

User Manual

Version 1.0 – UMWT4530



Evercode™ WT Mega 384

v4

For use with
ECWT4530



Legal Notices

This document and its contents are proprietary to Parse Biosciences, Inc. ("Parse Biosciences") and are intended solely for use by its customers in connection with the use of the product(s) described herein and for no other purpose. The products may be used solely FOR RESEARCH PURPOSES, AND MAY NOT BE USED IN ANY DIAGNOSTIC OR THERAPEUTIC USE IN HUMANS OR ANIMALS. This document and its contents shall not be used or distributed for any other purpose and/ or otherwise communicated, disclosed or reproduced in any way whatsoever without the prior written consent of Parse Biosciences.

No rights are granted under this document with respect to any of Parse Biosciences' intellectual property rights. The license to use of any products described herein is subject to a separate written agreement between Parse Biosciences and the applicable user.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. Parse Biosciences shall have no liability for any direct, indirect, consequential or incidental damages arising out of any failure to use the product(s) in strict compliance with the terms herein.

This document may contain references to third-party sources of information, hardware or software, products, or services and/ or third-party web sites (collectively "Third Party Information"). Parse Biosciences does not control, and is not responsible for, any Third Party Information. The inclusion of Third Party Information in this document does not imply endorsement by Parse Biosciences of the Third Party Information or the third party in any way.

The product(s) described in this document are provided for one-time use by the purchaser and may not be re-used, refurbished or resold. In addition, such product(s) may not be altered, changed or modified by anyone other than Parse Biosciences and its authorized agents, and Parse Biosciences will not be liable for any such alterations, changes or modifications. The product(s) described herein may be covered by one or more of the following patents:

U.S. Pat. No. 10,900,065

U.S. Pat. No. 11,168,355

U.S. Pat. No. 11,427,856

U.S. Pat. No. 11,634,751

U.S. Pat. No. 11,639,519

U.S. Pat. No. 11,680,283

Patents pending in the U.S. and other countries

PARSE and EVERCODE are trademarks of Parse Biosciences, Inc. The information made available herein is the sole property of Parse Biosciences and its licensors.

Copyright (c) 2024-2026 Parse Biosciences, Inc. All Rights Reserved.

Table of Contents

Overview	5
Workflow	5
Protocol Timing.....	11
Important Guidelines Mega 384	12
Part List	20
User Supplied Equipment and Reagents.....	24
Section 1: In situ Cell/Nuclei Barcoding.....	28
1.1 Sample Set Up and Barcoding Round 1.....	28
1.1a Set Up for Low Input Fixation Samples	29
1.1b Set Up for Standard Fixation Samples	36
1.2. Pooling Round 1 Plates.....	41
1.3. Barcoding Round 2	45
1.4. Barcoding Round 3	50
1.5. Lysis and Sublibrary Generation.....	53
Section 2: cDNA Capture and Amplification.....	55
2.1. cDNA Capture.....	55
2.2. cDNA Template Switch	59
2.3. cDNA Amplification.....	62
2.4. Post-Amplification Purification	65
2.5. cDNA Quantification	67
Section 3: Sequencing Library Preparation.....	68

3.1. Fragmentation and End Prep.....	68
3.2. Post-Fragmentation and End Prep Size Selection	71
3.3. Adapter Ligation.....	73
3.4. Post-Ligation Purification	75
3.5. Barcoding Round 4	76
3.6. Post-Barcoding Round 4 Size Selection	79
3.7. Sequencing Library Quantification.....	81
Appendices	82
Appendix A: Sublibrary Generation Table	82
Appendix B: Sequencing Information	83
Appendix C: Thermocycling Programs	86
Appendix D: Revision History.....	91

Overview

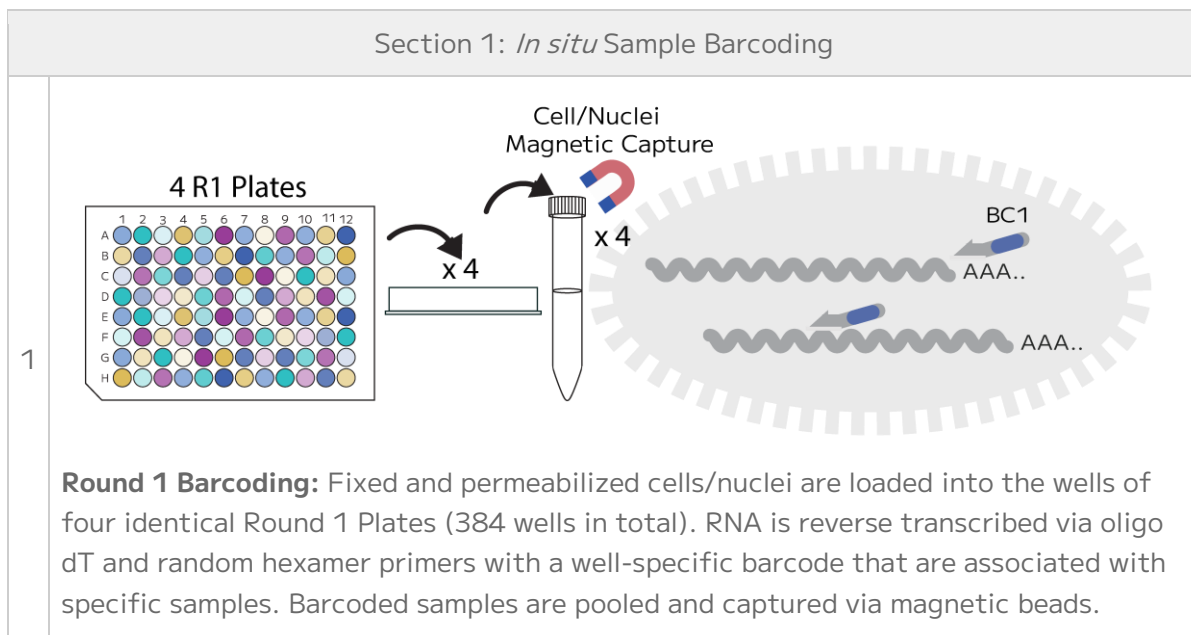
Workflow

Evercode split-pool combinatorial barcoding brings a simple workflow to large-scale single cell RNA-seq experiments. The Evercode WT Mega 384 v4 kit can profile up to 1,000,000 cells across up to 384 different biological samples or experimental conditions.

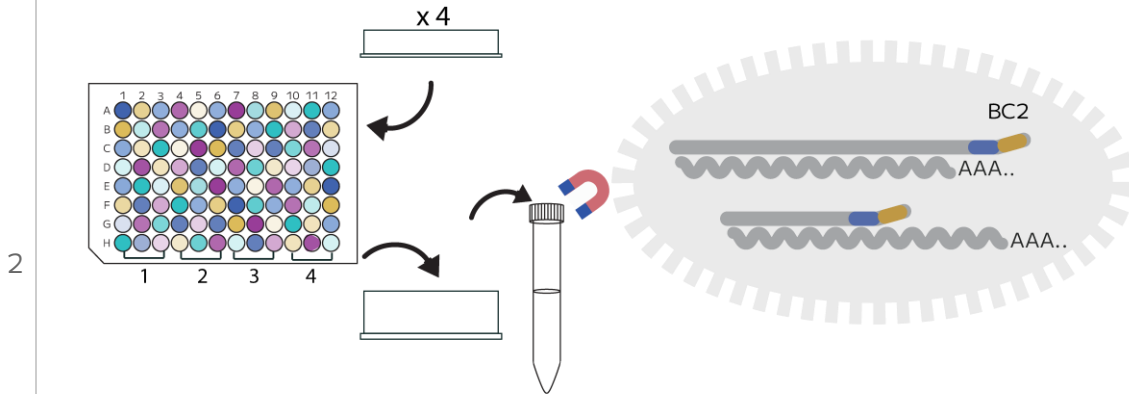
Evercode Fixation kits fix and permeabilize cells/nuclei so they act as individual reaction compartments, which eliminates the need for dedicated microfluidics instrumentation. Through four rounds of barcoding, the transcriptome of each fixed cell/nuclei is uniquely labeled. The four rounds of barcoding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

After sequencing, the Parse Biosciences Analysis Pipeline or the Trailmaker platform assigns reads that share the same 4 barcode combination to a single cell/nuclei.

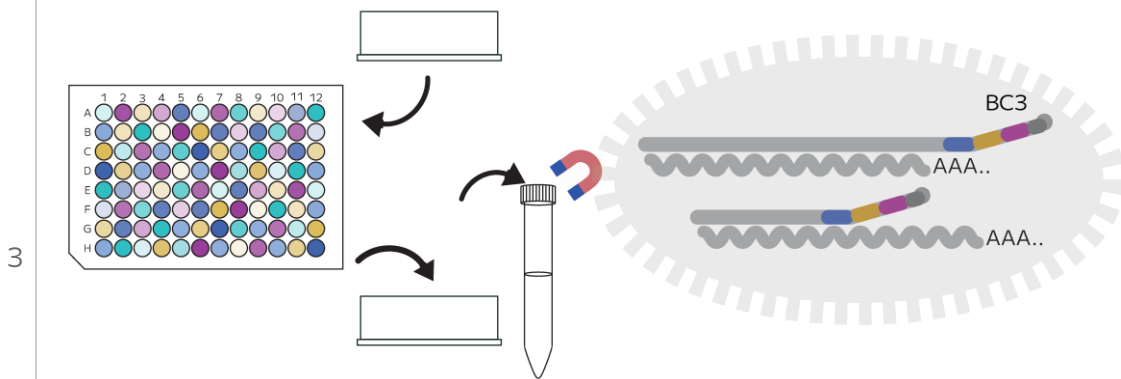
The tables below provide a high-level overview of the whole transcriptome workflow.



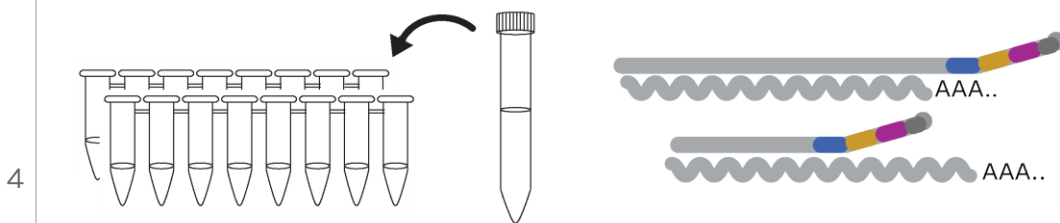
Section 1: *In situ* Sample Barcoding



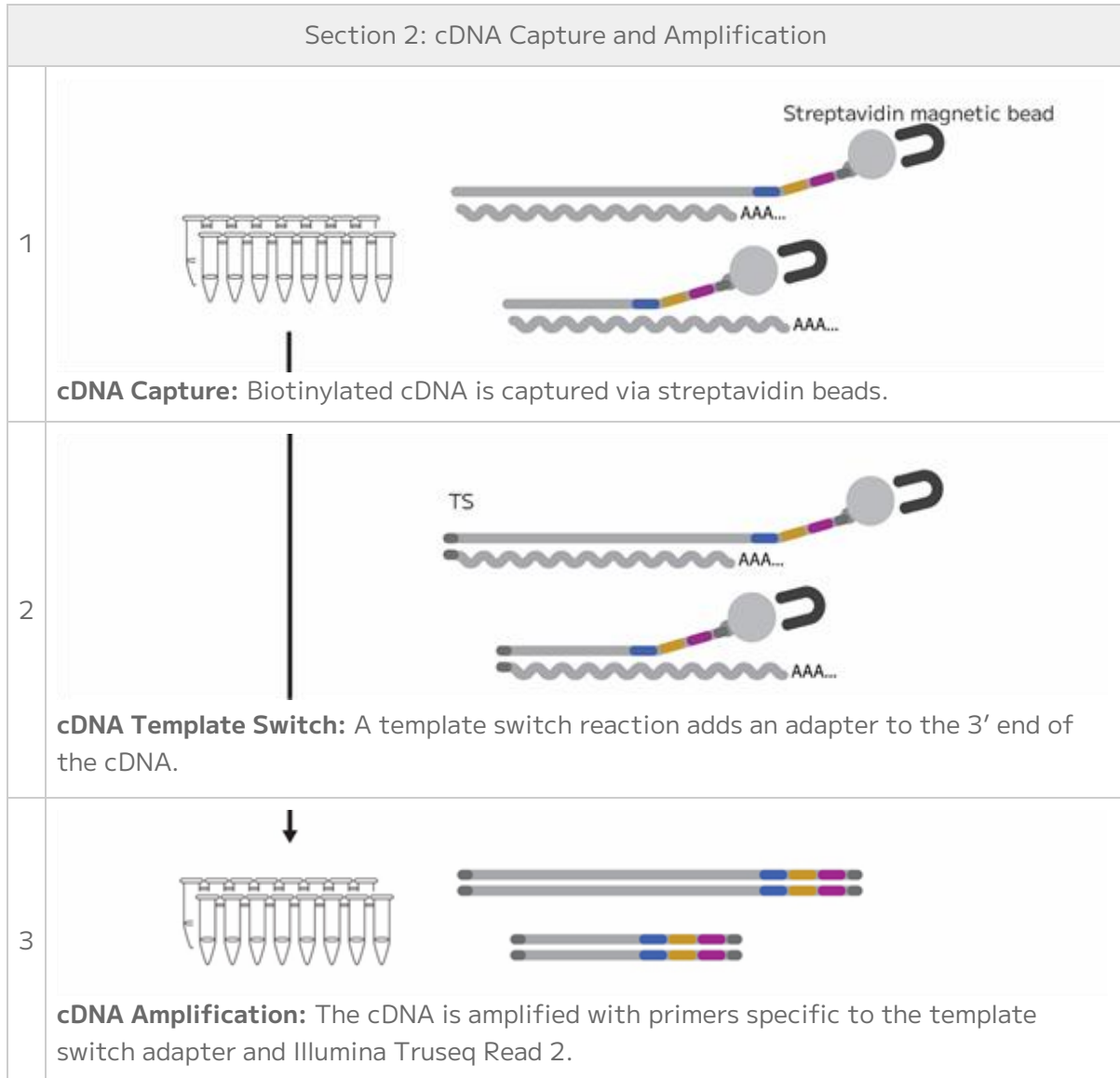
Round 2 Barcoding: The pooled cells/nuclei are loaded into the Round 2 Plate. An adapter with a well-specific barcode is ligated to the first barcode. Cells/nuclei are pooled and then captured via magnet.



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



Lysis and Sublibrary Generation: Cells/nuclei are split across 16 sublibraries and lysed.



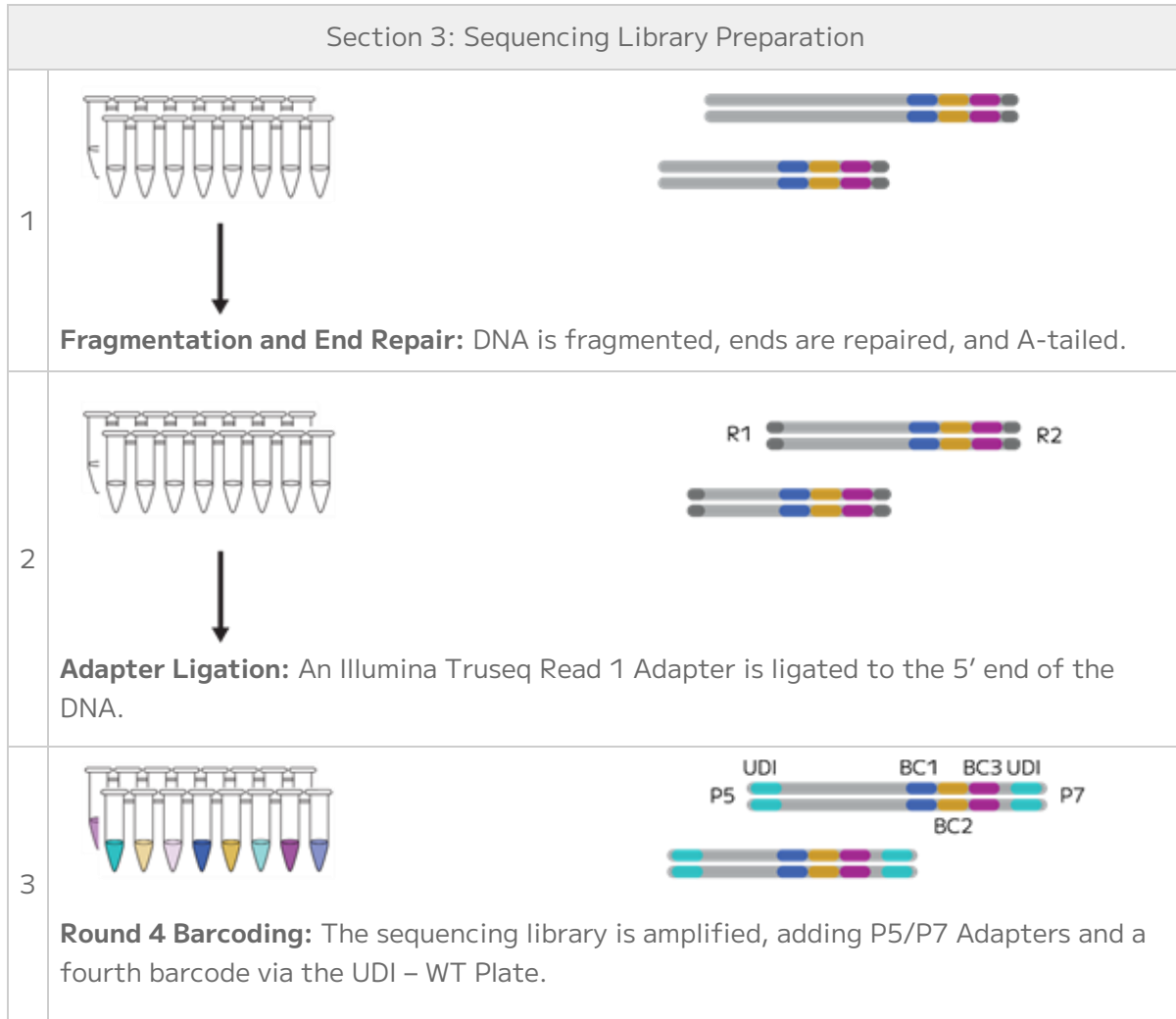


Figure 1 shows the Mega 384 workflow. This kit can barcode up to 1 million cells/nuclei across 384 samples in a single reaction. The Mega 384 utilizes four identical Round 1 Plates, each holding a unique set of 96 samples, totaling 384 samples. These samples are pooled from the four Round 1 Plates into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

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

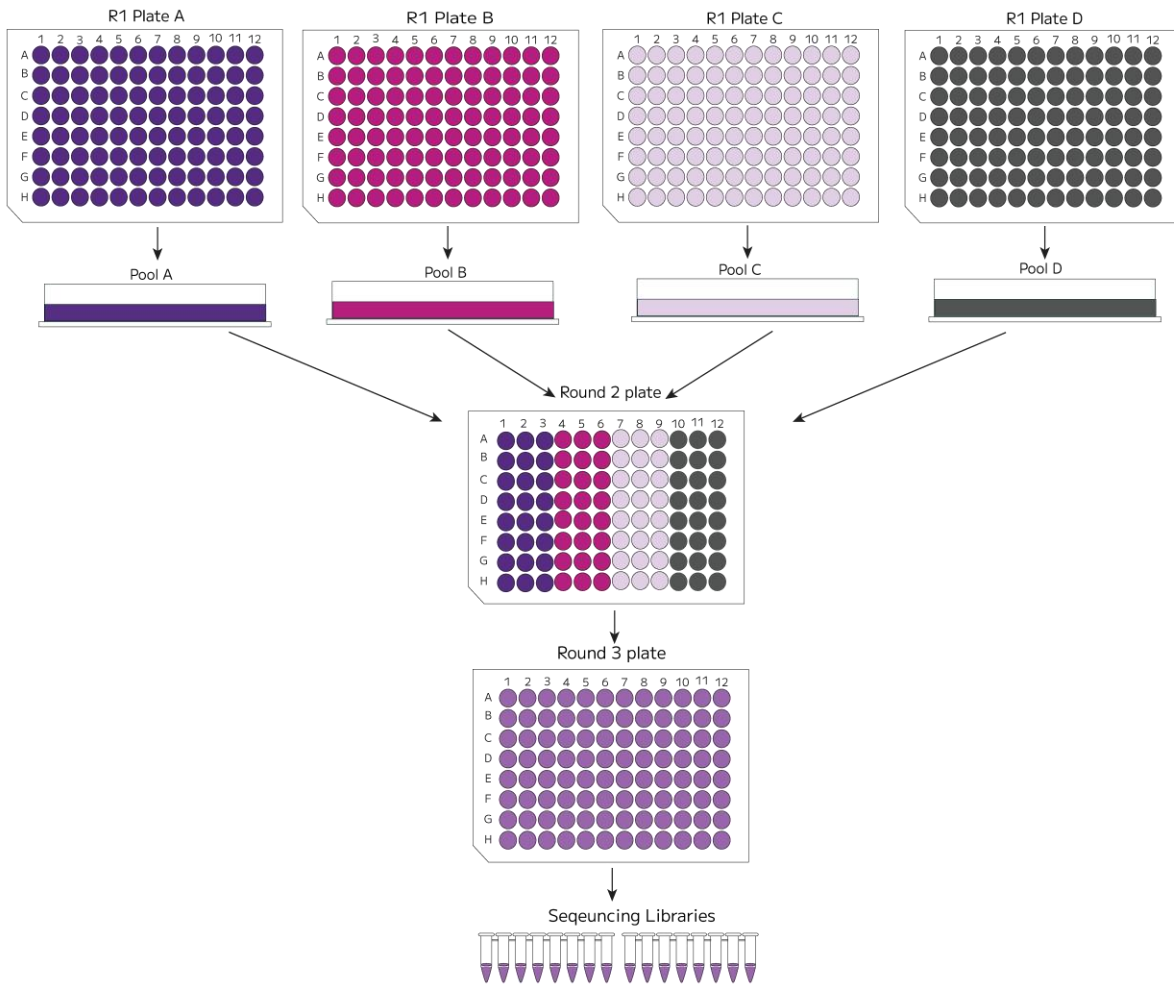


Figure 1: Evercode™ WT Mega 384 workflow pools 384 samples from four Round 1 Barcoding plates into one Round 2 and Round 3 Barcoding Plates respectively, generating 16 Sequencing Libraries

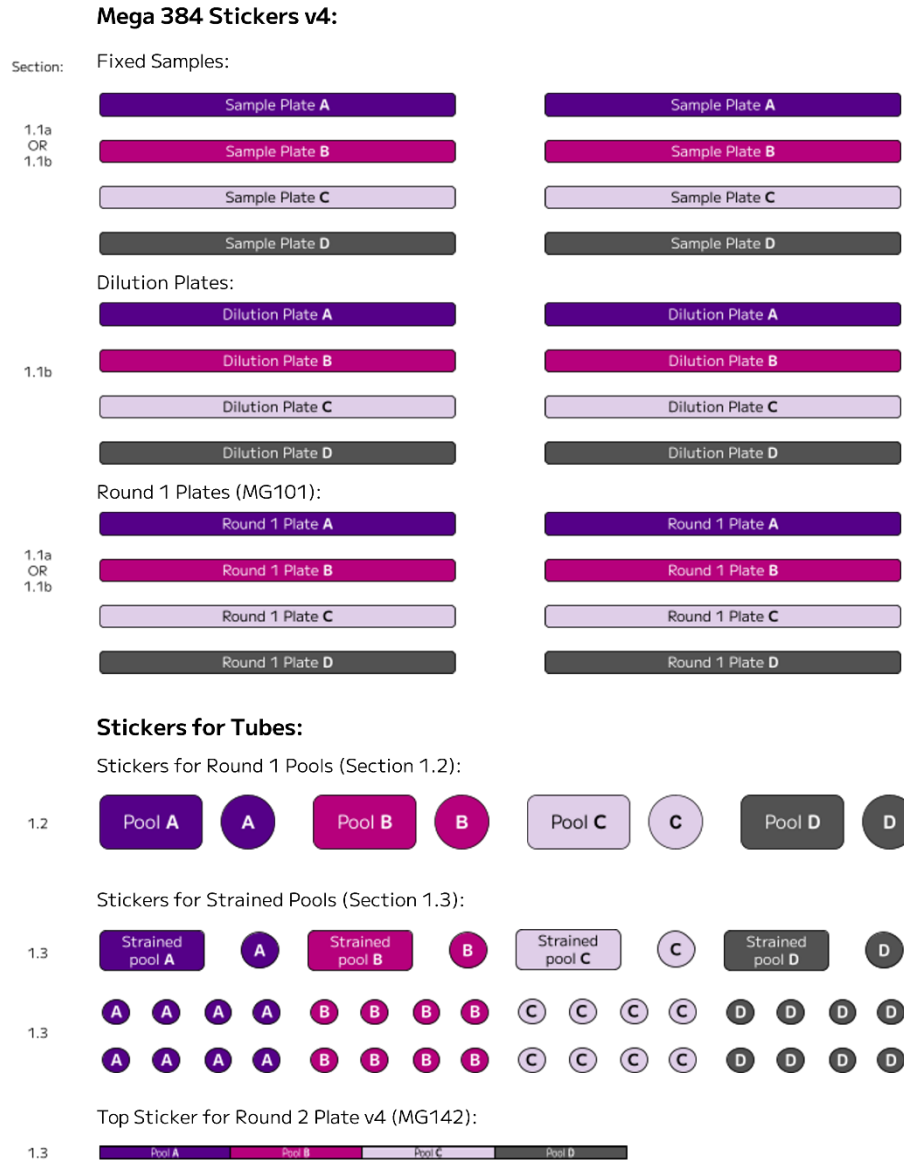


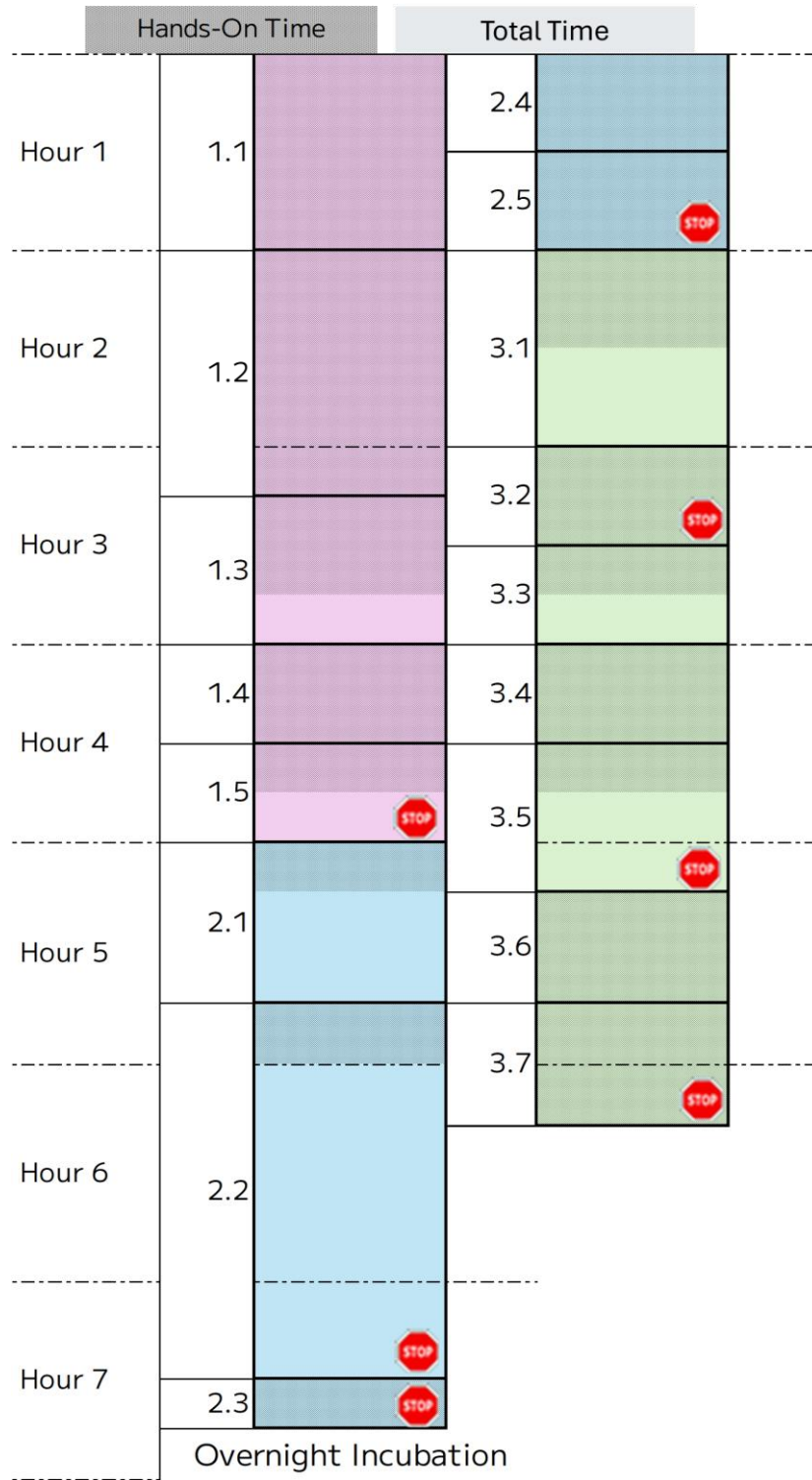
Figure 2: Plates are easy to handle with labeled color-coded stickers throughout the Round 1 Barcoding section.

Protocol Timing

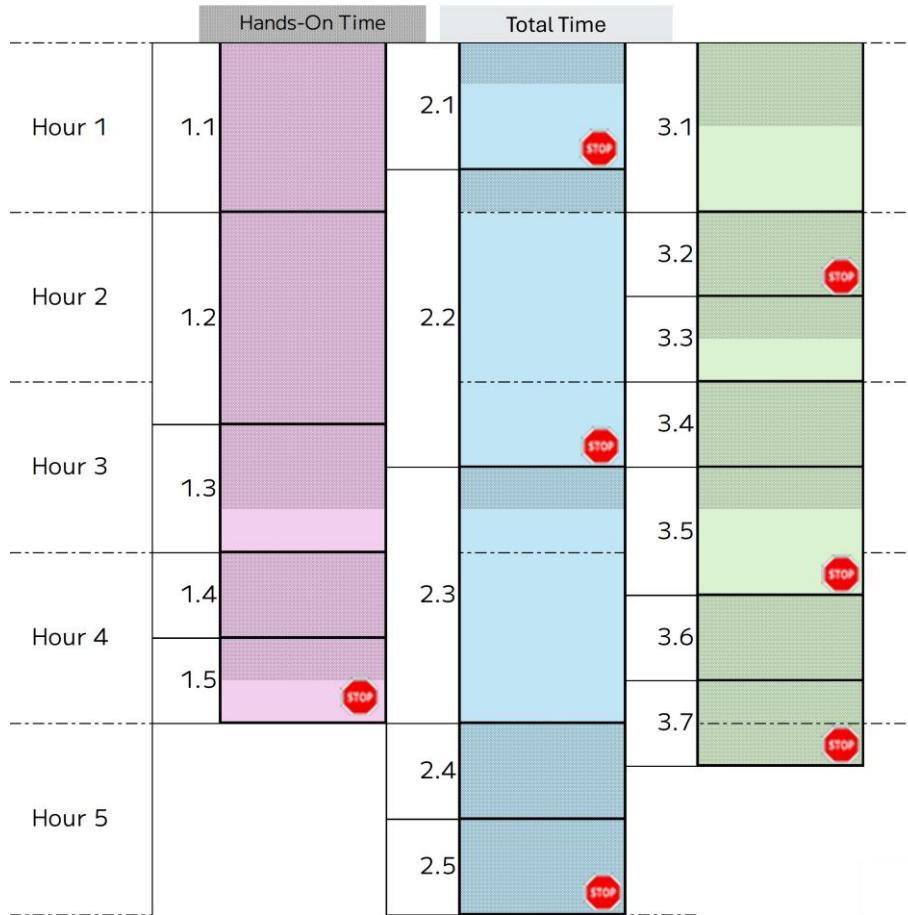
The table below provides details of the total and hands-on time required for the whole transcriptome workflow. A visual representation of the workflow is shown below the table.

DESCRIPTION	TIME	HANDS-ON-TIME	STOPPING POINTS
Section 1: In Situ Cell/Nuclei Barcoding			
1.1 Set up and Sample Counting	Variable (60-90 min)	Variable (60-90 min)	
1.2 Pooling Round 1 Plates	75 min	75 min	
1.3 Barcoding Round 2	40 min	25 min	
1.4 Barcoding Round 3	30 min	25 min	
1.5 Lysis and Sublibrary Generation	30 min	15 min	-80°C ≤ 6 months
Section 2: cDNA Capture and Amplification			
2.1 cDNA Capture	45 min	10 min	
2.2 cDNA Template Switch	105 min	15min	4°C ≤ 18 hrs
2.3 cDNA Amplification	90 min	10 min	4°C ≤ 18 hrs in the thermocycler
2.4 Post-Amplification Purification	30 min	30 min	
2.5 cDNA Quantification	30 min	30 min	4°C ≤ 48 hrs or -20°C ≤ 3 months
Section 3: Sequencing Library Preparation			
3.1 Fragmentation and End Prep	60 min	30 min	
3.2 Fragmentation and End Prep Size Selection	30 min	30 min	4°C ≤ 18 hrs or -20°C ≤ 2 weeks
3.3 Adapter Ligation	30 min	15 min	
3.4 Post-Ligation Purification	30 min	30 min	
3.5 Barcoding Round 4	45 min	15 min	4°C ≤ 18 hrs in the thermocycler
3.6 Post-Barcoding Round 4 Size Selection	30 min	30 min	
3.7 Sequencing Library Quantification	30 min	30 min	-20°C ≤ 3 months

2-days workflow



3-days workflow



Important Guidelines

The following guidelines provide additional information to obtain optimal performance. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at <https://support.parsebiosciences.com/>.

Sample Input

- This protocol begins with cells or nuclei fixed with an Evercode Cell Fixation v3 and v4 Kit, Evercode Nuclei Fixation v3 and v4 kit, Evercode Low-Input Cell Fixation Kit or Evercode Low-Input Nuclei Fixation v3 and v4 kits, or the Whole Blood Fixation kit.
- For the Standard Fixation kit, aliquot the samples after fixation and count the aliquots the day before using an Evercode kit. Refer to the guidelines in the Evercode Fixation User Manuals 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 thermocycler set to 37°C and counted with an automated counting device. Counts should be recorded in the Sample Loading Table, and any remaining counting aliquot should be discarded.
- For the Standard Fixation kit, after recording the concentrations of the counting aliquots in the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA, create Sample Dilution Plates as shown on the "Dilution and R1 Plate Config." tab (see Section 1.1b for more information).
- For Low Input Fixation samples, use recommended volumes of sample input and dilution buffer from Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA based on prefixation cell input. We do not recommend counting the samples directly post cell capture due to time constraints.
- For low input fixed samples, bead and storage buffer volumes vary based on cell/nuclei input. With 384 samples, loading can become complex and time-consuming. To streamline bead capture and keep it within a manageable timeframe, we strongly recommend normalizing cell/nuclei counts across sets of 12 or 8 prior to fixation.
- Once fixed samples have been thawed, they should not be refrozen.

Cell/Nuclei Counting and Quality Assessment

- For the Standard Fixation kit we recommend using a plate based automated cell counter to count cells/nuclei from the counting plates of Standard Fixation samples due to the high-throughput nature of the workflow.

- For Low Input samples, we recommend counting pre-fixation and optionally spot-counting post fixation.
- Fluorescent sample counting is recommended for low input samples after beads have been added for ease of counting.
- When first using Evercode Whole Transcriptome kits, we suggest saving images at each counting step.
- To assess sample quality, we recommend using viability stains like trypan blue or acridine orange and propidium iodide (AO/PI).
- Examples of AO/DAPI and trypan blue stained fixed cells are shown below. High quality fixed samples have single distinct cells/nuclei with <5% cell aggregation and no debris. Higher levels of aggregation will lead to elevated doublets after sequencing. When quantifying fixed samples, it is critical to avoid counting cell debris to avoid overestimating the number of cells/nuclei.

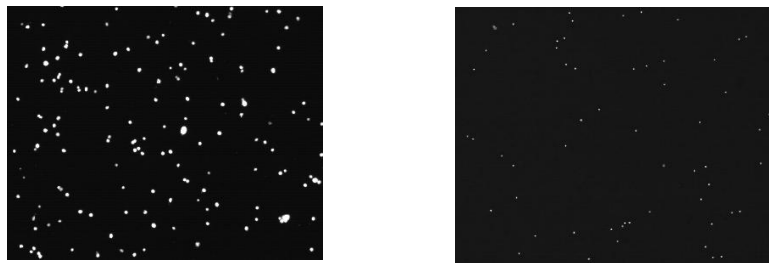


Figure 3: Low Input Fixation: example of post-bind AO/DAPI stained HEK cells (left) and PBMCs (right).

High Quality Sample

Aggregation

Debris

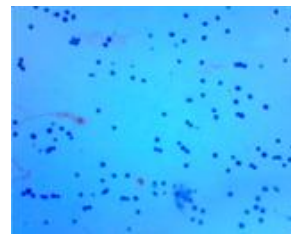


Figure 4: Example of trypan blue stained fixed cells.

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.
- Filtered pipette tips should be used to reduce RNase contamination from pipettes.

Optimizing Cell/Nuclei Recovery

- It is critical to thoroughly resuspend the cells/nuclei after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting up and down until no clumps are visible. Due to cell/nuclei adherence to tubes, carefully pipette up and down along the bottom and sides of tubes to minimize cell/nuclei loss.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell/nuclei pellets adequately.
- Ensure that the 15 mL centrifuge tubes that will be used are polypropylene, as polystyrene tubes will lead to substantial sample loss.
- The first time using an Evercode Whole Transcriptome kit, we recommend retaining supernatants after each barcoding step. In the unlikely event of unexpectedly high sample loss, these supernatants can be analyzed to identify points for optimization.
- For Low Input Fixation samples only: if the bead/cell pellet is disturbed during the bead binding aspiration step, dispense all liquid back into the wells and wait an additional 2 minutes for the bead-bound cells to bind to the magnet again before re-aspirating.

Sample Loading Table

- The Sample Loading Table should be completed before starting the experiment. Be sure to use either the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA or the Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA, since workflow requirements are different.
- 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, choose to either:
 - Decrease the "Max number barcoded cells" until the Sample Loading Table no longer gives an error, which will maintain the desired proportion between samples but barcode fewer cells.

- Add 14 μ L of undiluted sample into each designated well of the Round 1 Plate, which will result in more overall barcoded cells but change the desired proportion between samples.
- In the unlikely event that samples are too highly concentrated according to the Sample Loading Table, choose to either:
 - Standard Fixation Samples: dilute samples upstream of adding them into the Sample Dilution Plates. This can be done using the left over Cell/Nuclei Storage Buffer from Fixation.
 - For Low Input fixation, do not input more than 100,000 cells/nuclei per fixation sample because it will negatively impact the subsequent accuracy of the Evercode WT Mega 384 and Penta 384 Low Input Fixation Sample Loading Table.
- Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA only: sample concentrations used to input into the Sample Loading Table should be obtained from spot counts during fixation while samples are in Prefixation Buffer. Averaged spot count can be used to estimate the number of cells per well throughout the plate. Account for up to 10% cell/nuclei loss that can happen during freeze-thaw.

Cell Strainers

- A cell strainer with an appropriately sized mesh should be used throughout the protocol. Although 30-40 μ m is appropriate for many cell types, the mesh size should be chosen based on the sample type.
- To maximize cell retention with cell strainers, press the pipette tip directly against the mesh. Ensure ample pressure is applied to hold contact between the tip and the strainer to force liquid through in \sim 1 second. An example video can be found in our support site.

Vortexing

- Unless specified in an individual step, we strongly discourage vortexing samples or enzymes throughout the protocol.

Plate Sealing

- While 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 the PCR plate seal while applying downward pressure on the plate to keep it in the PCR tube rack.

Magnetic Racks and Bead Cleanups

- The Evercode WT v4 workflow uses magnetic beads to clean up cells/nuclei after each round of barcoding. A magnetic rack capable of holding 15 mL conical tubes and one capable of holding 1.5 mL tubes are needed throughout Section 1 of this protocol.
- Three validated 15 mL conical tube magnets are described in the "User Supplied Equipment and Reagents" section of this manual to choose from.
- The Parse Biosciences Magnetic Rack uses powerful rare earth magnets for rapid and efficient magnetic bead purification for 0.2 mL tubes. The rack has high and low magnet positions important for optimal yield at key steps. We do not recommend substituting alternative racks.
- To alternate between the positions, the rack can be flipped upside down so the magnet is closer to the top (high) or bottom (low) of the 0.2 mL tubes. See the figures below for clarification.

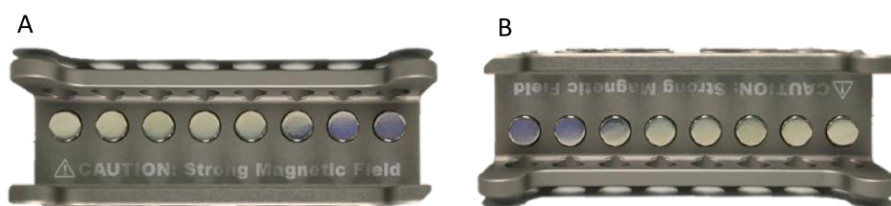


Figure 5: Parse Biosciences Magnetic Rack at (A) high and (B) low magnet positions.



Figure 6: Clear supernatant with compact bead pellets.

- The Low Input Fixation kit uses a plate magnetic rack. We validated the plate magnetic racks listed in the "User Supplied Equipment and Consumables". We do not recommend substituting alternative racks.

Sublibrary Loading

- The Evercode WT Mega 384 kit generates 16 sublibraries with distinct Illumina indexing barcodes, which can be processed and sequenced independently or concurrently after Section 1 of the protocol.

- The number of cells or nuclei per sublibrary is determined when the cells are divided into sublibraries in Section 1.5 according to the guidelines in Appendix A.
- When working with a new sample type, it can be beneficial to process a single sublibrary to optimize PCR conditions before running the remaining sublibraries.
- Sublibraries can be loaded with different numbers of cells, and the maximum number of cells that can be analyzed is the sum of cells/nuclei across all sublibraries.
- Asymmetric sublibrary loading can enable cost-effective sequencing quality control. One sublibrary can be loaded with a few hundred cells/nuclei and sequenced very deeply. This data can be used to choose an appropriate sequence depth for the remaining sublibraries.

Indexing Primers

- The UDI Plate - WT is a 96-well plate containing 48 unique dual indexing (UDI) primers. Each well is a single-use reaction sufficient for one Barcoding Round 4 PCR reaction for a single sublibrary. Thus, the UDI Plate - WT can be used for multiple Evercode Whole Transcriptome kits.
- The UDI Plate - WT is sealed with a pierceable foil to minimize cross-contamination. The plate seal should be wiped with 70% ethanol and pierced with a new pipette tip immediately prior to use. Avoid splashing or mixing the liquid between individual wells. Once a well has been used, it should not be resealed or reused.
- We recommend selecting UDIs column-wise from left to right (starting with indices 1-8). UDI sequences can be found in Appendix B.












Thermocycling Programs
















- We recommend pre-programming the thermocycling programs required for the entire workflow. A summary of these can be found in Appendix C.

Part List

The Evercode™ WT Mega 384 v4 kit includes the following boxes. Safety Data Sheets for these reagents can be provided upon request.



-20°C Reagents Store -20°C, MGT400

LABEL	ITEM	PN	FORMAT	QTY
	Round 1 Plate	MG101	Green semi-skirted 96 well plate	4
	Round 2 Plate v4	MG142	Blue semi-skirted 96 well plate	1
	Round 3 Plate	MG103	Yellow semi-skirted 96 well plate	1
	Sample Dilution Buffer	MG132	5 mL tube	1
	Bead Prep Buffer	PT136	5 mL tube	1
	Barcoding Buffer	PT137	15 mL tube	3
	Round 1 Stop Mix	PTT138	5 mL tube	1
	Ligation Enzyme	MG140	1.5 mL tube	1
	Ligation Buffer	MG134	15 mL tube	1
	Round 2 Stop Mix	MG139	2 mL tube	1
	Final Stop Mix	MG141	5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Pre-Lysis Dilution Buffer	MG112	2 mL tube	1
	Pre-Lysis Wash Buffer	MG110	5 mL tube	1
	Lysis Enzyme	MG103	1.5 mL tube	1
	Lysis Solution	MG146	1.5 mL tube	1
	Wash Buffer A	MG143	5 mL tube	1
	Wash Buffer B	MG144	5 mL tube	1
	Bead Wash Buffer	MG114	5 mL tube	1
	Binding Buffer	MG118	1.5 mL tube	1
	Template Switch Buffer	MG148	2 mL tube	1
	Template Switch Enzyme	MG121	1.5 mL tube	1
	Template Switch Primer	MG122	1.5 mL tube	1
	PCR Additive	MG150	1.5 mL tube	1
	cDNA Amp Mix	MG123	1.5 mL tube	1
	cDNA Amp Primers	MG124	1.5 mL tube	1
	Fragm/End Prep Buffer	MG125	1.5 mL tube	1

LABEL	ITEM	PN	FORMAT	QTY
	Fragm/End Prep Enzymes	MG126	1.5 mL tube	1
	Ligation Adapter	MG127	1.5 mL tube	1
	Adapter Ligation Buffer	MG128	1.5 mL tube	1
	Adapter Ligation Enzyme	MG129	1.5 mL tube	1
	Library Amp Mix	MG130	1.5 mL tube	1

4°C Reagents. Store 4°C, MGT500

LABEL	ITEM	PN	FORMAT	QTY
	Sample Binding Beads	PT135	2 mL tube	1
	Streptavidin Beads	MG201	2 mL tube	1

4°C Reagents and Stickers. Store at room temperature, MGT500

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

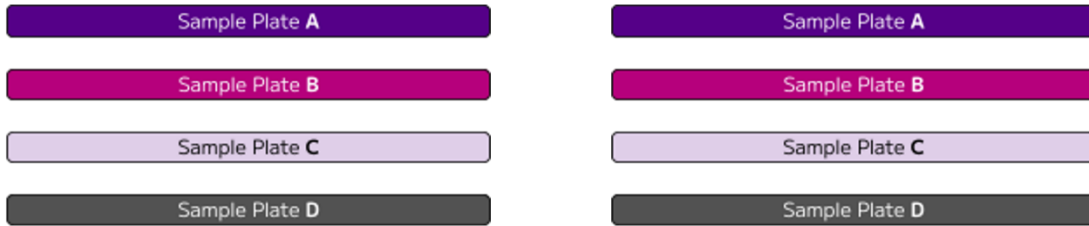
To avoid confusion while handling the plates, specifically labeled color-coded stickers are provided with the kit. When working with four fixed sample plates, we recommend labeling each as Fixation Plate A, Fixation Plate B, Fixation Plate C, and Fixation Plate D respectively.

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

Section:

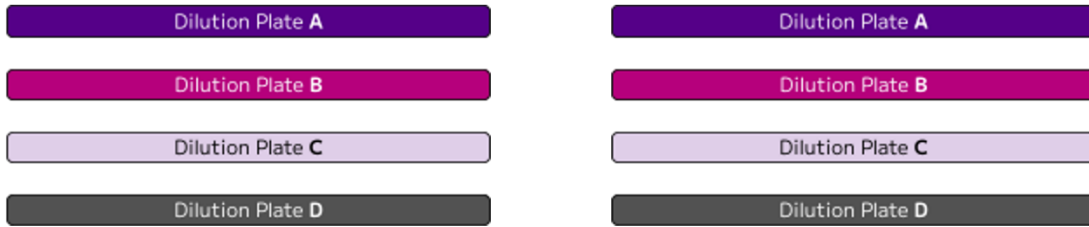
Fixed Samples:

1.1a
OR
1.1b



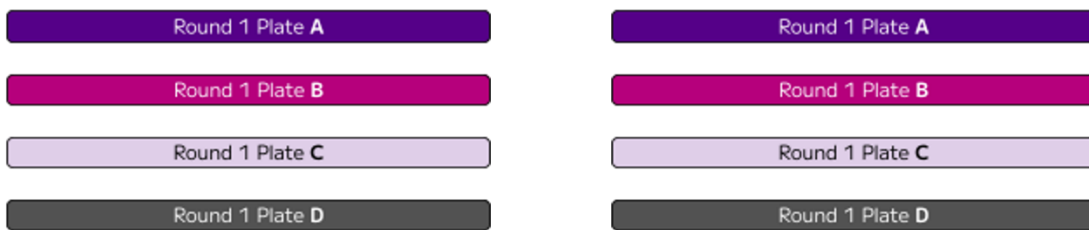
Dilution Plates:

1.1b



Round 1 Plates (MG101):

1.1a
OR
1.1b



Stickers for Tubes:

Stickers for Round 1 Pools (Section 1.2):

1.2



Stickers for Strained Pools (Section 1.3):

1.3



1.3



Top Sticker for Round 2 Plate v4 (MG142):

1.3



User Supplied Equipment and Reagents

The following materials and equipment are required to perform the protocol, but are not provided within the kit. Note that this list does not include standard laboratory equipment, such as freezers.

Equipment

ITEM	SUPPLIER	PN	NOTES
Parse Biosciences Magnetic Rack	Parse Biosciences	SB1004	We do not recommend alternative 0.2 mL PCR tube racks, as they may result in lower transcript and gene detection.
Permagen Ring Magnet	Permagen®	S500	Needed only for processing low input samples.
6-Tube Magnetic Separation Rack	New England Biolabs®	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
15 mL Conical Tube Magnet (choose one)	Permagen	MSR6X15	6 X 15 mL Centrifuge Magnetic Separation Rack.
	Millipore	LSKMAGS15	PureProteome Magnetic Stand.
	STEMCELL Technologies	18103	EasyEights™ EasySep™ Magnet.
Centrifuge with Swinging Bucket Rotors	Various Suppliers	Varies	Compatible with 96 well plates and capable of reaching 4°C.
Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL and 0.2 mL tubes.
Water bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device. We recommend validating alternatives relative to a hemocytometer.
Fluorescence Cell Counter	Various Suppliers	Varies	
PCR tube rack	Various Suppliers	Varies	Capable of holding semi-skirted 96 well PCR plates.
Plate Seal Applicator	Various Suppliers	Varies	Capable of adhering plate sealing films to 96 well plates.

ITEM	SUPPLIER	PN	NOTES
Single Channel Pipettes: P20, P200, P1000 12-channel: P20, P200	Various Suppliers	Varies	Or 8-channel pipettes can be substituted for 12-channel pipettes.
2-4 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.
Vortex-Genie 2®	Scientific Industries®	SI-0236	Or alternative vortex and adapter for 96 well plates, or a thermomixer or alternative shaker that can be set to 800-1000 RPM.
6-inch Platform	Scientific Industries	146-6005-00	
Microplate Foam Insert	Scientific Industries	504-0235-00	
Qubit™ Flex Fluorometer	Thermo Fisher Scientific®	Q33327	
2100 Bioanalyzer	Agilent®	G2939BA	Choose one.
4200 TapeStation System	Agilent	G2991BA	
Serological Pipette Controller	Various Suppliers	Varies	Optional.

Consumables

ITEM	SUPPLIER	PN	NOTES
Reagent basins	Various Suppliers	Varies	Sterile, nuclease-free, 10 mL or 25 mL polypropylene reagent basins.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning®	352097	Or equivalent 15 mL polypropylene centrifuge tubes. Do not substitute polystyrene centrifuge tubes, as it will lead to substantial cell loss.
SWiSH™ Mini Cell Strainer	Stellar Scientific®	TC70-SWM-20 TC70-SWM-40 TC70-SWM-70 TC70-SWM-100	Choose one or an equivalent sterile cell strainer with an appropriate mesh size for the cell type(s) being fixed (20 µm, 40 µm, 70 µm, 100 µm).
pluriStrainer® Mini	pluriSelect®	43-10020-40 43-10040-40 43-10070-40 43-10100-40	We do not recommend FlowMi Cell Strainers (SP Bel-Art).

ITEM	SUPPLIER	PN	NOTES
Falcon® Cell Strainer	Corning®	431750 431751 431752	
EASYstrainer™, small	Greiner Bio-One™	542120 542140 542170 542100	
DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf®	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
DNA LoBind® Tubes, 2 mL, Snap Cap	Eppendorf	022431048	Or equivalent DNA low-binding, nuclease-free 2 mL tubes.
DNA LoBind® Tubes, 5 mL, Snap Cap	Eppendorf	030108310	Or equivalent DNA low-binding, low retention, nuclease-free 5 mL tubes.
TempAssure® PCR 8-Tube Strips, 0.2 mL	USA Scientific®	1402-4700	Or equivalent nuclease-free 0.2 mL PCR tubes.
SealPlate®	Excel Scientific	100-SEAL-PLT	Or equivalent PCR plate seals.
Pipette Tips TR LTS 20 µL, 200 µL, 1000 µL	Rainin®	17014961 17014963 17014967	Or appropriate DNA low-binding, low retention, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
5 mL serological pipettes	Various Suppliers	Varies	Optional. Use DNase/RNase-free pipettes.

Reagents

ITEM	SUPPLIER	PN	NOTES
SPRIselect Reagent	Beckman Coulter®	B23317 (5mL) B23318 (60mL)	Choose one. We do not recommend substituting other magnetic beads.
AMPure® XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (60mL)	
KAPA® Pure Beads	Roche®	KK8000 (5 mL) KK8001 (30mL)	
MAGFLO NGS Size Selection	INTEGRA	7000 (1 mL) 7002 (5 mL)	
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Ethyl alcohol, Pure	Sigma-Aldrich	459844	Or equivalent ethanol.
TE Buffer pH 8.0	Various Suppliers	Varies	Optional. Choose one that is RNase-free.
Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
AO/PI	Various Suppliers	Varies	Or alternative viability dyes.
Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent DNA quantifier.
High Sensitivity DNA Kit	Agilent	5067-4626	Choose one that corresponds to the chosen Bioanalyzer or TapeStation.
High Sensitivity D5000 ScreenTape and Reagents	Agilent	5067-5592 (screen tape) 5067-5593 (sample buffer and ladder)	
High Sensitivity D1000 ScreenTape and Reagents	Agilent	5067-5584 (screen tape) 5067-5585 (sample buffer and ladder)	

Section 1: In situ Cell/Nuclei Barcoding

1.1 Sample Set Up and Barcoding Round 1

Prior to starting barcoding, fixed samples are thawed and prepared.

If the Low-Input Fixation workflow was performed, set up and prepare samples following Section 1.1a and using the "Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA".







If the Standard Fixation workflow was performed, set up and prepare samples following Section 1.1b and using the "Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA".

1.1a Set Up for Low Input Fixation Samples

Capture and barcoding of Low-Input samples requires planning to minimize sample processing time. Prior to barcoding, cells or nuclei are thawed and captured using magnetic beads. Due to the precise nature of the bead capture, sample input into Round 1 barcoding can be calculated based on known sample input prior to fixation. This is determined by the Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA. In the following protocol, the four plates with fixed samples will be labeled as A, B, C, and D. To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

To set up for barcoding:

1. Open the "Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
2. Cool a centrifuge with plate swinging bucket rotors to 4°C.
3. Fill a bucket with ice.
4. Set the thermocyclers at the temperatures needed.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 1 Plate	-20°C Reagents	4	Place directly on ice.
 Ligation Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
 Cell/  Nuclei Binding Beads	Low Input Prefixation and Binding Reagents (4°C)	4*	Gently pulse-vortex or pipette mix until completely resuspended and store at room temperature. Do not let it settle for >3 minutes before pipetting.
 Cell/Nuclei Binding Bead Wash Buffer	Low Input Fixation Reagents (-20°C)	4*	Thaw at room temperature then store on ice. Mix by inverting 3x.
 Storage Buffer	Low Input Fixation Reagents (-20°C)	8**	Thaw at room temperature then immediately store on ice. Mix by inverting. Do not vortex.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
Sticker Sheet	4°C Reagents and Stickers	1	Remove sheet and follow instructions for plate and tube labeling.

*The high throughput Low Input Fixation kit can prepare up to 96 samples. Reagents from four Low Input Fixation kits are needed to process 384 samples.

** The eight tubes come from four different Low Input Fixation kits.



Note: For every 96 samples being processed, a 1.5 mL tube is needed to resuspend and wash the beads. Therefore, up to 4 tubes would be needed to process all 384 samples.

- Gently pulse-vortex (1-2 seconds per pulse) or pipette mix the ● Cell/● Nuclei Binding Beads until completely resuspended.
- Add **1.44 mL** of ● Cell/● Nuclei Binding Beads to **four** new 1.5 mL tubes.

BINDING BEADS VOLUMES (4)		
Number of Samples	1	96
● Cell/● Nuclei Binding Beads	15 µL	1.44 mL

- Place the four tubes on the 1.5 mL tube magnetic rack until the solution clears (~ 2 minutes).
- With the tubes still on the magnetic rack, remove and discard the supernatant.
- Remove the tubes from the magnetic rack and fully resuspend each bead pellet.
- Add **1.44 mL** of ○ Cell/Nuclei Binding Bead Wash Buffer to each tube.

CELL/NUCLEI BINDING BEAD WASH BUFFER (4)		
Number of Samples	1	96
○ Cell/Nuclei Binding Bead Wash Buffer	15 µL	1.44 mL



Note: Ensure no beads are stuck to the sides of the 1.5 mL tube(s).

- Place the tubes on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).

13. With the tubes still on the magnetic rack, remove and discard the supernatant.
14. Repeat steps 11-13 twice for a total of 3 washes.
15. Remove the tubes from the magnetic rack. Fully resuspend each pellet in the appropriate volume of **○** Storage Buffer following the table below.

STORAGE BUFFER (4)		
Number of Samples	1	96
○ Storage Buffer	15 μ L	1.44 mL



CRITICAL! Do not discard the **○** Storage Buffer after this step as it is used again in the following steps.

16. Using a P200 pipette set to 110 μ L, aliquot **110 μ L** of beads resuspended in **○** Storage Buffer into each well of **four** sets of 12 PCR tubes.



CRITICAL! Ensure beads are thoroughly mixed several times before and throughout the aliquoting process.

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

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

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

19. Remove the Round 1 Plates from the centrifuge, store on ice, and label the four Round 1 Plates uniquely with "Round 1-Plate [A-D]" using the stickers provided.



Note: To minimize the processing time, we recommend capturing, loading and initiating Round 1 barcoding reactions for plates A and B first, then repeating the process for plates C and D.

20. Remove **two** of the four plates of fixed cells/nuclei from -80°C storage. Set the thermocycler to the following protocol and thaw cells/nuclei:

THAW CELLS/NUCLEI (2)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

21. To thaw, place the plate of frozen cells/nuclei in the thermocycler and start the program.
22. Once the thaw protocol has finished, check that all wells are fully thawed. If ice remains, place the plate back into the thermocycler for another minute at 37°C. Once fully thawed, place the plate into a PCR plate holder, and store on ice.
23. Label the plates with the corresponding adhesive labels provided in the kit, following designations as filled out in the Sample Loading Table (i.e. "Sample Plate-[A-D]").

24. Use the table below as guidance for loading the binding beads into each sample starting with "Sample Plate A". See the Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA for a visual diagram of loading the appropriate amount of beads to each sample.

CELL/NUCLEI BINDING BEADS ADDITION	
Cell/Nuclei Input Number	Cell/Nuclei Binding beads (μL)
10,000-49,999	4
50,000-74,999	6
75,000-89,999	9
90,000-100,000	12



Note: Be sure to mix beads by pipetting thoroughly to fully resuspend before using them for cell/nuclei capture.

25. With a P200 multichannel pipette set to 120 μL , pipette 3x to ensure beads are fully suspended in cell/nuclei samples, switching tips. The beads will begin binding to the cells/nuclei almost immediately.

26. Place the plate on a plate magnet and bind at room temperature for **5 minutes**.

27. During the bind step, add **20 mL** of **O** Storage Buffer to a clean basin (refilling with more volume as needed). The Storage Buffer will be used in a later step.

28. With the plate still on the magnet, remove the supernatant from the wells, without disturbing the pellet with a multichannel P200 set to 200 μL and switching tips between columns.



CRITICAL! Move quickly to resuspend beads at this step to minimize time beads are out of liquid (<5 minutes).

29. Remove the plate from the magnetic rack and place it on ice.

30. Keep plate on ice while vigorously resuspending cells/nuclei in the **O** Storage Buffer with a P200 multichannel pipette to ensure no pellets remain. Switch tips between wells. Refer to the Sample Loading Table to determine **O** Storage Buffer volumes (ranges from 20-100 μL).



CRITICAL! Resuspending the beads in less than the volume required for resuspension will harm the barcoding chemistry and result in a loss of transcript detection.

31. Repeat steps 24-30 for the second sample plate ("Sample Plate B").
32. Before loading the first and second sample plates (A and B) into the corresponding Round 1 Plate, remove the third and fourth sample plates (C and D) from the -80°C storage and thaw according to the following protocol. Once the program has completed, store plates on ice and label plates with corresponding adhesive labels.

THAW CELLS/NUCLEI (2)		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	200 µL*	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

* Or maximum volume allowed by the thermocycler.

33. Remove the plastic seal from a thawed Round 1 Plate (labeled "Round 1-Plate A").



Note: PCR plate seals may be difficult to remove. Carefully peel the seal while applying downward pressure to keep the plate from moving to minimize cross-contamination.

34. With the Round 1 Plate on ice, add **14 µL** of "Sample Plate A" to the appropriate wells of "Round 1 Plate A" as defined in the Sample Loading Table. Mix immediately after dispensing each sample by pipetting 3x.



CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells into a sample or different well.



Note: Refer to the "Evercode WT Mega384 Penta384 Low Input Fixation v4 Sample Loading Table RevA" Plate Configuration tab for visual reference on how to load samples into Round 1 plates.

35. While secured in a PCR plate holder on a flat surface, add a new plate seal and place back on ice.
36. Repeat steps 33-35 for "Sample Plate-B" with "Round 1 Plate B".

37. Place both Round 1 Plates (A and B) into thermocyclers and run the program below for both plates using two separate thermocyclers. Once the program has been started, proceed immediately to the next step.

BARCODING ROUND 1 (2)			
Run Time	40 minutes		
Lid Temperature	70°C		
Sample Volume	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	1

38. Repeat steps 20-37 to load third and fourth sample plates (C and D) with the corresponding Round 1 Plates (C and D).
39. Once the Round 1 Plates A and B have finished the "Barcoding Round 1" program, place them on ice.
40. When there are **10 minutes** remaining in "Barcoding Round 1" for Round 1 Plates C and D, proceed with Section 1.2, individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B ([Section 1.2](#)).

1.1b Set Up for Standard Fixation Samples

Prior to barcoding, users are expected to count cells or nuclei using a counting plate in order to ascertain the concentration of all 384 samples. This will allow for using variable Sample Dilution Buffer volumes to dilute with a constant volume of fixed samples. This will streamline the loading process into the Round 1 Plate and minimize dedicated processing time. In the following protocol, we will designate the four plates of fixed samples as A, B, C and D. Appropriate dilutions, loading concentrations, and loading positions are determined by the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA. Dilution plates may be made up to 48 hours in advance of barcoding.

Dilution plate set up:


1. Prepare an automated counter, hemocytometer, flow cytometer, or other plate-based counting device.
2. Using a counting plate aliquot of fixed samples, count the number of cells/nuclei in the sample with an automated cell counter or alternative cell counting device. Record the cell/nuclei count.
3. Fill out the "Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
4. Fill a bucket with ice and cool a centrifuge with plate swinging bucket rotors to 4°C.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Sample Dilution Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
Sticker Sheet	4°C Reagents	1	Remove sheet and follow instructions for plate and tube labeling.

6. Label four (user-provided) PCR Plates uniquely with stickers "Dilution Plate [A-D]" provided in the 4°C Reagents and Stickers box.
7. Create the corresponding dilution plates of ○ Sample Dilution Buffer using the Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA.



Note: If there are errors in the Sample Loading Table, see the Important Guidelines section for suggestions.

 **Safe Stopping Point:** Dilution plates can be made in advance, sealed, and frozen at -20°C for 48 hours prior to barcoding.

Barcoding plate set up:

8. Open the "Evercode WT Mega384 Penta384 Standard Fixation v4 Sample Loading Table RevA", which will guide sample dilutions and plate loading in later steps.
9. Fill a bucket with ice.
10. Have two or four thermocyclers on standby.



Note: If only 2 thermocyclers are available, process Plates A and B first, keeping Plates C and D on ice.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
<input type="checkbox"/> Round 1 Plate	-20°C Reagents	4	Place directly on ice.
<input type="radio"/> Round 1 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
<input type="radio"/> Ligation Buffer	-20°C Reagents	1	
<input checked="" type="radio"/> Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

12. If the Dilution Plates are stored at -20°C, thaw the four pre-prepared Dilution Plates in the thermocyclers according to the following protocol. Then gently remove the Dilution Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.

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

13. Place the Dilution Plates on ice, and label with corresponding adhesive labels, if not done previously.
14. Thaw the four Round 1 Plates in the thermocyclers according to the following protocol:

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

15. Gently remove the Round 1 Plates from the thermocyclers, place in 0.2 mL tube racks, and centrifuge for **1 minute** at 100 x g at 4°C.
16. Remove the Round 1 Plates from the centrifuge, and store on ice.
17. Label Round 1 Plates with a sticker “Round 1 Plate [A-D]” sticker provided, taking care that each plate is associated with a unique letter from A-D.



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

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

THAW CELLS/NUCLEI		
Run Time	3 minutes	
Lid Temperature	40°C	
Sample Volume	100 µL	
Step	Time	Temperature
1	3 min	37°C
2	Hold	4°C

19. Gently remove the sample plate from the thermocycler and place on ice. Label sample plate with "Sample Plate A" sticker provided.



Note: At this point, there will be 9 plates on ice: four Dilution Plates, four Round 1 Plates, and the Sample Plate A. Be sure to have each plate labeled for traceability using the provided sticker sheet. To avoid confusion, when not in use, Dilution Plates and Round 1 Plates can be stored in the fridge at 4°C.

20. Using a multichannel P200 set to 40 μL , mix cells/nuclei 3x in the Sample Plate A, Row A. Then, using a multichannel P20 set to 14 μL , transfer **14 μL** from Sample Plate A, Row A into Dilution Plate A, Row A.

21. Mix 3x with the same pipette tips to ensure even mixing, immediately transfer **14 μL** of the diluted cells/nuclei into the corresponding wells of the Round 1 Plate A, Row A and mix again 3x. Example of the workflow is shown in the diagram below. See Sample Loading Table plate configuration tab for sample location.

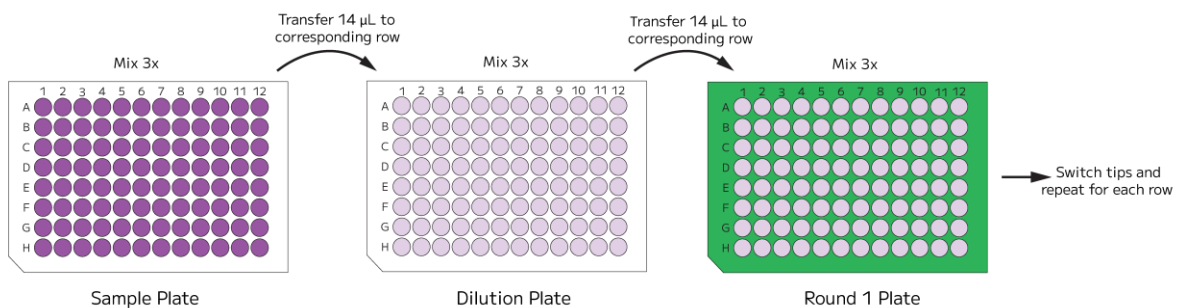


Figure 7: Samples in the sample plate are diluted and then transferred to the Round 1 Plate.

22. Repeat the process for the rest of rows B-H until all samples from Sample Plate A are diluted and transferred into Round 1 Plate A, switching tips after every transfer.



CRITICAL! Do not reuse any tips that have entered the Round 1 Plate wells. Do not reuse tips across non-correlating rows.

23. Place the Round 1 Plate A into the thermocycler and run the program below.

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

24. Repeat steps 18-23 for Plates B, C, and D labeling the plates accordingly.

25. When there are **10 minutes** remaining in "Barcoding Round 1" for Round 1 Plates C and D, proceed with Section 1.2, individually pooling Round 1 Plate A into Pool A and Round 1 Plate B into Pool B (Section 1.2).



Note: If only 2 thermocyclers are available, store the completed Plates A and B on ice, while Plates C and D undergo Barcoding Round 1.

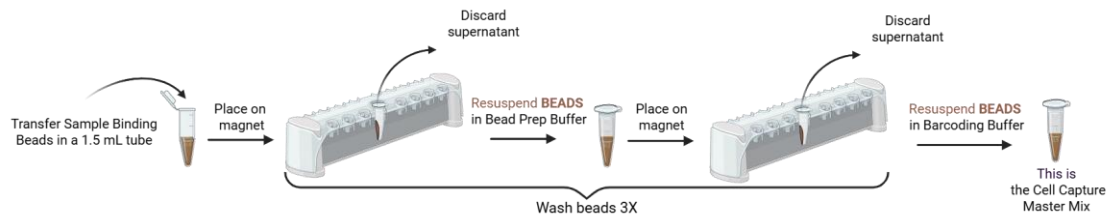
1.2. Pooling Round 1 Plates

After the Round 1 barcoding, the reverse transcription reaction is stopped, cells/nuclei are pooled into four Pools, and then cell/nuclei are resuspended.

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
○ Bead Prep Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Round 1 Stop Mix	-20°C Reagents	1	
● Ligation Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
○ Barcoding Buffer	-20°C Reagents	1	Thaw in 37°C water bath, then store on ice.
● Sample Binding Beads	4°C Reagents	1	Keep at room temperature. Ensure beads are not settled before use.

2. **If using low input samples**, the cells/nuclei are already attached to the binding beads and don't need further washing. **Proceed directly to step 12.**
3. **If using standard fixation samples**, proceed to the next step to prepare the ● Sample Binding Beads to capture the cells/nuclei. See workflow below.



Cell Capture Master Mix: Sample Binding Beads are washed three times, then Barcoding Buffer is added.

4. While the thermocycling program is still running, gently pulse-vortex ● Sample Binding Beads to resuspend and store at room temperature. Do not let them settle for >3 minutes before pipetting. Transfer **800 μ L** of ● Sample Binding Beads into two 1.5 mL Eppendorf tubes (for a total of **1,600 μ L** of ● Sample Binding Beads used).
5. Place the tubes on the magnetic rack compatible with 1.5 mL tubes and wait for the solution to clear (~2 minutes).

6. While still on the magnet, remove and discard the supernatants.
7. Remove the tubes from the magnetic rack and fully resuspend each bead pellet in **800 μ L** of **○ Bead Prep Buffer**.
8. Place the tube on the magnetic rack until the solution clears (~2 minutes).
9. While still on the magnet, remove and discard the supernatant.
10. Repeat steps 7-9 twice for a total of 3 washes.
11. Remove the tubes from the magnetic rack. Fully resuspend each pellet in **800 μ L** of **○ Barcoding Buffer** and store on ice. This is your Cell Capture Master Mix (for a total of 1,600 μ L of resuspended beads).



Note: Save the Barcoding Buffer as it will be used later in this section.

12. Proceed to make the Ligation Master Mix for both Round 2 and Round 3 Ligation in a new tube:

LIGATION MASTER MIX	
○ Ligation Buffer	9.8 mL
● Ligation Enzyme	44 μ L

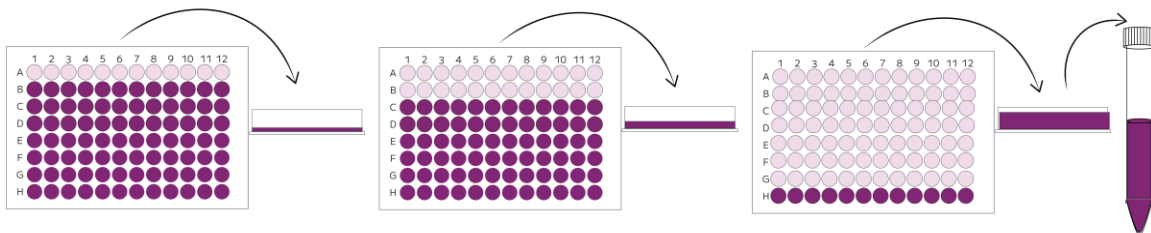
13. Mix the Ligation Master Mix by inverting the tube 10x. Store on ice.



CRITICAL! **4 mL** will be used for the Round 2 ligation barcoding step, and later **4 mL** will be used for the Round 3 ligation barcoding step.

14. Remove the Round 1 Plate A from the thermocycler, place it in a PCR tube rack on the benchtop. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 1 Plate A on ice.
15. Transfer all the **○ Round 1 Stop Mix** into a new basin with a P1000.
16. With a 12-channel multi-pipette transfer **10 μ L** of **○ Round 1 Stop Mix** into Row A of the Round 1 Plate A, still keeping the plate on ice.
17. Switching tips between each row, repeat step 16, **7 more times** for Rows B-H to dispense the **○ Round 1 Stop Mix** into all wells of the Round 1 Plate A.

18. Switch tips between plates and repeat steps 16-17 with Plates B, C, and D to add the **○ Round 1 Stop Mix** to all samples. Keep plates on ice.
19. Label four 15 mL conical tubes with the provided stickers as Pool A, Pool B, Pool C, and Pool D.
20. Start pooling the samples from Round 1 Plate A **into Pool A tube** as follows:
 - a. With a multichannel P200 set to 50 μ L, mix the sample in row A by pipetting 3x.
 - b. Transfer **50 μ L** from **row A** into a clean basin (bubbles will be generated, this will not impact experimental results).
 - c. Repeat steps b-c for **rows B-H**.
 - d. Once cells from all wells are in the basin, using a P1000 transfer the mix into a new 15 mL conical tube. Keep on ice.
 - e. Repeat steps a-d with the remaining three Round 1 Plates B-D to generate four separate pools of cells/nuclei: Pool A, Pool B, Pool C, and Pool D.



Round 1 Plate [A-D] pooling. Pool Round 1 Plate A sample into Tube A, store on ice, then proceed with [B-D].

21. **If using low input fixation samples**, cells/nuclei have been already captured. Place the 15 mL conical tube **magnet on ice** and wait **1 minute** to let it pre-cool. **Proceed directly to step 27.**
22. **If using standard fixation samples**, proceed to the next step to capture the cells/nuclei.
23. Resuspend any settled beads in the Cell Capture Master Mix from step 12 by pipette mixing.
24. Using a P1000, transfer **384 μ L** of the Cell Capture Master Mix into each Pool [A-D] tube containing the pooled Round 1 cells/nuclei. Mix each pool 10x with the P1000 set to 900 μ L. Switch pipette tips between each Pool.

25. Incubate the cells/nuclei on ice for **5 minutes** to allow beads to bind.



CRITICAL! Let beads incubate for the **entire 5 minutes**.

26. While cells are incubating, place the magnet on ice to pre-cool.



Note: In the next step, if the magnet cannot accommodate all four Pools simultaneously, **process one or two Pools at a time, keeping the others on ice**.

27. Remove or loosen the cap, then place the Pool tubes on the 15 mL conical tube magnet to allow magnetic beads to bind to the magnet. If the cap is adjusted afterward, the rotational motion may cause the pellet to shift. Keep magnet on ice the entire time, covering the tube in ice up to the liquid level to keep the tube cold. Allow beads to bind to the magnet for **10 minutes**.

28. With a P1000 set to 1000 μ L or a 5 mL serological pipette and without touching the beads, hold the tube steady and remove and discard the supernatant while the 15 mL Pool tube is still on the magnet. Use a P1000 to remove any excess liquids.

29. With the tube still on the magnet, add **5 mL** of **O Barcoding Buffer**. Do not resuspend. Incubate on ice for **1 minute** before removing and discarding the supernatant with a P1000 or serological pipette.

30. Remove tube from magnet and resuspend the beads-bound cells/nuclei in **1.2 mL** of Ligation Master Mix. Using a P1000 set to 1000 μ L, pipette mix 10x to fully resuspend the beads into the Ligation Master Mix. Keep the sample on ice before proceeding to the next step.

31. If processing one or two Pools at the time, repeat steps 27-30 with the remaining Pools. Then proceed to Section 1.3.



CRITICAL! Do not throw away Ligation Master Mix after use, keep it on ice: the remainder will be used in Section 1.3.

1.3. Barcoding Round 2

The four Pools of beads-bound cells/nuclei are loaded **column-wise** into the Round 2 Plate. An *in situ* ligation reaction adds a well-specific barcode to the 5' end of the cDNA. The ligation reaction is quenched with Round 2 Stop Mix, and the cells/nuclei are pooled and strained.

To add Round 2 barcodes:

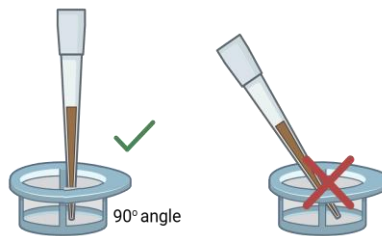
1. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 2 Plate v4	-20°C Reagents	1	Place directly on ice.
 Round 2 Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.

2. Label four new 1.5 mL tubes with the provided stickers.
3. Pipette the entire content of each Pool sample in Ligation Master Mix (**1.2 mL** each Pool) through a cell strainer into the corresponding 1.5 mL tube with a P1000.



CRITICAL! To ensure that all of the liquid passes through the strainer, keep the tip or the pipette straight up and against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.



4. Label four 8-tubes PCR strips with the provided stickers. You can either label only the first tube of each strip or all 8 tubes in the strip.
5. Using a P200 single-channel, aliquot **139 µL** of the resuspended pool (from Pool A) into 8 labeled PCR tubes (strip format), mixing between each transfer step.
6. Repeat previous step for the remaining three resuspended pools corresponding to Pools B-D, changing tips between each pool.

7. Place the Round 2 Plate into a thermocycler and run the following program. Proceed immediately to the next step while the program is still running.

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

8. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.
9. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 2 Plate and apply the provided appropriate adhesive labels to assist with loading. Store on ice.
10. With the labeled Round 2 Plate on ice and PCR strip tubes on ice, transfer the cells in Ligation Master Mix to each well in the Round 2 Plate as follows:
 - a. Mix Pool A in the PCR strip tube by pipetting 2x with a multichannel P200 set to 40 µL.
 - b. Transfer **40 µL** of the mix to column 1 of the Round 2 Plate and mix by pipetting 2x.
 - c. Switching tips between each column, repeat steps a-b to mix Pool A in the basin then transfer to columns 2-3.
 - d. Repeat steps a-c to add the remaining 3 samples to columns 4-6, 7-9, and 10-12, respectively.



CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.

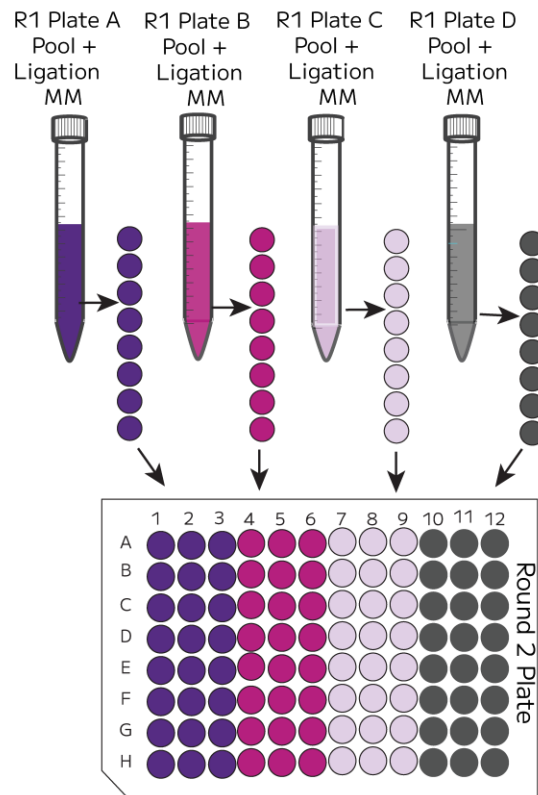


Figure 8: After resuspending the four Round 1 pools with Ligation Master Mix, each pool is strained and aliquoted across **four** 8-tubes PCR strips respectively. Each pool is then loaded into three columns of the Round 2 Plate.



Note: If when loading column 3, 6, 9, and 12 (last column loaded for each pool), the volume remaining is less than 40 μ L, the wells can be partially loaded without impacting experimental results.

11. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 2 Plate.

12. Place the Round 2 Plate into a thermocycler and run the following program. Upon completion, proceed immediately to the next step.

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

13. Briefly vortex the ● Round 2 Stop Mix and ensure there is no precipitate. Transfer **1 mL** of this tube to a new basin with a P1000.
14. Remove the Round 2 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
15. With the Round 2 Plate on ice and the basin on the bench, transfer the contents of the Round 2 Barcoding plate into the ● Round 2 Stop Mix as follows:
 - a. With a multichannel P200 set to 50 µL, mix the sample in row A by pipetting 3x.
 - b. Transfer the entire content (**50 µL**) from row A to the basin containing ● Round 2 Stop Mix.
 - c. Repeat steps a-b for rows **B-H** to mix the sample then transfer to the basin. The same pipette tips may be used while pooling.
16. Transfer the Round 2 pool into a 15 mL conical tube and apply to the magnet. Loosen or remove the cap to avoid a rotational motion may cause the pellet to shift. Keep the magnet on ice.
17. Allow beads to bind to the magnet for **5 minutes**, or until supernatant is clear.
18. With a P1000 set to 1000 µL or a 5 mL serological pipette and without touching the pellet, remove the supernatant while the 15 mL tube is still on the magnet. Use a P1000 to remove any excess liquids.







19. Keep the 15 mL conical tube on the magnet on ice and with a P1000 set to 1000 μ L or a 5 mL serological pipette, add **5 mL** of the **O** Barcoding Buffer to rinse the pellet. Do not resuspend. Incubate on ice for **1 minute** to ensure all beads are bound.
20. Remove and discard the supernatant with a P1000 set to 1000 μ L or a 5 mL serological pipette.
21. Remove tube from magnet and resuspend the beads with **4.2 mL** of the Ligation Master Mix and mix the full volume with the P1000. Store the sample on ice and proceed to section 1.4.

1.4. Barcoding Round 3

The cells are strained and loaded into the Round 3 Plate. A second in situ ligation reaction adds a third well-specific barcode, the Illumina Truseq Read 2 sequence, and a biotin. The ligation reaction is stopped, and the sample is then pooled and strained.

To add round 3 barcodes:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
 Round 3 Plate	-20°C Reagents	1	Place directly on ice.
 Final Stop Mix	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by vortexing.
 Pre-Lysis Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
 Pre-Lysis Dilution Buffer	-20°C Reagents	1	
 Lysis Enzyme	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.
 Lysis Solution	-20°C Reagents	1	Thaw and store at room temperature.

2. Place the Round 3 Plate into a thermocycler and run the following program. Proceed immediately to the next step while the program is still running.

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

3. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, and centrifuge for **1 minute** at 100 x g at 4°C.
4. While secured in a PCR tube rack on a flat surface, remove the plate seal from the Round 3 Plate.

5. Pipette all of the sample in Ligation Master Mix (**4.2 mL**) through a cell strainer into a new basin with a P1000.



CRITICAL! To ensure that all of the liquid passes through the strainer, keep the tip of the pipette straight up and against the mesh to create a tight seal and press the pipette plunger down steadily. All the liquid should pass through the strainer in ~1 second.

6. With the Round 3 Plate on ice and the basin on the bench, transfer the cells in Ligation Master Mix to each well in the Round 3 Plate as follows:
 - a. Mix the sample in the basin by pipetting 2x with a multichannel P200 set to 40 μL .
 - b. Transfer **40 μL** of the mix to row **A** of the Round 3 Plate and mix by pipetting 2x.
 - c. Changing tips for each row, repeat steps a-b to mix the sample in the basin then transfer to rows **B-H**.



CRITICAL! Do not reuse any tips during this step. Different tips must be used when pipetting cells into each well of the plate. Never place a tip that has entered one of the wells back into the basin or a different well.



Note: If the volume is insufficient to transfer the last row with a multichannel, tilt the basin and transfer the remaining volume with a single channel pipette. If the volume is still insufficient to fill every well, a few can be left empty without impacting experimental results.

7. While secured in a PCR tube rack on a flat surface, add a new plate seal to Round 3 Plate.
8. Place the Round 3 Plate into a thermocycler and run the following program.

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

9. Briefly vortex the ○ Final Stop Mix and ensure there is no precipitate. Transfer **2 mL** to a new basin with a P1000.
10. Remove the Round 3 Plate from the thermocycler, place in a PCR tube rack, remove the plate seal, and store on ice.
11. With the Round 3 Plate on ice and the basin on the bench, transfer all the liquid in the Round 3 Plate into the basin with the ○ Final Stop Mix as follows:
 - a. With a multichannel P200 set to 50 μ L, mix the sample in row A by pipetting 3x.
 - b. Transfer **50 μ L** from row **A** to the basin and mix 2x.
 - c. Repeat steps a-b for rows **B-H** to mix the sample then transfer to the basin.
12. Pipette the pooled sample through a cell strainer into a new 15 mL tube with a P1000. Before each transfer, gently mix the cells in the basin by pipetting 2x and tilt the basin to recover as much liquid as possible.
13. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind to the magnet for **5 minutes**, or until supernatant is clear.
14. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 μ L.
15. Remove the 15 mL tube from the magnet and fully resuspend the cells/nuclei in **4 mL** of ○ **Pre-Lysis Wash Buffer**. Do not invert.
16. Place the 15 mL tube on a magnet on ice and cover the tube in ice up to the liquid level. Allow beads to bind for **5 minutes**, until supernatant is clear.
17. With the 15 mL tube still on the magnet, remove the supernatant with a P1000 set to 1000 μ L or serological pipette.
18. Remove the tube from the magnet and resuspend the cells in **200 μ L** of ● Pre-Lysis Dilution Buffer.



Note: Do not discard ● Pre-Lysis Dilution Buffer as it will be used in another step.

19. Proceed to Lysis and Sublibrary Generation Step 1.5.

1.5. Lysis and Sublibrary Generation

The cell/nuclei pool resuspended in Pre-Lysis Dilution Buffer is counted and divided into sublibraries based on the Sublibrary Generation Table in Appendix A. These sublibraries are lysed, transferred into new tubes, and stored at -80°C.

1. While minimizing time on ice, count the number of cells/nuclei in the sample with a fluorescent (DAPI) cell counting device. Record the cell/nuclei count.



CRITICAL! We strongly recommend carefully mixing the sample before collecting an aliquot for counting to ensure accurate sublibrary loading.

2. Decide how to divide cells/nuclei across the sublibraries. See the “Sublibrary Loading” in the Important Guidelines section for strategies. Use the Sublibrary Generation Table in Appendix A to determine the volume of sample and ● Pre-Lysis Dilution Buffer that should be added to each sublibrary.



CRITICAL! Do not add more than 62,500 cells/nuclei to a sublibrary. Adding additional cells/nuclei will result in an increased multiplet rate.

3. Ensure the cells/nuclei are in suspension by pipetting 5x with a P200 set to 200 μL prior to each transfer. Add the appropriate volume of sample to 16 different 0.2 mL PCR tubes.
4. Keeping the samples on ice, add the appropriate volume of ● Pre-Lysis Dilution Buffer to the 0.2 mL tubes for a total volume of **25 μL** .
5. Prepare the Lysis Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 3x with a P1000 set to 425 μL . Store at room temperature.

LYSIS MASTER MIX	
● Lysis Solution	440 μL
● Lysis Enzyme	88 μL
Total	528 μL



Note: Ensure that there is no precipitate before using the ● Lysis Solution.



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

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



7. Vortex the 0.2 mL tube(s) for **1 minute**. Briefly centrifuge.
8. Place the tube(s) into a thermocycler and run the following program. If continuing to Section 2 without freezing the sample, proceed to Section 2 while the program is still running.

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

9. Remove samples from the thermocycler and place the tubes on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
10. While still on the magnetic rack, transfer **55 μ L** of the **supernatant** containing lysed cells into new 0.2 mL tubes.



CRITICAL! Do NOT discard the supernatant as it contains the lysed cells/nuclei.

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



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

Section 2: cDNA Capture and Amplification

2.1. cDNA Capture

The barcoded cDNA is captured with streptavidin-coated magnetic beads and washed to remove cellular debris.



CRITICAL! Always use new tips for each sublibrary.

To capture the cDNA:

1. Fill an ice bucket.
2. Gather the following equipment.
 - a. Magnetic rack for 1.5 mL tubes
 - b. Parse Biosciences magnetic rack for 0.2 mL PCR tubes
 - c. Vortex with an adapter for 96 well plates
3. Gather the following items and handle as indicated below:

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Streptavidin Beads	4°C Reagents	1	Keep at room temperature.
○ Bead Wash Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.
○ Wash Buffer A	-20°C Reagents	1	
○ Wash Buffer B	-20°C Reagents	1	
● Binding Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Mix by inverting 3x.

4. Vortex ● Streptavidin Beads until fully mixed. Add the appropriate volume of ● Streptavidin Beads to a new 1.5 mL tube as follows, depending on the number of lysates being processed:

Lysates being processed	1	16
● Streptavidin Beads	66 µL	1056 µL

5. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).
6. Remove and discard the supernatant.
7. Remove the tube from the magnetic rack and fully resuspend the bead pellet in the appropriate volume of ○ Bead Wash Buffer as follows:

Lysates being processed	1	16 μ L
○ Bead Wash Buffer	50 μ L	800 μ L



Note: Ensure no beads are stuck to the sides of the 1.5 mL tube.

8. Place the tube on the magnetic rack for 1.5 mL tubes until the solution clears (~2 minutes).
9. Remove and discard the supernatant.
10. Repeat steps 7-9 twice for a total of 3 washes.
11. Remove the tube from the magnetic rack. Fully resuspend the pellet in the appropriate volume of ● Binding Buffer as follows and store at room temperature.

Lysates being processed	1	16
● Binding Buffer	55 μ L	880 μ L

12. Remove the desired tube(s) of lysate from the thermocycler (if continuing directly from Section 1) or from storage at -80°C.

13. If previously frozen, incubate the tube(s) in water bath or thermocycler at 37°C for **5 minutes**.

THAW LYSATES		
Run Time	5 minutes	
Lid Temperature	40°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C



Note: Ensure there is no precipitation before proceeding. Otherwise, an additional 5 minutes at 37°C should be sufficient.

14. Briefly centrifuge the lysates and store at room temperature.
15. Add **50 µL** of ● Streptavidin Beads in ● Binding Buffer to each tube of lysate.
16. Place the tube(s) into a 96 well PCR tube rack, press to secure, and ensure the caps are secured tightly. Place the lid on the rack.
17. Place the rack onto a vortex mixer with a plate adapter. Push to secure. Vortex plastic holder on 100% power for **1 minute**.
18. Vortex on 20% power (~800-1000 RPM) for **30 minutes** at room temperature.



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

19. Remove the tube(s) from the vortex mixer.
20. Briefly vortex the tube(s) on a standard vortex adapter. Briefly centrifuge without letting beads collect at the bottom of the tube(s).

21. Place the tube(s) on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).



CRITICAL! Ensure the supernatant is completely clear before proceeding. Discarding any beads in the supernatant will result in a reduction of transcripts and genes detected per cell. See "Magnetic Racks and Bead Cleanups" in the Important Guidelines section for representative images.

22. While still on the magnetic rack, remove and discard the supernatant.

23. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 μ L \emptyset Wash Buffer A** by vortexing for **10 seconds** (100% power).



Note: Save \emptyset Wash Buffer A to use for optional storage before cDNA amplification.

24. Incubate for **1 minute** at room temperature.

25. Proceed immediately to Section 2.2.

2.2. cDNA Template Switch

After an additional wash, the Template Switch Master Mix is added to the captured cDNA. The template switch reaction adds a 5' adapter to the cDNA.



CRITICAL! Always use new tips for each sublibrary.

To perform template switch:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Template Switch Buffer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting 5x . Briefly centrifuge before use.
● Template Switch Primer	-20°C Reagents	1	Thaw at room temperature then place on ice. Mix by pipetting 3x . Briefly centrifuge before use.
● Template Switch Enzyme	-20°C Reagents	1	Keep on ice. Briefly centrifuge before use.



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

2. Prepare the Template Switch Master Mix in a new 2 mL tube as follows, depending on the number of sublibraries being processed. Mix by pipetting 10x and store on ice.

TEMPLATE SWITCH MASTER MIX		
Number of Samples	1	16
● Template Switch Buffer	87 µL	1392 µL
● Template Switch Primer	3 µL	48 µL
● Template Switch Enzyme	6 µL	96 µL
Total	96 µL	1536 µL

3. Place each tube of captured cDNA from Section 2.1 on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).

4. While still on the magnetic rack, remove and discard the supernatant.
5. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 μL O Wash Buffer B** by vortexing for **10 seconds** (100% power).



CRITICAL! Do not discard the O Wash Buffer B as it will be used in another step.

6. Place the tube(s) on the high magnet position of the Parse Biosciences Magnetic Rack, so the magnet is closer to the top of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the **O Wash Buffer B**.
8. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 μL** nuclease-free water by vortexing for **10 seconds**, followed by a brief centrifugation.
9. Add **80 μL** of the Template Switch Master Mix to each resuspended pellet and mix 5x with a P200 set to 80 μL .



Note: Because the Template Switch Master Mix is viscous, it may take time to fully mix the beads in solution.

10. Briefly centrifuge without letting beads collect at the bottom of the tube(s).
11. Incubate for **30 minutes** at room temperature.
12. Fully resuspend each bead pellet by mixing 5x with a P200 set to 75 μL .
13. Place the tube(s) into a thermocycler and run the following program.

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

14. Proceed immediately to Section 2.3. Alternatively, proceed to step 15 to store samples prior to cDNA amplification.
15. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).



Note: Beads may need to be resuspended if they have settled prior to placing on the magnet.

16. While still on the magnetic rack, remove and discard the supernatant.
17. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 µL** Wash Buffer A by vortexing for **10 seconds** (100% power).



Safe stopping point: Template switched cDNA can be stored in Wash Buffer A at 4°C for up to 18 hours. Do not freeze.

2.3. cDNA Amplification

The captured cDNA is washed and amplified with Template Switch Primer and Illumina Truseq Read 2 specific primers.



CRITICAL! Always use new tips for each sublibrary.

To amplify the cDNA:

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

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	
● PCR Additive	-20°C Reagents	1	

2. Equilibrate 80 μL of SPRI beads per lysate to room temperature. Immediately proceed to step 3 after placing SPRI beads at room temperature. These will be used later.
3. Prepare the cDNA Amplification Master Mix in a new 2 mL tube as follows. Mix by pipetting 10x and store on ice.

cDNA AMPLIFICATION MIX		
Number of Sublibraries	1	16
● cDNA Amp Mix	60 μL	960 μL
● cDNA Amp Primers	57.5 μL	920 μL
● PCR Additive	2.5 μL	40 μL
Total	120 μL	1920 μL

4. Place each tube of template switched cDNA from Section 2.2 on the high position of the Parse Biosciences Magnetic Rack. Incubate until the solution clears (~2 minutes).



Note: You may need to pipette mix to resuspend settled beads so they separate appropriately prior to placing them on the magnet.

5. While still on the magnetic rack, remove and discard the supernatant.
6. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **125 μ L O Wash Buffer B** by vortexing for **10 seconds** (100% power).
7. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes)
8. While still on the magnetic rack, remove and discard the O Wash Buffer B.
9. Remove tube(s) from the magnetic rack. Fully resuspend each bead pellet with **100 μ L** of the Amplification Master Mix. Store on ice.
10. 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 Sublibrary	High RNA content such as cell lines	Low RNA content such as PBMCs	Nuclei
200-1,000	11	13	12
1,000-2,000	9	11	10
2,000-6,000	7	9	8
6,000-12,500	6	8	7
12,500-25,000	4	6	5
25,000-62,500	3	5	4

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

cDNA AMPLIFICATION			
Run Time		50-70 minutes	
Lid Temperature		105°C	
Sample Volume		100 µL	
Step	Time	Temperature	Cycle
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.



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

2.4. Post-Amplification Purification

Amplified cDNA is purified with a single sided SPRI bead cleanup.



CRITICAL! Always use new tips for each sublibrary.

To purify the cDNA:

1. Gather **400 μL** of freshly prepared 85% ethanol for each tube of amplified cDNA.
2. Gather room temperature SPRI beads (**80 μL** per tube of amplified cDNA).



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

3. Place each tube of amplified cDNA from Section 2.3 on the high position of the Parse Biosciences Magnetic Rack. Incubate until the solution clears (~2 minutes).



Note: If beads remain in solution after 2-3 minutes, remove from the magnet and pipette 3x in the bottom of the PCR tube with a P200 set to 40 μL . Then return to the magnet and incubate until the solution clears.

4. While still on the magnetic rack, transfer **90 μL** of the supernatant containing the cDNA into a new 0.2 mL tube(s). Store at room temperature.
5. Vortex the SPRI beads until fully mixed. Add **72 μL** of SPRI beads to each tube with amplified cDNA.
6. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
7. Incubate for **5 minutes** at room temperature.
8. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
9. While still on the magnetic rack, remove and discard the supernatant.
10. While still on the magnetic rack, add **180 μL** of 85% ethanol to each tube.
11. Incubate for **1 minute** at room temperature.
12. While still on the magnetic rack, remove and discard the supernatant.

13. Repeat steps 10-12 once for a total of 2 washes. Remove any residual ethanol with a P20.

14. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).



CRITICAL! Do not over-dry the beads, which can lead to a substantial loss in yield. "Cracking" of the beads is a sign of over-drying.

15. Remove the tube(s) from the magnetic rack. With a pipette, fully resuspend each bead pellet with **25 μ L** of nuclease-free water.

16. Incubate for **5 minutes** at room temperature.

17. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).



CRITICAL! Do not over-dry the beads, which can lead to a substantial loss in yield. "Cracking" of the beads is a sign of over-drying.

18. While still on the magnetic rack, transfer 25 μ L of the supernatant containing the purified cDNA into new 0.2 mL tube(s). Store on ice.



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 immediately to Section 3.

2.5. cDNA Quantification

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

To quantify the cDNA:

1. Measure the concentration of each tube of purified cDNA from Section 2.4 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.

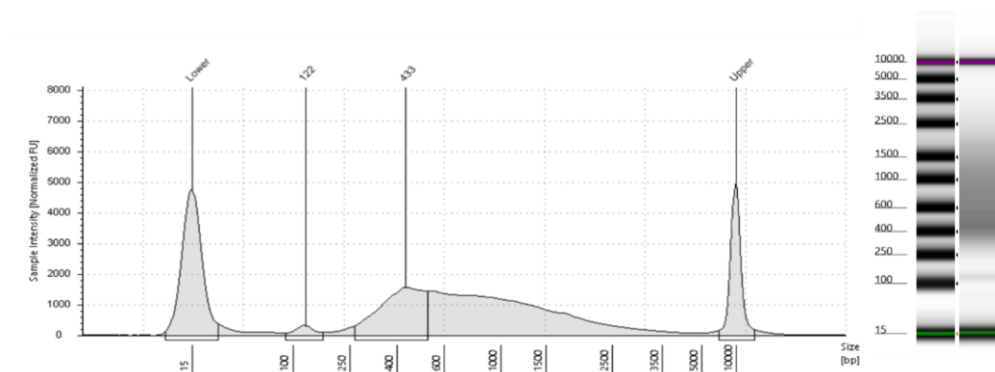


Figure 9: Expected post-amplification sublibrary cDNA size distribution. Example trace of Human cDNA run on a TapeStation.

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

3.1. Fragmentation and End Prep

Barcoded and amplified cDNA is fragmented, end repaired and A-tailed in a single reaction.



CRITICAL! Always use new tips for each sublibrary.

To prepare for fragmentation and end prep:

1. For each sublibrary, prepare **1.2 mL** of 85% ethanol with nuclease-free water.
2. Equilibrate **180 µL** of SPRI beads per sublibrary to room temperature.
3. Fill an ice bucket.
4. Take out the Parse Biosciences magnetic rack for 0.2 mL PCR tubes.
5. Obtain recorded cDNA concentrations from Section 2.5.
6. Gather the following items and handle as indicated below.

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● Fragm/End Prep Buffer	-20°C Reagents	1	Thaw at room temperature then store on ice. Vortex to mix.
● Fragm/End Prep Enzymes	-20°C Reagents	1	Place directly on ice. Briefly centrifuge before use.

7. Vortex the tube(s) of cDNA for **5 seconds**. Briefly centrifuge.
8. Prepare Diluted cDNA in new 0.2 mL tube(s) as follows to a final volume of **35 µL** and store on ice. Store any remaining purified cDNA at -20°C.

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

9. Start the following program to ensure the thermocycler is cool prior to use.

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



Note: * This hold ensures that the thermocycler is cooled and ready for step 13.

10. Mix ● Fragm/End Prep Buffer by vortexing for **5 seconds**. Store on ice.



Note: Confirm the ● Fragm/End Prep Buffer is fully thawed and mixed without precipitation.

11. Prepare the Fragmentation and End Prep master mix in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

FRAGMENTATION AND END PREP MASTER MIX		
Number of Sublibraries	1	16
● Fragm/End Prep Buffer	6 µL	96 µL
● Fragm/End Prep Enzymes	12 µL	192 µL
Total	18 µL	288 µL

12. Add **15 µL** of Fragmentation and End Prep master mix to each tube of diluted cDNA. Mix by pipetting 10x with a P200 multichannel pipette set to 40 µL. Briefly centrifuge.

13. Place the tube(s) into the cooled thermocycler. Skip the 4°C hold in Step 1 so the thermocycler proceeds to Step 2 of the Fragmentation and end prep program.



Note: Ensure the thermocycler has cooled to 4°C prior to adding the tubes.

14. As soon as the program reaches step 4 of the thermocycling program (4°C), store the tube(s) on ice and proceed immediately to Section 3.2.

3.2. Post-Fragmentation and End Prep Size Selection

The fragmented and end prepped DNA are size selected with a double sided SPRI cleanup.



CRITICAL! Always use new tips for each sublibrary.

To size select the fragmented and end prepped DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads.



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

3. Vortex the SPRI beads until fully mixed. Add **30 μ L** of SPRI beads to each tube of fragmented and end prepped DNA.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **75 μ L** of the supernatant containing the fragmented and end prepped DNA into new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **20 μ L** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).



CRITICAL! Ensure the solution is completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.

13. While still on the magnetic rack, add **180 μ L** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.
15. While still on the magnetic rack, remove and discard the supernatant.
16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.
17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).



CRITICAL! Do not over-dry the beads, which can lead to substantial loss in yield. "Cracking" of the beads is a sign of over-drying.

18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **50 μ L** of nuclease-free water.
19. Incubate for **5 minutes** at room temperature.
20. Place the tube(s) on the high position of the magnetic rack. Incubate until the solution clears (~2 minutes).
21. While still on the magnetic rack, transfer **50 μ L** of the supernatant into new 0.2 mL tube(s).



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.

3.3. Adapter Ligation

Adapters with an Illumina Truseq Read 1 sequence are ligated to the 5' end of the fragmented and end prepped DNA.



CRITICAL! Always use new tips for each sublibrary.

To ligate adapters:

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

ITEM	SOURCE	QTY	HANDLING AND STORAGE
● 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.
● 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	-20°C Reagents	1 sealed well per sublibrary	Thaw at room temperature then place on ice.

2. Prepare the Adapter Ligation Master Mix in a new 1.5 mL tube as follows. Mix by pipetting 10x and store on ice.

ADAPTER LIGATION MASTER MIX		
Number of Sublibraries	1	16
Nuclease-free water	19.25 µL	308 µL
● Adapter Ligation Buffer	22 µL	352 µL
● Adapter Ligation Enzyme	11 µL	176 µL
● Ligation Adapter	2.75 µL	44 µL
Total	55 µL	880 µL

3. Add **50 μ L** of Adapter Ligation Master Mix to each tube of purified fragmented and end prepped DNA from Section 3.2. Mix by pipetting 10x with a P200 multichannel pipette set to 80 μ L. Briefly centrifuge.
4. Place the tube(s) into a thermocycler and run the program below.

ADAPTER LIGATION		
Run Time	15 min	
Lid Temperature	30°C*	
Sample Volume	100 μ L	
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.

5. As soon as the program reaches 4°C, store the adapter ligated DNA on ice and proceed immediately to Section 3.4.

3.4. Post-Ligation Purification

Adapter ligated DNA is size selected with a single sided SPRI bead cleanup.



CRITICAL! Always use new tips for each sublibrary.

To purify the ligated DNA:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~**100 μ L** per sublibrary).



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

3. Vortex the SPRI beads until fully mixed. Add **80 μ L** of SPRI beads to each tube of adapter ligated DNA from Section 3.3.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, remove and discard the supernatant.
8. While still on the magnetic rack, add **180 μ L** of 85% ethanol to each tube.
9. Incubate for **1 minute** at room temperature.
10. While still on the magnetic rack, remove and discard the supernatant.
11. Repeat steps 8-10 once for a total of 2 washes. Remove any residual ethanol with a P20.
12. While still on the magnetic rack, air dry the SPRI beads (~2 minutes).
13. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **23 μ L** of nuclease-free water.
14. Incubate for **5 minutes** at room temperature.

15. Place the tube(s) on the low magnet position of the magnetic rack, so the magnet is closer to the bottom of the 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
16. While still on the magnetic rack, transfer exactly **21 μ L** of the supernatant containing the purified adapter ligated DNA into new 0.2 mL tube(s). Store on ice.
17. Proceed immediately to Section 3.5.

3.5. Barcoding Round 4

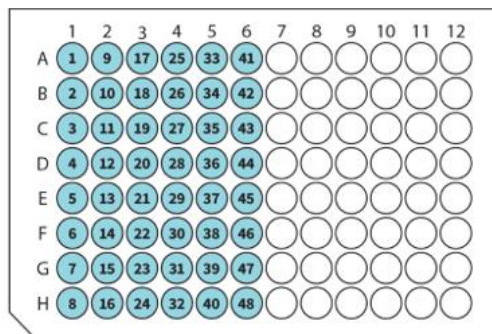
Purified adapter ligated DNA is PCR amplified with Illumina Truseq Read 1 and Read 2 primers. This indexing PCR generates sequencing libraries and adds i5/i7 UDIs that act as a fourth cell barcode.



CRITICAL! Always use new tips for each UDI Plate's well and corresponding sublibrary.

To add round 4 barcodes:

1. Centrifuge the UDI Plate - WT at 100 x g for 1 minute.
2. Wipe the surface of the plate with 70% ethanol and allow it to dry.
3. 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.



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

- 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 tube of purified adapter ligated DNA from Section 3.4.



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

- If any unused wells remain in the UDI Plate - WT, store the plate at -20°C. Do not reuse wells.
- Add **25 µL** of ● Library Amp Mix to each tube. Mix by pipetting 10x with a P200 multichannel pipette set to 25 µL. Briefly centrifuge.
- 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 as recorded in Section 2.5.

NUMBER OF PCR CYCLES	
cDNA Input (ng)	PCR Cycles
10-24	13
25-49	12
50-99	11
100-199	10
200-399	9
400-999	8
1,000 or more	7

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

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



CRITICAL! If processing sublibraries with different cDNA input, they should be amplified in separate thermocyclers according to the recommendations above.



Safe stopping point: Prior to size selection, sequencing libraries can be stored at 4°C for up to 18 hours.

3.6. Post-Barcoding Round 4 Size Selection

The sequencing libraries are size-selected with a double sided SPRI cleanup



CRITICAL! Always use new tips for each sublibrary.

To size select the sequencing libraries:

1. Gather freshly prepared 85% ethanol.
2. Gather room temperature SPRI beads (~**50 μ L** per sublibrary).



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

3. Vortex the SPRI beads until fully mixed. Add **30 μ L** of SPRI beads to each sequencing library tube.
4. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
5. Incubate for **5 minutes** at room temperature.
6. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~2 minutes).
7. While still on the magnetic rack, transfer **75 μ L** of the supernatant containing the DNA into a new 0.2 mL tube(s). Discard the tube(s) with bead pellet(s).
8. Add **10 μ L** of SPRI beads to each tube.
9. Vortex the tube(s) for **5 seconds**. Briefly centrifuge.
10. Incubate for **5 minutes** at room temperature.
11. Place the tube(s) on the high position of the magnetic rack for 0.2 mL tubes. Incubate until the solution clears (~3 minutes).



CRITICAL! This may take longer due to the low volume of beads. Ensure the solution is completely clear before proceeding.

12. While still on the magnetic rack, remove and discard the supernatant.

13. While still on the magnetic rack, add **180 μ L** of 85% ethanol to each tube.
14. Incubate for **1 minute** at room temperature.
15. While still on the magnetic rack, remove and discard the supernatant.
16. Repeat steps 13-15 once for a total of 2 washes. Remove any residual ethanol with a P20.
17. While still on the magnetic rack, air dry the SPRI beads (~30 seconds).
18. Remove the tube(s) from the magnetic rack. Fully resuspend each bead pellet with **20 μ L** of nuclease-free water or TE Buffer.
19. Incubate for **5 minutes** at room temperature.
20. Place the tube(s) on the low position of the magnetic rack. Incubate until the solution clears (~2 minutes).
21. While still on the magnetic rack, transfer the supernatant into a new 0.2 mL tube(s). Store on ice.



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

3.7. Sequencing Library Quantification

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

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.

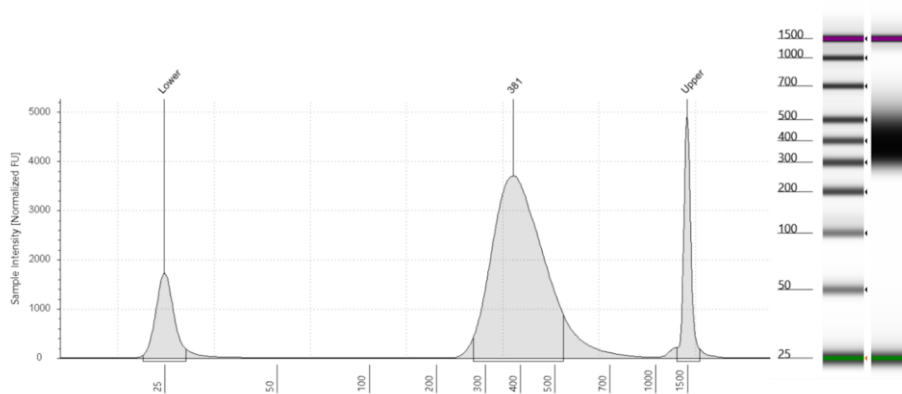


Figure 10: Expected Size Distribution before Illumina Sequencing. Example trace of Human DNA from indexed sublibraries run on a TapeStation.

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 300-450 bp. The prominence of the trace is dependent on the amount of DNA loaded into the TapeStation. Sublibraries with minor deviations can still produce high quality data.



Note: If using a Bioanalyzer, there may be an additional peak present. This typically occurs if products are overamplified, but it should not impact sequencing or data quality (assuming there is still a peak present at 300-450 bp). Do not use this additional peak when estimating amplicon size.

Appendices

Appendix A: Sublibrary Generation Table

The Sublibrary Generation Table provides guidance for sample dilution in Section 1.5 to achieve a target sublibrary concentration.

Green text (top): Volume of cell suspension in μL (from step 1.5.10) to add to each sublibrary.

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

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

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

Cell Stock Conc. (cells/ μL)	Target Sublibrary Cell Count (cells/sublibrary)													
	200	500	1,000	2,000	5,000	10,000	12,000	12,500	15,000	20,000	25,000	30,000	31,250	62,500
50	4	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	21	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
100	2	5	10	20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	23	20	15	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
200	Dilute	2.5	5	10	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	22.5	20	15	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
400	Dilute	Dilute	2.5	5	12.5	25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	22.5	20	12.5	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
600	Dilute	Dilute	Dilute	3.33	8.33	16.67	20	20.83	25	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	21.67	16.67	8.33	5	4.17	0	N/A	N/A	N/A	N/A	N/A
800	Dilute	Dilute	Dilute	2.5	6.25	12.5	15	15.63	18.75	25	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	22.5	18.75	12.5	10	9.37	6.25	0	N/A	N/A	N/A	N/A
1,000	Dilute	Dilute	Dilute	2	5	10	12	12.5	15	20	25	N/A	N/A	N/A
	N/A	N/A	N/A	23	20	15	13	12.5	10	5	0	N/A	N/A	N/A
1,200	Dilute	Dilute	Dilute	Dilute	4.17	8.33	10	10.42	12.5	16.67	20.83	25	N/A	N/A
	N/A	N/A	N/A	N/A	20.83	16.67	15	14.58	12.5	8.33	4.17	0	N/A	N/A
1,400	Dilute	Dilute	Dilute	Dilute	3.57	7.14	8.57	8.93	10.71	14.29	17.86	21.43	22.32	N/A
	N/A	N/A	N/A	N/A	21.43	17.86	16.43	16.07	14.29	10.71	7.14	3.57	2.68	N/A
1,600	Dilute	Dilute	Dilute	Dilute	3.13	6.25	7.5	7.81	9.38	12.5	15.63	18.75	19.53	N/A
	N/A	N/A	N/A	N/A	21.87	18.75	17.5	17.19	15.63	12.5	9.38	6.25	5.47	N/A
1,800	Dilute	Dilute	Dilute	Dilute	2.78	5.56	6.67	6.94	8.33	11.11	13.89	16.67	17.36	N/A
	N/A	N/A	N/A	N/A	22.22	19.44	18.33	18.06	16.67	13.89	11.11	8.33	7.64	N/A
2,000	Dilute	Dilute	Dilute	Dilute	2.5	5	6	6.25	7.5	10	12.5	15	15.63	N/A
	N/A	N/A	N/A	N/A	22.5	20	19	18.75	17.5	15	12.5	10	9.38	N/A
2,500	Dilute	Dilute	Dilute	Dilute	2	4	4.8	5	6	8	10	12	12.5	25
	N/A	N/A	N/A	N/A	23	21	20.2	20	19	17	15	13	12.5	0
3,000	Dilute	Dilute	Dilute	Dilute	3.33	4	4.17	5	6.67	8.33	10	10.42	20.83	
	N/A	N/A	N/A	N/A	21.67	21	20.83	20	18.33	16.67	15	14.58	4.17	
3,500	Dilute	Dilute	Dilute	Dilute	2.86	3.43	3.57	4.29	5.71	7.14	8.57	8.93	17.86	
	N/A	N/A	N/A	N/A	22.14	21.57	21.43	20.71	19.29	17.86	16.43	16.07	7.14	
4,000	Dilute	Dilute	Dilute	Dilute	2.5	3	3.13	3.75	5	6.25	7.5	7.81	15.63	
	N/A	N/A	N/A	N/A	22.5	22	21.88	21.25	20	18.75	17.5	17.19	9.38	
4,500	Dilute	Dilute	Dilute	Dilute	2.22	2.67	2.78	3.33	4.44	5.56	6.67	6.94	13.89	
	N/A	N/A	N/A	N/A	22.78	22.33	22.22	21.67	20.56	19.44	18.33	18.06	11.11	
5,000	Dilute	Dilute	Dilute	Dilute	2	2.4	2.5	3	4	5	6	6.25	12.5	
	N/A	N/A	N/A	N/A	23	22.6	22.5	22	21	20	19	18.75	12.5	
5,500	Dilute	Dilute	Dilute	Dilute	2.18	2.27	2.73	3.64	4.55	5.45	5.68	11.36		
	N/A	N/A	N/A	N/A	22.82	22.73	22.27	21.36	20.45	19.55	19.32	13.64		
6,000	Dilute	Dilute	Dilute	Dilute	2	2.08	2.5	3.33	4.17	5	5.21	10.42		
	N/A	N/A	N/A	N/A	23	22.92	22.5	21.67	20.83	20	19.79	14.58		
7,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.14	2.86	3.57	4.29	4.46	8.93	
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.86	22.14	21.43	20.71	20.54	16.07	
8,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.5	3.13	3.75	3.91	7.81
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.5	21.88	21.25	21.09	17.19
9,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2.22	2.78	3.33	3.47	6.94
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.78	22.22	21.67	21.53	18.06
10,000	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	Dilute	2	2.5	3	3.13	6.25
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	23	22.5	22	21.88	18.75

Appendix B: Sequencing Information

We recommend a minimum sequencing depth of 10,000 reads per cell for whole transcriptome libraries. However, ideal sequencing depth is dependent on the sample type and experimental goals. For example, if trying to capture rare cell types in a heterogenous population, deeper sequencing is required. Alternatively, analyzing a homogenous population, more shallow sequencing may be appropriate.

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

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

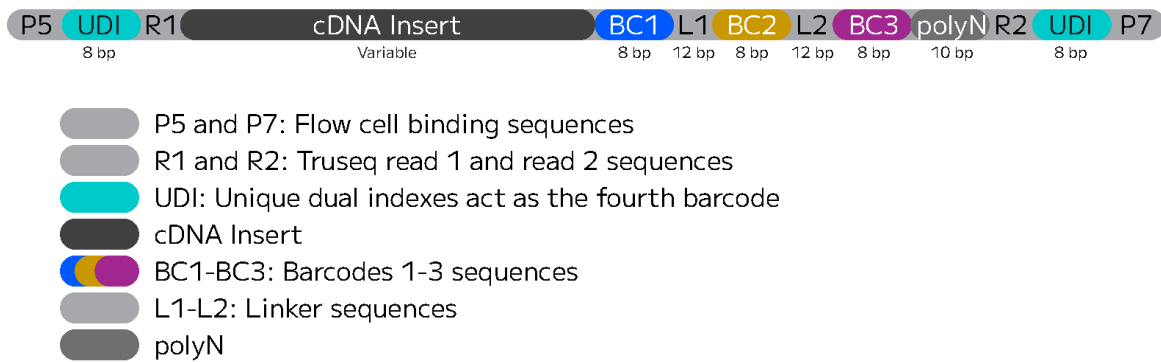


Figure 11: Whole transcriptome sequencing library structure.

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

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

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

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_1	A1	CAGATCAC	ATGTGAAG	CTTCACAT
UDI_Plate_WT_2	B1	ACTGATAG	GTCCAACC	GGTTGGAC
UDI_Plate_WT_3	C1	GATCAGTC	AGAGTCAA	TTGACTCT
UDI_Plate_WT_4	D1	CTTGTAAT	AGTTGGCT	AGCCAACT
UDI_Plate_WT_5	E1	AGTCAAGA	ATAAGGCG	CGCCTTAT
UDI_Plate_WT_6	F1	CCGTCCTA	CCGTACAG	CTGTACGG
UDI_Plate_WT_7	G1	GTAGAGTA	CATTCATG	CATGAATG
UDI_Plate_WT_8	H1	GTCCGCCT	AGATACGG	CCGTATCT
UDI_Plate_WT_9	A2	GTGAAACT	TACAGACT	AGTCTGTA
UDI_Plate_WT_10	B2	TCATTCCCT	AATGCCTG	CAGGCATT
UDI_Plate_WT_11	C2	GGTAGCAT	TGCTTGCC	GGCAAGCA
UDI_Plate_WT_12	D2	ACTTGATC	TTTGGGTG	CACCCAAA
UDI_Plate_WT_13	E2	ATGAGCAT	GAATCTGA	TCAGATTC
UDI_Plate_WT_14	F2	GCGCTATC	CGACTGGA	TCCAGTCG
UDI_Plate_WT_15	G2	TGACCAGT	ACATTGGC	GCCAATGT
UDI_Plate_WT_16	H2	TATAATCA	ACCACTGT	ACAGTGGT
UDI_Plate_WT_17	A3	CAAAAGTC	CGGTTGTT	AACAACCG
UDI_Plate_WT_18	B3	CGATGTCA	CATGAGGA	TCCTCATG
UDI_Plate_WT_19	C3	CTCAGAGT	TGGAGAGT	ACTCTCCA
UDI_Plate_WT_20	D3	TAATCGAC	TGACTTCG	CGAAGTCA
UDI_Plate_WT_21	E3	CATTTTCT	GGAAGGAT	ATCCTTCC
UDI_Plate_WT_22	F3	CTATACTC	TGTTTCGAG	CTCGAACA
UDI_Plate_WT_23	G3	CACTCACA	AAGGCTGA	TCAGCCTT
UDI_Plate_WT_24	H3	CTCGAACA	CTCGAGTG	CACTCGAG
UDI_Plate_WT_25	A4	CTCTATCG	ATCGGTGG	CCACCGAT
UDI_Plate_WT_26	B4	TCCTCATG	AGGTCTTG	CAAGACCT
UDI_Plate_WT_27	C4	AACAACCG	AGGAAGCG	CGCTTCCT
UDI_Plate_WT_28	D4	GCCAATGT	ACATGTGT	ACACATGT
UDI_Plate_WT_29	E4	TGGTTGTT	ATACAGTT	AACTGTAT
UDI_Plate_WT_30	F4	TCTGCTGT	ATCGCCTT	AAGGCGAT
UDI_Plate_WT_31	G4	TTGGAGGT	TTCGACGC	GCGTCGAA
UDI_Plate_WT_32	H4	TCGAGCGT	TGTCGTTC	GAACGACA
UDI_Plate_WT_33	A5	TGCGATCT	TCCATAGC	GCTATGGA

Sublibrary Index ID	Well Position	i7 Forward Sequence	i5 Reverse Complement Sequence	i5 Forward Sequence
UDI_Plate_WT_34	B5	TTCCTGCT	TAAGTGTC	GACACTTA
UDI_Plate_WT_35	C5	TTCCATTG	CTGGCATA	TATGCCAG
UDI_Plate_WT_36	D5	TAACGCTG	CTGAGCCA	TGGCTCAG
UDI_Plate_WT_37	E5	TTGGTATG	CTCAATGA	TCATTGAG
UDI_Plate_WT_38	F5	TGAACTGG	CGCATACA	TGTATGCG
UDI_Plate_WT_39	G5	TCCAGTCG	CCGAAGTA	TACTTCGG
UDI_Plate_WT_40	H5	TGTATGCG	CCAGTTCA	TGAACTGG
UDI_Plate_WT_41	A6	TGGCTCAG	CAGCGTTA	TAACGCTG
UDI_Plate_WT_42	B6	TATGCCAG	CAATGGAA	TTCCATTG
UDI_Plate_WT_43	C6	GGTTGGAC	ATCCTGTA	TACAGGAT
UDI_Plate_WT_44	D6	GACACTTA	AGCAGGAA	TTCCTGCT
UDI_Plate_WT_45	E6	GAACGACA	ACGCTCGA	TCGAGCGT
UDI_Plate_WT_46	F6	AAGGCGAT	ACAGCAGA	TCTGCTGT
UDI_Plate_WT_47	G6	ATGCTTGA	ACAAGCTA	TAGCTTGT
UDI_Plate_WT_48	H6	AGTATCTG	CATCAAGT	ACTTGATG

Appendix C: Thermocycling Programs

Section 1: In situ Cell/Nuclei Barcoding

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

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

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

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

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

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

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

Section 2: cDNA Capture and Amplification

THAW LYSATES		
Run Time	5 minutes	
Lid Temperature	40°C	
Sample Volume	55 µL	
Step	Time	Temperature
1	5 min	37°C
2	Hold	4°C

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

cDNA AMPLIFICATION			
Run Time		50-70 minutes	
Lid Temperature		105°C	
Sample Volume		100 µL	
Step	Time	Temperature	Cycle
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

Section 3: Sequencing Library Preparation

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

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

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

Appendix D: Revision History

Version	Description	Date
1.0	Initial release	March 2026



parsebiosciences.com

support@parsebiosciences.com

