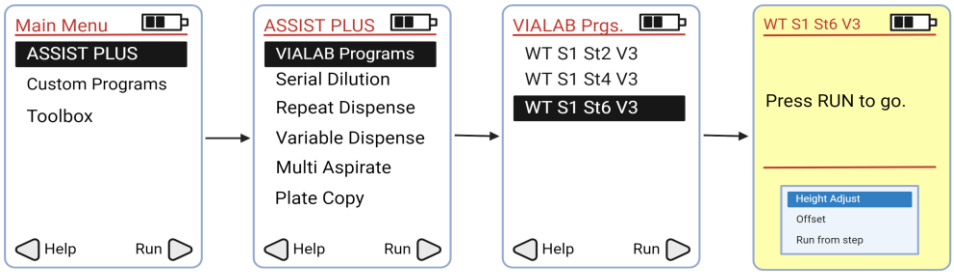


STEPS	ACTION
1	Initial Volumes

STEPS	ACTION
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/nuclei
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/nuclei to right basin

Section 1.6. Round 3 Ligation

WT S1 St6 V3

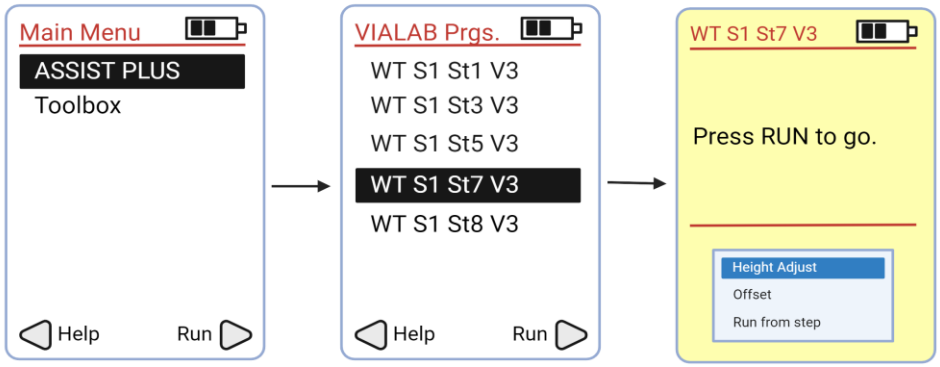


STEPS	ACTION
1	Initial Volumes
2-4	Mix sample in basin
5-12	Add sample to Round 3 Plate
13	"Seal and incubate for R3 Ligation" message

STEPS	ACTION
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

WT S1 St7 V3

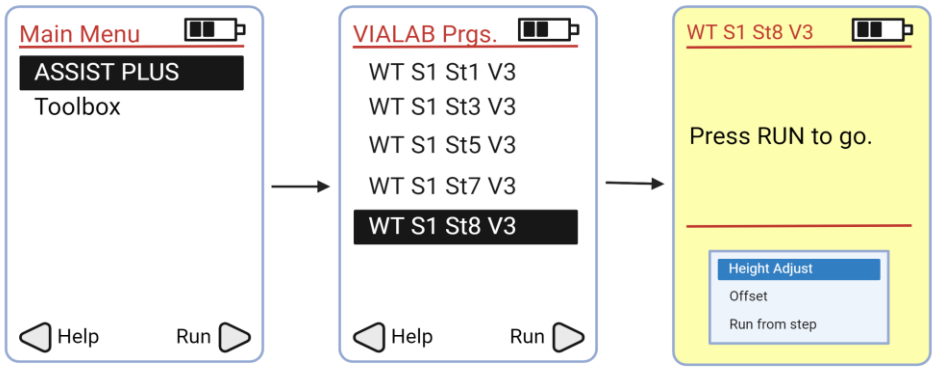


STEPS	ACTION
1	Initial Volumes
2	“Move Basin Holder to Deck B” message
3	"Insert cell strainer in 10 mL tube" message
4	Wash basin
5	Volume change
6-12	Strain cells
13	“Move Basin Holder to Deck A” message
14	"Remove cell strainer" message
15	Add Spin Additive

STEPS	ACTION
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

WT S1 St8 V3

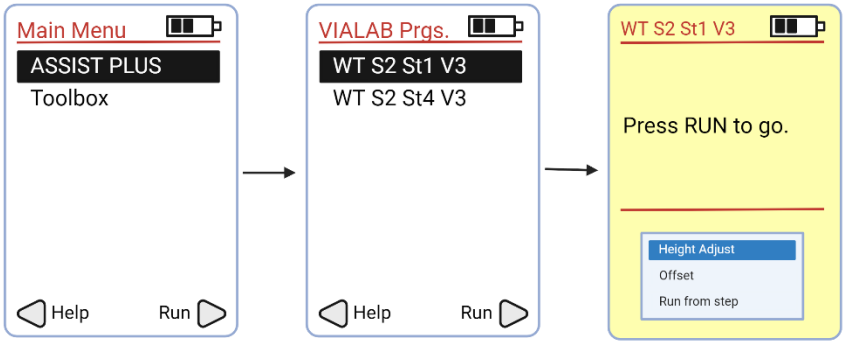


STEPS	ACTION
1	Initial Volumes
2-3	Aliquot 25 µL of sample
4	Lysis Mastermix
5	Mix Lysis Mastermix

STEPS	ACTION
6	Add Lysis Mastermix to sample
7	"Vortex and centrifuge samples" message

Section 2.1. Reagent Plating

WT S2 St1 V3

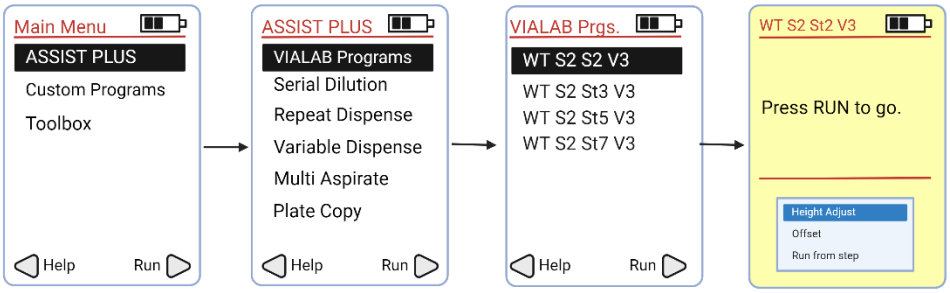


STEPS	ACTION
1	Initial Volumes
2	Dispense Binder Beads
3	Dispense SPRI Beads
4	Dispense Binding Buffer
5-7	Dispense Bead Wash Buffer
8	"Thaw Lysates" message
9-10	Dispense Wash Buffer 1
11-12	Dispense Wash Buffer 2
13-14	Dispense Wash Buffer 3
15	"Load Lysates–" message
16	Enhancer Addition
17	"Cap and store SPRI beads at RT" message

STEPS	ACTION
18	“Proceed immediately to S2 St2” message

Section 2.2. cDNA Capture

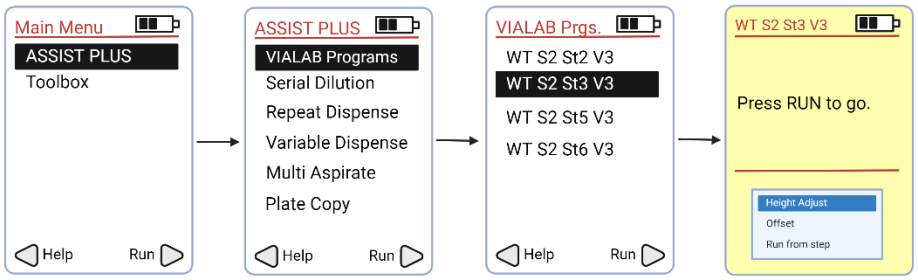
WT S2 St2 V3



STEPS	ACTION
1	Initial Volumes
2	Raise Magnet
3	Mix Lysates
4-5	Remove Binder Bead Supernatant
6-14	1st Bead Wash
15-22	2nd Bead Wash
23-30	3rd Bead Wash
31-32	Add Binding Buffer and Mix
33-34	Add Mix Binder Beads to Sample
35	“Vortex at 800-1k rpm for 30 mins” message

Section 2.3. Binder Beads Wash

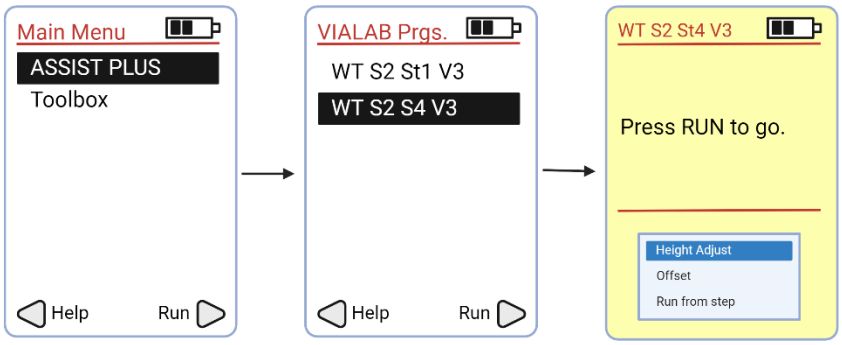
WT S2 St3 V3



STEPS	ACTION
1	Initial Volumes
2-5	Supernatant Removal
6-13	1st Wash 1
14-21	2nd Wash 1
22-28	Wash 2
29	Wash 3
30	"Proceed immediately to S2 St4" message

Section 2.4. Master Mixes Preparation

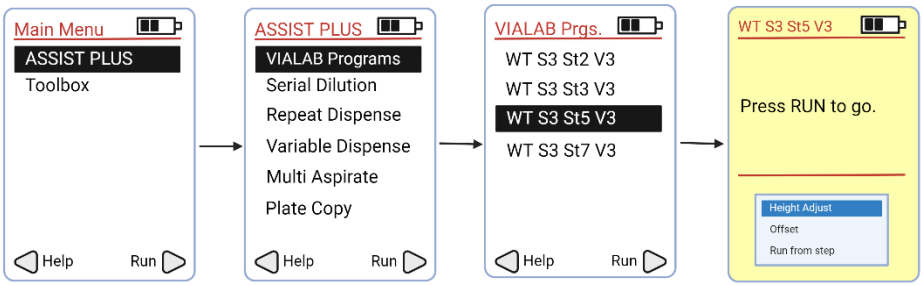
WT S2 St4 V3



STEPS	ACTION
1	Initial Volumes
2-4	cDNA Amp Mix Prep
5	Dispense cDNA Amp Mix to Strip Tubes
6	“Cap and store cDNA Amp on ice–” message
7-11	Template Switch Mix Prep
12	Dispense Template Switch to Strip Tubes
13	“Proceed immediately to S2 St5” message

Section 2.5. Template Switch and cDNA Amplification

WT S2 St5 V3

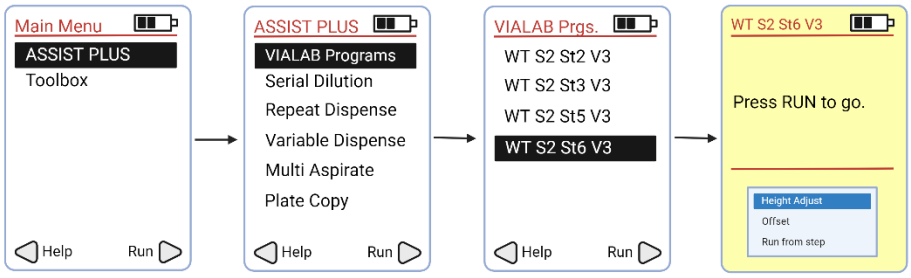


STEPS	ACTION
1	Initial Volumes
2-5	Remove Wash 3 Supernatant
6-7	Adds Template Switch Mix to Samples
8	“Seal and incub at RT for 30 mins” message
9	“Reload samples on Heamag–” message
10-11	Mix samples
12	“Seal and run TS on thermocycler” message
13	“Reload samples on Heatmag–” message
14-18	Remove Template Switch Supernatant

STEPS	ACTION
19-27	Wash 2
28-29	Wash 3
30	“Load Amp Mix on Deck A–” message
31-32	Remove Wash 3 Supernatant
33-34	Add cDNA Amp Mix to Sample
35	“Run cDNA Amp on the Thermocycler” message

Section 2.6. Post-Amplification Purification

WT S2 St6 V3

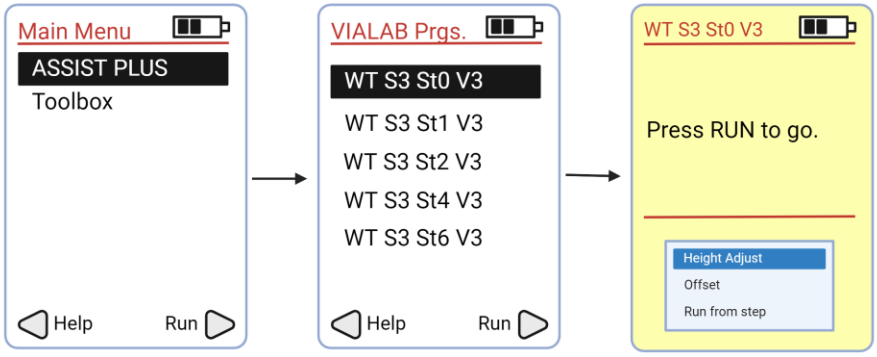


STEPS	ACTION
1	Initial Volumes
2	Mix Samples
3	Activate Magnet
4-5	Mix SPRI Beads
6-8	Transfer Sample to Tubes on HEATMAG
9-11	Add SPRI Beads to Samples
12-16	Remove Supernatant
17-22	1st EtOH Wash
23-30	2nd EtOH Wash
31-39	Elution

STEPS	ACTION
40	“Samples on HEATMAG Column 9” message

Section 3.0. cDNA Normalization

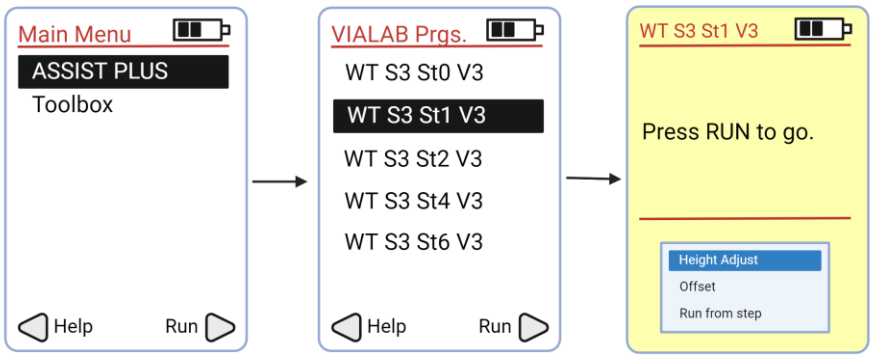
WT S3 St0 V3



STEPS	ACTION
1	Initial Volumes
2	Normalize samples

Section 3.1. SPRI Bead Plating

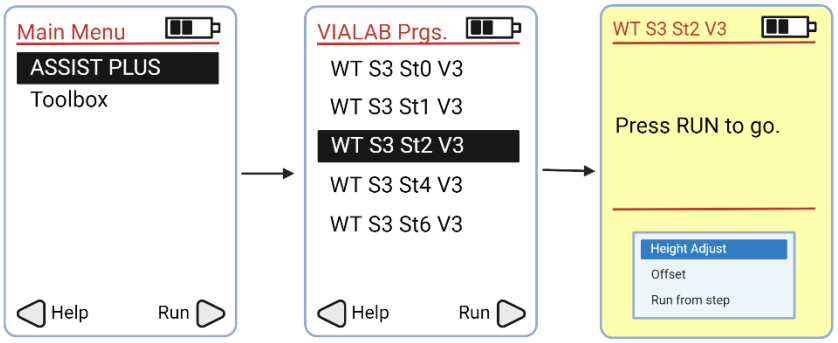
WT S3 St1 V3



STEPS	ACTION
1	Initial Volumes
2	Plate out beads into columns 1 & 3
3	Plate out beads into column 2

Section 3.2. Fragmentation Mix Creation and Plating

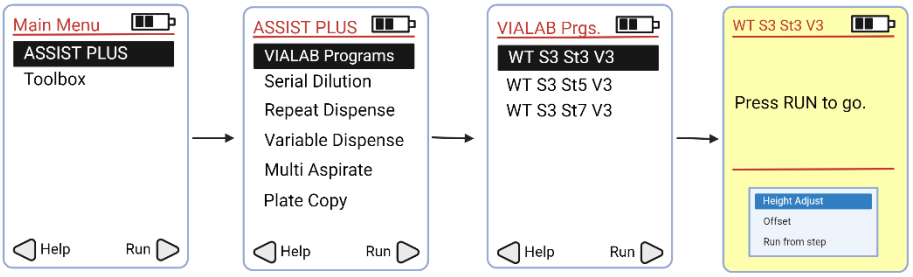
WT S3 St2 V3



STEPS	ACTION
1	Initial Volumes
2	Pre-chill thermal cycler message
3	Create Fragmentation Mix
4	Plate out Fragmentation Mix into column 11
5	Proceed to S3 St3 message

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

WT S3 St3 V3

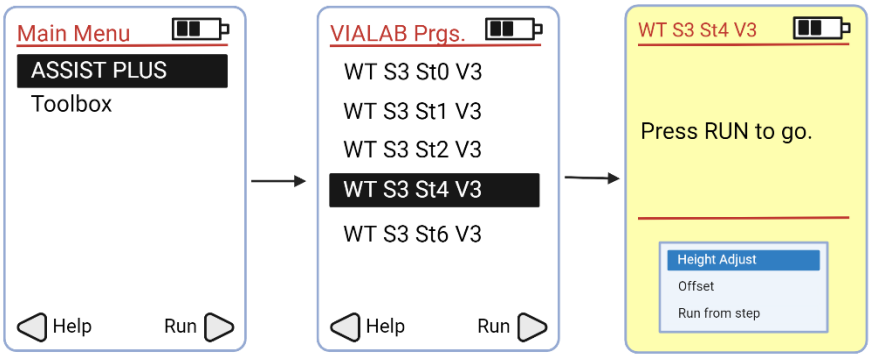


STEPS	ACTION
1	Initial Volumes
2	Stamp Fragmentation Mix into samples
3-7	Deck loading messages
8	Volume change
9	Ensure magnet is deactivated
10-11	Mix SPRI beads
12	Add SPRI beads to samples
13	5 minute bead incubation
14	Activate magnet
15	2 minute bead immobilization
16	Transfer supernatant
17	Deactivate magnet
18	Add SPRI beads to samples
19	5 minute bead incubation
20	Activate magnet
21	3 minute bead immobilization
22	Volume change

STEPS	ACTION
23	Discard supernatant
24-25	Ethanol addition 1
26	1 minute ethanol incubation
27-28	Discard ethanol
29-30	Ethanol addition 2
31	1 minute ethanol incubation
32-33	Discard ethanol
34	Air dry delay
35	Deactivate magnet
36	Resuspend beads in water
37-38	Offset mixing to ensure full resuspension
39	5 minute bead incubation
40	Activate magnet
41	2 minute bead immobilization
42	Transfer eluate
43	Deactivate magnet

Section 3.4. Ligation Mix Creation and Plating

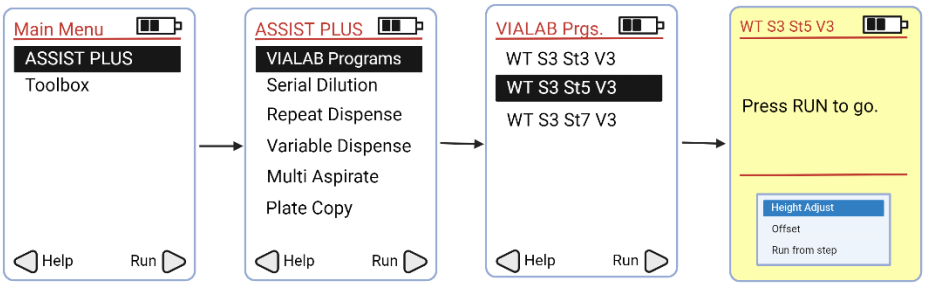
WT S3 St4 V3



STEPS	ACTION
1	Initial Volumes
2	Create Ligation Mix
3	Slow mix to reduce volume stuck in tip
4	Plate out Ligation Mix into column 10
5	Proceed to S3 St5 message

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

WT S3 St5 V3

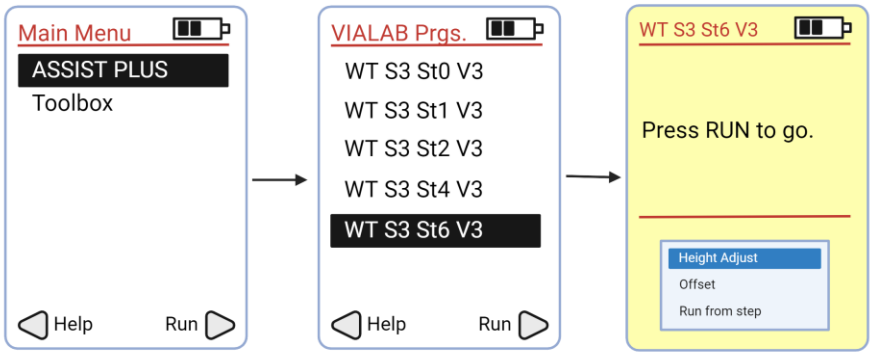


STEPS	ACTION
1	Initial Volumes
2	Stamp Ligation Mix into samples
3-5	Deck loading messages
6	Ensure magnet is deactivated
7-8	Mix SPRI beads
9	Add SPRI beads to samples
10	5 minute bead incubation
11	Activate magnet
12	5 minute bead immobilization
13	Volume change

STEPS	ACTION
14-15	Discard supernatant
16-17	Ethanol addition 1
18	1 minute ethanol incubation
19-20	Discard ethanol
21-22	Ethanol addition 2
23	1 minute ethanol incubation
24-25	Discard ethanol
26	Air dry delay
27	Deactivate magnet
28	Resuspend beads in water
29	5 minute bead incubation
30	Activate magnet
31	2 minute bead immobilization
32	Transfer eluate
33	Deactivate magnet

Section 3.6. Barcoding Round 4

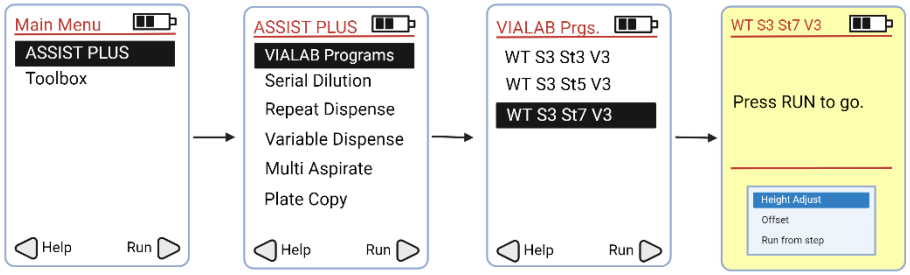
WT S3 St6 V3



STEPS	ACTION
1	Initial Volumes
2	Add UDIs message
3	Plate out Amplification Mix into column 9
4	Proceed to S3 St7 message

Section 3.7. Library Amp Mix Addition and Size Selection

WT S3 St7 V3



STEPS	ACTION
1	Initial Volumes
2	Stamp Amplification Mix into samples
3-4	Deck loading messages
5	Ensure magnet is deactivated
6-7	Mix SPRI beads
8	Add SPRI beads to samples
9	5 minute bead incubation
10	Activate magnet
11	2 minute bead immobilization
12	Transfer supernatant
13	Deactivate magnet

STEPS	ACTION
14	Add SPRI beads to samples
15	5 minute bead incubation
16	Activate magnet
17	3 minute bead immobilization
18	Volume change
19	Discard supernatant
20-21	Ethanol addition 1
22	1 minute ethanol incubation
23-24	Discard ethanol
25-26	Ethanol addition 2
27	1 minute ethanol incubation
28-29	Discard ethanol
30	Air dry delay
31	Deactivate magnet
32	Resuspend beads in water
33-34	Offset mixing to ensure full resuspension
35	5 minute bead incubation
36	Activate magnet
37	2 minute bead immobilization
38	Transfer eluate
39	Deactivate magnet

Appendix B: 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 C: Revision History

Version	Description	Date
1.0	Initial release of Full Solution Workflow	October 2024
1.1	Section 1.8.1: Revised reagents storage and handling. Section 2.1.11: Revised step to include 2 PCR strip tube.	November 2024

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