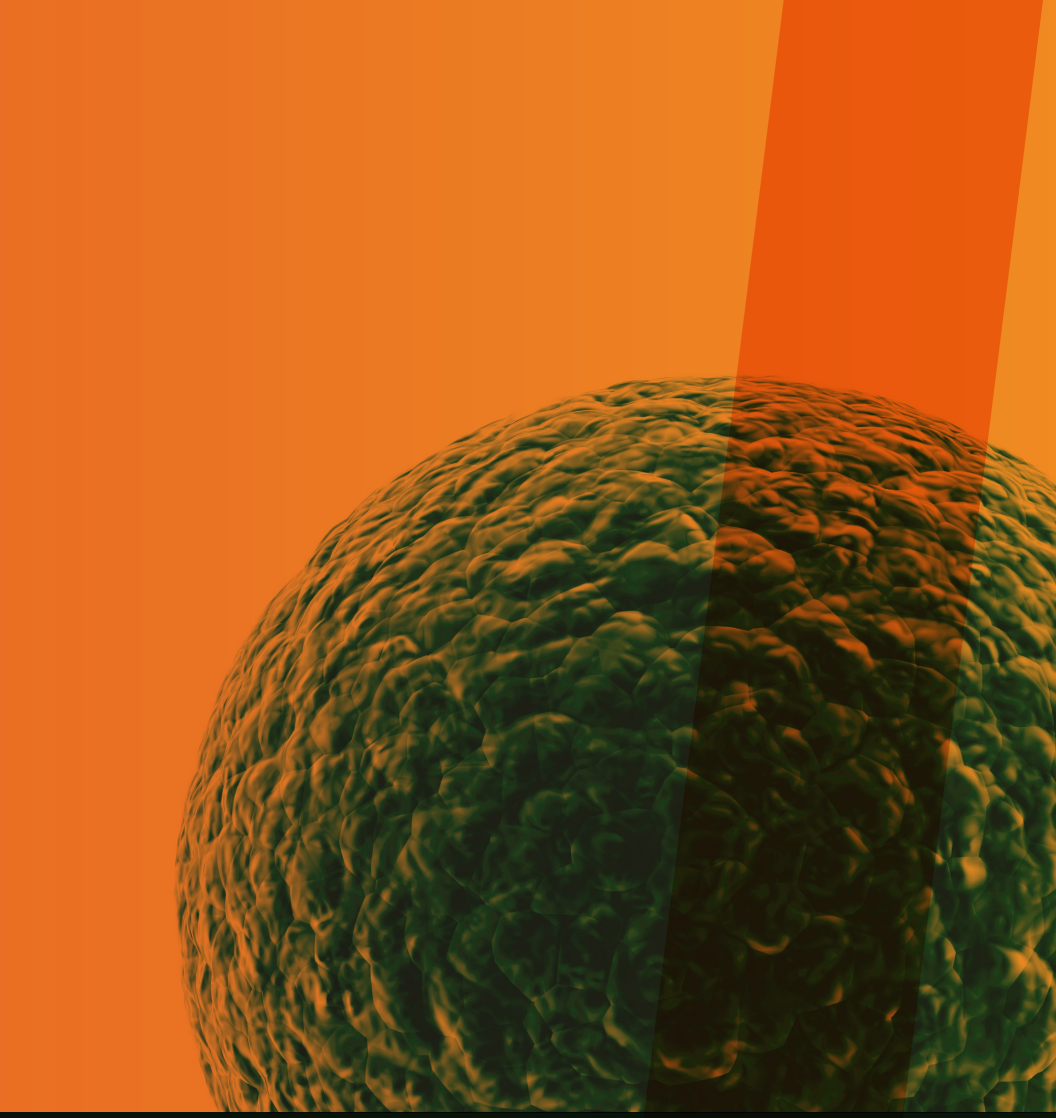


HONEYCOMB™

HIVE™ scRNAseq v1 Processing Kit with Molecular Controls

User Protocol - Revision A

v22.09 | September 2022 | Revision A

This product is for research use only.
Not for use in diagnostic procedures.

HIVE™ scRNAseq Processing Kit User Protocol:

///// Product Overview

HIVE™ scRNAseq is a complete solution transforming single-cells to NGS libraries. The HIVE is a portable, handheld, single-use device that enables gentle capture, easy storage, and scalable processing for the analysis of single-cell samples. Cell-loaded HIVEs can be stored or shipped until ready for simplified and scalable HIVE processing and library prep workflow.

The HIVE™ scRNAseq workflow is divided into two parts: sample capture and sample processing to create a sequencing library. The following protocol guides users through the sample processing and library preparation workflow with four Molecular Controls in addition to user generated cell-loaded HIVEs. Ideal for training first time users, or verifying the workflow.

///// Kit Overview

The **HIVE™ scRNAseq Processing Kit** is comprised of three boxes: HIVE Parts & Reagents, Spin Parts, and Library Prep Reagents. Required products include **HIVE™ Accessories** and **Index Plate**. The **Vacuum Kit, Plate Kit,** and **Lysis Boxes** contain additional materials needed for new users.

The **HIVE™ scRNAseq Processing Kit** can be used with **Molecular Controls** and cell-loaded HIVEs from the **HIVE™ scRNAseq Sample Capture Kit** for training and workflow validation. There are enough parts and reagents to either a) train one user and run 4 samples, or b) train two users and run 2 samples.

///// Kit Contents & Storage

HIVE™ scRNAseq Processing

HIVE™ Parts & Reagents: Ambient*

8 rxns
Storage Wash Solution
Lysis Stock
Hybridization Buffer
Bead Recovery Solution
Wash A
Water
SPRI Beads
~~SPRI Wash Concentrate~~ (no longer needed)

Spin Parts: Ambient

Library Prep Reagents: -20°C

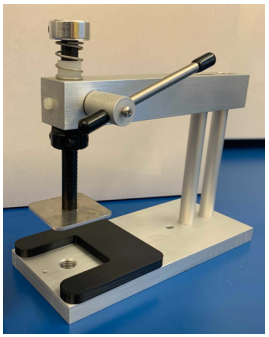
8 rxns	Cap Color
Reducing Solution	White
1st Strand Wash x2	Pink
1st Synthesis Buffer	Blue
1st Strand Enzyme	Blue
Clean-Up Buffer	Green
Clean-Up Enzyme	Green
10X NaOH	Clear
2nd Synthesis Buffer	Violet
2nd Strand Oligo	Violet
2nd Strand Enzyme	Violet
WTA Oligo	Orange
PCR Enzyme x2	Orange
Read 1 seq Primer	Red
Read 2 seq Primer	Red
Index 1 seq Primer	Red
Index 2 seq Primer	Red

Molecular Controls: -20°C

2 doses
Pre-1st Strand Control
Post-1st Strand Control
WTA Input Control
Index PCR input Control

HIVE Accessories: Ambient

Closure Tool



This tool is used for
1. HIVE sealing &
2. Bead Recovery

WARNING: Potential pinch point. Keep hands clear during operation

Filter Plate Adaptor

This adaptor convert thermocyclers into incubators for filter plate steps.
It may work for heating blocks with grooves between wells, but not those with a continuous flat surface.
It also decreases the height clearance betweenthe heating block and the lid.
Check the heating block contour and lid clearance before use.

///// Revision History

Version	Date	Description
v21.10	October 2021	Product Launch
v22.09 | Revision A	September 2022	Addition of WTA SPRI Clean-Up, revised Index PCR and Index SPRI Clean-Up

///// User Supplied Materials

Reagents

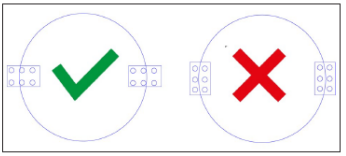
- Molecular biology grade ethanol, absolute
- Wescodyne® (bleach alternative)

- **Disposables**

- Reagent reservoirs for 25-50 mL
- Paper towels
- Optional: Nunc™ Square BioAssay Dishes. Thermo Scientific (CAT# 240845)

Equipment

Radial (Not Perpendicular) Plate Orientation



- -20°C freezer
- 4°C refrigerator and ice bucket
- Biosafety cabinet (optional)
- Oven, for 37°C and 50°C incubations
- Bench-top vortex
- Centrifuge with plate rotor (or swinging-bucket rotor with plate adaptors), e.g. Eppendorf 5810™ with Rotor S-4-104 and MTP/Flex buckets
  Critical Requirements:
  - 1,800 RCF capacity
  - Deep-well plate (DWP) compatible
  - Radial (not perpendicular) plate orientation (see Diagram above)
- Thermocycler for 96-well plate
- Bar magnet for 96 well plates, e.g. Invitrogen DynaMag™-96 Side Skirted (CAT# 12027)
- DNA quantification device, e.g. Thermo Scientific QuBit™ 4 Fluorometer (CAT# Q33238)
- DNA capillary electrophoresis device, e.g. Tapestation™, Bioanalyzer,™ or LabChip GX Touch™ (plus kit for >1,000 bp DNA smear)

Pipets & Tips

- Pipet aid (optional) - 5 mL - 25 mL serological pipettes
- Single-channel 1000 µL - 1000 µL tips
- 8-channel and single-channel 200 µL, single-channel 20 µL - 200 µL tips
- 8-channel and single-channel 10 µL - 10 µL tips

Currently Available

Plate Kit (sufficient for 2 experiments)

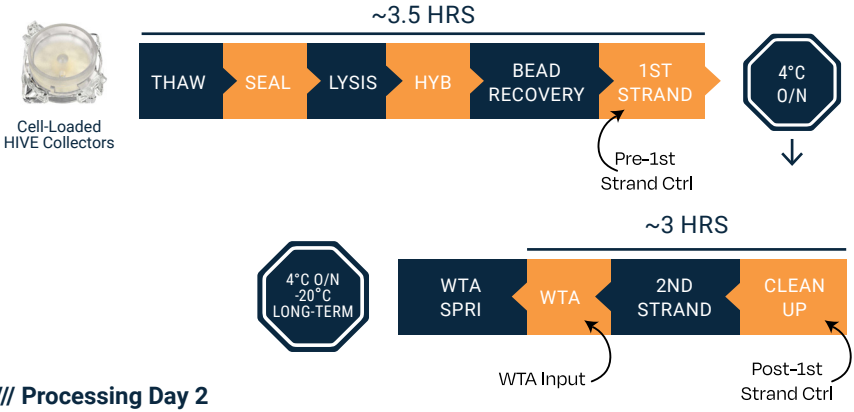
- 96-well filter plates. Millipore MultiScreen™ Filter Plates (CAT# MSHVN4B10)
- 96-well deepwell plate. Fisher Sci round well, V/U/conical bottom, >0.8 mL well capacity, natural polypropylene (CAT# AB0765)
- 96-well full-height PCR plate, 0.3 mL metric capacity. Thermo Fisher semi-skirted, flat deck, black lettering (CAT# AB1400L)
- Evaporation resistant adhesive PCR plate sealing films. Biorad Microseal™ 'B' adhesive film (CAT# MSB1001)
- Adhesive foil PCR plate seal. Excel Scientific, eXTReme™ FoilSeal™ (CAT# XTR-FOIL-100) **Vacuum**

Kit

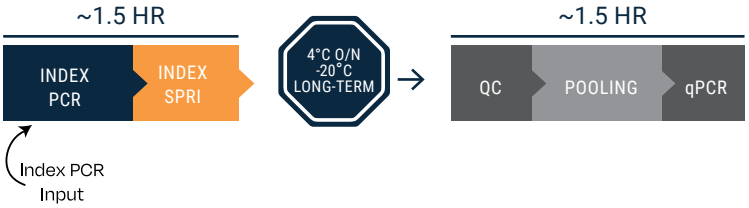
- Vacuum pump/line* (optional), e.g. Cole-Parmer Air diaphragm vacuum/pressure pump, 0.37 cfm, 115 VAC (CAT# EW-79202-00)
- Vacuum aspiration reservoirs, e.g. VWR Vactrap™ Vacuum Trap System for Aspiration and Vacuum Protection (CAT# 76207-602)
- 96 well vacuum manifold, e.g. Millipore MultiScreen™ Vacuum Manifold 96-well (CAT# MAVM0960R)

*can reach vacuum of at least 15 in Hg (381 mm Hg), and fit tubing with inner diameter of 0.25 inches (0.63 cm)

//// Processing Day 1



//// Processing Day 2



10 MINS | TOTAL 100 MINS

STEP A: Thaw & Seal Cell-Loaded HIVE

Materials

- Cell-Loaded HIVE Collector
- Storage Wash Solution
- HIVE Top with Protective Cover
- Closure Tool (HIVE Accessories)

User Provided

- Aspirator (optional)
- 1 mL pipet & tips
- 50°C oven

! **IMPORTANT:** Keep HIVEs flat at all times, only tilt when directed to do so during liquid removal
! **IMPORTANT:** Pre-heat oven to 50°C

1. Remove Cell-Loaded HIVE Collector from freezer and from packaging
2. Thaw for 60 minutes at room temperature

HIVE Parts Practice: During thaw use a HIVE Collector from Sample Capture Training to practice through Bead Recovery with parts from scRNAseq Processing Kit

3. Remove Stopper from port, and discard

STEPS 4-5 One HIVE at a Time

4. Tilt HIVE Collector, remove thawed **Cell Preservation Solution** (~1mL) through port
   DO NOT MIX Cell Preservation Solution with BLEACH
5. With HIVE Collector flat, add 1mL of **Storage Wash Solution** through the port

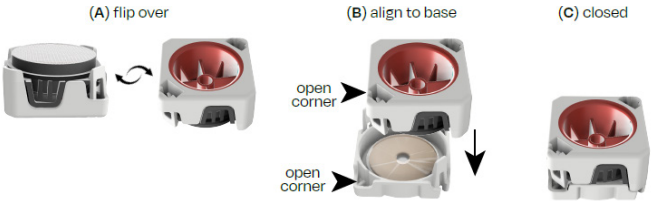
STEPS 6-12 One HIVE at a Time

6. Tilt HIVE Collector, remove **Storage Wash Solution** through port
   DO NOT MIX Storage Wash Solution with BLEACH
7. Remove Cell Loader, gently push out on HIVE Base tabs while lifting up on Cell Loader wings

8. Discard the Cell Loader and move immediately to the next steps
9. Squeeze HIVE Top + Protective Cover from the top and bottom, until fully clicked in place

10. Bend out the Cover arms to remove and dispose Cover, move immediately to the next step

11. Seal the HIVE
    a. flip HIVE Top over so the membrane is facing down
    b. align and place HIVE Top to HIVE Base, open corners to open corners, do not click closed
    c. Slide into Closure Tool (from HIVE Accessories), close slowly, pressing through resistance



12. Repeat with any remaining HIVE Collectors, working one at a time
13. Incubate Closed HIVEs for 30 minutes at 50°C to seal membrane onto the array
14. Prepare Lysis Solution during incubation (see STEP B)

STEP B: Lysis & Hybridization

10 MINS | TOTAL 55 MINS

Materials

- Lysis Stock
- **White Cap** - Reducing Solution
- Hybridization Solution
- Spin Plate and HIVE Blanks
- Lysis boxes (from Starter Bundle)

User Provided

- Auto pipette and serological pipets (optional, if working with more than one HIVE)
- Pipets & tips

! **IMPORTANT:** Place Sealed HIVE and Blanks on Plates to ensure balance when placed in centrifuge
! **IMPORTANT:** Don't let membrane dry out, add Lysis Solution immediately after opening HIVEs

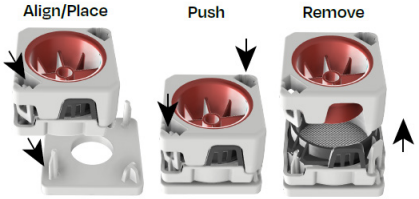
1. Prepare **Lysis Solution**, thaw **Reducing Solution** until white pellet is dissolved. Vortex if necessary.

	1 HIVE	8 HIVEs	_ HIVEs
Lysis Solution	**x1**	**x8**	**x_**
Lysis Stock	1 mL	8 mL	
Reducing Solution	100 µL	800 µL	
Total Volume	**~1 mL**	**~8 mL**	

2. Write plate number on provided Spin Plates (Spin Parts)
3. Record Sample ID and/or HIVE serial number and corresponding Spin Plate position

Spin Plate Number

Plate Position	1	2	3	4
A1				
A2				
A3				
B1				
B2				
B3				



(whole Plate not shown)

4. **Align** sealed HIVE open corners to Plate pins, and place on pins, repeat for all HIVEs

STEPS 5-9 Six HIVEs (or One Plate) at a Time

5. **Push sealed** HIVE down onto Plate by the open corners
6. **Remove** Plunger (lift up if doesn't pop up on own), membrane + frame will stay attached
7. Discard white top and orange plunger and move immediately to next step
8. Add 1mL **Lysis Solution** directly onto membrane, swirl plate
9. Incubate for **exactly** 15 minutes at room temperature

STEPS 10-12 Six HIVEs (or One Plate) at a Time

10. Remove **Lysis Solution**, tilt plate to pipette out (DO NOT MIX WITH BLEACH)
11. Add 1mL **Hybridization Solution** to membrane, swirl plate
12. Incubate 30 seconds

STEPS 13-15 Six HIVEs (or One Plate) at a Time

13. Remove **Hybridization Solution**, tilt plate to pipette out (DO NOT MIX WITH BLEACH)
14. Add 1mL **Hybridization Solution** to membrane, swirl plate
15. Incubate 30 minutes at room temperature
16. During incubation
    a. Prepare Pre-1st Strand Control (see STEP C)
    b. Prepare 1st Strand Wash and 1st Strand Synthesis Reaction (see STEP D)
    c. Check Vacuum Setup:
        Cover all wells of a filter plate foil seal
        Place sealed filter plate on vacuum manifold
        Plug in and turn on pump
        Press down firmly on filter plate
        Gauge should register between 5-15 inHg
        Turn valve clockwise to increase vacuum, counterclockwise to decrease vacuum
        Turn off pump and let gauge return to 0 before removing filter plate

10 MINS | TOTAL 15 MINS

STEP C: Bead Recovery

Materials

- Spin Plates, HIVE Blanks, Spin Lids (Spin Parts)
- Bead Collectors
- Closure Tool (HIVE Accessories)
- Bead Recovery Solution
- Red Stoppers
- Filter Plate (Plate Kit)
- Pre-1st Strand Control (Molecular Controls)

User Provided

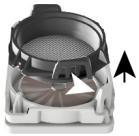
- Auto pipette and serological pipets (optional, if working with more than one HIVE)
- 1 mL pipet & tips
- Centrifuge (confirm g-force setting is accurately calculated)

! **IMPORTANT:** Place HIVE and Blanks on Plates to ensure balance when placed in centrifuge
! **IMPORTANT:** Move quickly through each step so membranes, arrays, and beads don't dry out

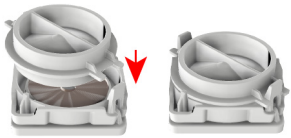
1. Thaw **Pre-1st Strand Control**, add 250 µL Bead Recovery Solution to re-suspend, keep on ice

STEPS 2-13 One Plate at a Time

2. Tilt Plate of HIVEs to pipette out and remove **Hybridization Solution** (DO NOT MIX WITH BLEACH)
3. Remove the Membrane Frame; gently pull out on front HIVE base tab, peal Frame up & off



4. Trash Membrane Frame
5. Align **Bead Collector** wings to HIVE Base tabs, place on top, do not click into place yet



6. Snap Bead Collector onto HIVE Base with **Closure Tool**, close slowly and firmly with 2 CLICKS
7. Balance With HIVE Blank(s) if needed
8. Add Spin Lid

9. Flip assembled HIVE(s)+plate+lid upside-down

10. Pierce Foil covered port with 1mL pipette tip until completely open
11. Add 3 x 1,000 µL Bead Recovery Solution pipetting quickly, keeping pipet tip vertical at port opening
12. Insert Red Stopper into port
13. Remove any residual Bead Recovery Solution around the Red Stopper

Repeat STEPS 1-12 for each additional Plate

14. Place Plates in centrifuge, Red Stoppers up
15. Balance centrifuge with additional plate/blank/lid assembly if needed
16. Spin At 1,800 RCF for 5 minutes
17. Remove foil seal from filter plate for one well per sample, including **Pre-1st Strand Control**
18. Remove Red Stoppers from port
19. Pipet set to 300 µL, remove bead pellet with tip gently touching bottom of Bead Collector
20. Transfer each bead pellet to filter plate, one sample per well, keeping track of each sample position
21. Mix re-suspended **Pre-1st Strand Control** beads by rapidly pipetting up and down ~10 times

STEP D: 1st Strand Synthesis

Materials

- Pink cap - 1st Strand Wash
- Blue cap - 1st Synthesis Buffer
- Blue cap - 1st Strand Enzyme
- Bead Recovery Solution
- Vacuum set-up (Vacuum Kit)
- Clear PCR plate seals (Plate Kit)
- Filter Plate Adaptor - optional (HIVE Accessories)

User Provided

- 1mL pipette & tips
- 1.5 mL microfuge tube
- Ice
- 37°C oven (or thermocycler - with sufficient lid clearance for the filter plate)

! **IMPORTANT:** Thaw 1st Strand Wash, 1st Synthesis Buffer at 37°C, vortex, keep on ice till use
! **IMPORTANT:** Set oven (or thermocycler) to 37°C

1. Make 1st Strand Synthesis Reaction, mix by pipetting, keep on ice until use
   Treat **Pre-1st Strand Control** as an additional sample, make enough 1st Strand Synthesis Reaction for this additional sample

	1 Sample	8 Samples +10%	_ Samples +10%
1st Strand Synthesis Reaction	**x1**	**x8.8**	**x_**
1st Synthesis Buffer	185 µL	1,628 µL	
1st Strand Enzyme	15 µL	132 µL	
Total Volume	200 µL	1,760 µL	

2. Place filter plate on vacuum manifold, turn pump on, press down filter plate, liquid will flow out
3. Turn off pump once liquid is gone, wait for vacuum to fully release ~30 seconds
4. Add 200 µL **Bead Recovery Solution**, mix by pipetting x3, turn pump on, press down filter plate
5. Turn off pump once liquid is gone, wait for vacuum to fully release ~30 seconds
6. Add 200 µL **Bead Recovery Solution** again, mix by pipetting x3, turn pump on, press down filter plate
7. Turn off pump once liquid is gone, wait for vacuum to fully release ~30 seconds
8. Add 200 µL 1st Strand Wash, mix by pipetting x3, turn pump on, press down filter plate

9. Turn Off pump once liquid is gone, wait for vacuum to fully release, remove filter plate
10. Pat dry filter plate bottom with paper towel and cover bottom firmly with clear PCR plate seal
11. Add 200 µL 1st Strand Synthesis Reaction to each well
12. Tilt Plate and mix beads by gently pipetting up and down 3 times, trying not to introduce bubbles
13. Seal the active wells firmly with a strip of clear PCR plate seal, and incubate at 37°C for 60 minutes
    optional: use Filter Plate Adaptor from HIVE Accessories to incubate in thermocycler, thermocycler lid needs to be heated to ~10°C higher than block temperature to avoid condensation
14. Prepare for Bead Clean-up if not pausing overnight after incubation (see STEP E)
    PAUSE POINT: Store overnight in sealed Filter Plate at 4°C

STEP E: Bead Clean-Up

Materials

- Green cap - Clean-Up Buffer
- Green cap - Clean-up Enzyme
- Wash A
- Vacuum set-up (Vacuum Kit)
- Clear PCR plate seals (Plate Kit)
- Filter Plate Adaptor - optional (HIVE Accessories)

User Provided

- Pipets & tips
- Reagent reservoir (optional: for multichannel pipet)
- 1.5 mL microfuge tube
- Ice
- Oven (or thermocycler) set to 37°C

1. Thaw Clean-Up Buffer at room temperature, ~15 minutes, keep on ice
2. Place Clean-Up Enzyme on ice until use
3. Prepare Clean-Up Reaction, mix by pipetting, keep on ice until use
   Treat **Post-1st Strand Control** as an additional sample, make enough Clean-Up Reaction for this additional sample

	1 Sample	8 Samples +10%	_ Samples +10%
Clean-Up Reaction	**x1**	**x8.8**	**x_**
Clean-Up Buffer	190 µL	1,672 µL	
Clean-Up Enzyme	10 µL	88 µL	
Total Volume	200 µL	1,760 µL	

4. Remove seal from the bottom of filter plate first, and then from the top of the active wells
5. Resuspend beads of **Post-1st Strand Control** by rapidly pipetting up and down ~5 times
6. Transfer entire tube (200 µL) of **Post-1st Strand Control** to new well in filter plate
7. Place filter plate on vacuum manifold, turn pump on, press down filter plate, liquid will flow out
8. Wash 3 times with 200 µL **Wash A** each time, allow liquid to flow through between washes
9. Turn Off pump once liquid is gone, wait for vacuum to fully release, remove filter plate
10. Pat dry filter plate bottom with paper towel and cover bottom firmly with clear PCR plate seal
11. Add 200 µL Clean-Up Reaction to each well
12. Tilt filter plate and mix beads by pipetting up and down 2-3 times, trying not to introduce bubbles
13. Seal the active wells firmly with a strip of clear PCR plate seal
14. Incubate at 37°C for 45 minutes (optional: use Filter Plate Adaptor from HIVE Accessories to incubate in thermocycler)
15. Prepare for 2nd Strand Synthesis during incubation (see STEP F)

STEP F: 2nd Strand Synthesis

10 MINS | TOTAL 40 MINS

Materials

- Violet cap - 2nd Synthesis Buffer
- Violet cap - 2nd Strand Oligo
- Violet cap - 2nd Strand Enzyme
- Clear cap - 10X NaOH
- Water
- Wash A
- Vacuum set-up (Vacuum Kit)
- Clear PCR plate seals (Plate Kit)
- Filter Plate Adaptor (HIVE Accessories - optional)

User Provided

- Pipets & tips
- Reagent reservoir (optional: for multichannel pipet)
- 1.5 mL microfuge tube, x2
- Ice
- 37 oven or thermocycler

! **IMPORTANT:** Prepare fresh 1X NaOH each time, 10X NaOH stock is good for 10 freeze-thaw cycles

! **IMPORTANT:** Make sure vacuum is OFF for 1X NaOH incubation, and let sit for exactly 5 minutes

1. Thaw 2nd Synthesis Buffer and 2nd Strand Oligo at room temp, ~15 minutes, transfer to ice
2. Place 2nd Strand Enzyme on ice until use
3. Thaw 10X NaOH, dilute 1:10 with Water to make 1X NaOH
4. Keep 1X NaOH at room temperature until use, return remaining 10X NaOH to freezer

	1 Sample	8 Samples +10%	_ Samples +10%
1x NaOH	**x1**	**x8.8**	**x_**
Water	180 µL	1,584 µL	
10x NaOH	20 µL	176 µL	
Total Volume	200 µL	1,760 µL	

5. Prepare 2nd Strand Synthesis Reaction on ice, mix by pipetting

	1 Sample	8 Samples +10%	_ Samples +10%
2nd Strand Synthesis Reaction	**x1**	**x8.8**	**x_**
Synthesis Buffer	185 µL	1,628 µL	
2nd Strand Oligo	10 µL	88 µL	
2nd Strand Enzyme	5 µL	44 µL	
Total Volume	200 µL	1,760 µL	

6. Remove seals from top of active wells and bottom of filter plate (may be some liquid on bottom seal)
7. Place filter plate on vacuum manifold, turn pump on, press down filter plate, liquid will flow out
8. Wash 3 times with 200 µL **Wash A**, allow liquid to flow out between washes
9. Turn Off pump once liquid is gone, wait for vacuum to fully release, remove filter plate
10. Pat dry filter plate bottom completely with paper towel
11. Place filter plate back on vacuum manifold **(do not turn pump on yet)**
12. Add 200 µL **1X NaOH** to each well

13. Incubate for **exactly** 5 minutes
14. Turn pump on, press down filter plate, liquid will flow out
15. Wash 3 times with 200 µL **Wash A**, allow liquid to flow out between washes
16. Turn Off pump once liquid is gone, wait for vacuum to fully release, remove filter plate
17. Pat dry filter plate bottom with paper towel and cover bottom firmly with clear PCR plate seal
18. Add 200 µL 2nd Strand Synthesis Reaction to each well
19. Tilt filter plate and mix beads by pipetting up and down 2-3 times, trying not to introduce bubbles
20. Seal the active wells firmly with a strip of clear PCR plate seal
21. Incubate at 37°C for 30 minutes
    optional: use Filter Plate Adaptor from HIVE Accessories to incubate in thermocycler
22. Thaw and prepare reagents for WTA Reaction during incubation (see STEP G)

STEP G1: Whole Transcriptome Amplification (WTA)

Materials

- Orange cap - WTA Oligo
- Orange cap - PCR Enzyme
- WTA Input Control (Molecular Controls)
- Wash A
- Water
- Vacuum Set-Up (Vacuum Kit)
- Clear PCR plate seals (Plate Kit)
- Full-height and deepwell PCR plates (Plate Kit)
- Filter Plate Adaptor (HIVE Accessories - optional)

User Provided

- Pipets & tips
- Reagent reservoir (optional: for multichannel pipet)
- 1.5 or 5 mL tube
- Ice
- Thermocycler

! **IMPORTANT:** Fully resuspend beads for transfer from filter to deepwell plate

1. Thaw WTA Oligo, PCR Enzyme, and **WTA Input Control** at room temp, keep on ice until use
2. Set-up Thermocycler program for WTA Reaction, heated lid, 100 µL reaction volume

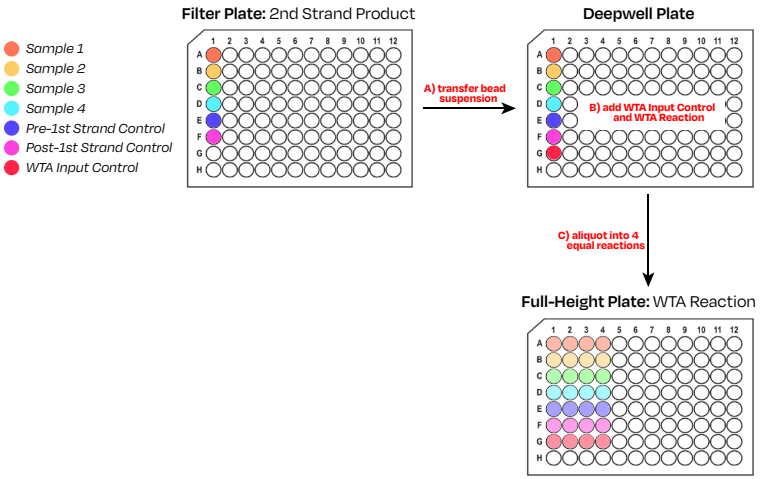
Temp	Time	# of Cycles
95°C	5 minutes	1
98°C	20 seconds	20
60°C	45 seconds	
70°C	1 minute	
72°C	2 minutes	1
4°C	hold	

3. Make WTA Reaction, mix by pipetting, keep on ice until use
   Treat **WTA Input Control** as an additional sample,make enough WTA Reaction for this additional sample

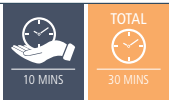
	1 Sample	8 Samples +10%	_ Samples +10%
WTA Reaction	**x1**	**x8.8**	**x_**
PCR Enzyme	200 µL	1,760 µL	
WTA Oligo	40 µL	352 µL	
Total Volume	240 µL	2,112 µL	

4. Remove seals from top of active wells and bottom of filter plate (may be some liquid on bottom seal)
5. Place filter plate on vacuum manifold, turn pump on, press down filter plate, liquid will flow out
6. Wash **6 times** with 200 µL **Wash A**, allow liquid to flow out between washes
7. Turn Off pump once liquid is gone, wait for vacuum to fully release, remove filter plate
8. Pat dry filter plate bottom with paper towel and cover bottom firmly with clear PCR plate seal
9. Add 160 µL **Water** to each filter plate well
10. Tilt filter plate and rapidly pipet up and down 5-10 times to resuspend beads immediately prior to transfer
11. Transfer 160 µL from each filter plate well to new wells in a deepwell plate
12. Add 160 µL of **WTA Input Control** to an additional deepwell plate well
13. Add 240 µL WTA Reaction to each deepwell plate well
14. Rapidly pipet up and down 5-10 times to resuspend beads in WTA Reaction, immediately prior to transfer
15. Aliquot 100 µL of beads suspension across 4 full-height PCR plate wells, making sure to resuspend before each aliquot as beads will settle very quickly
16. Seal the full-height PCR plate firmly with clear plate seal
17. Run on thermocycler with WTA Reaction program (~70 minutes)
18. Prepare for WTA SPRI Clean Up during incubation if continuing on (see STEP G2)

STOPPING POINT: Store overnight at 4°C , or long-term at -20°C



STEP G2: WTA SPRI Clean Up



Materials

- SPRI Beads
- Water

User Provided

- 96-well plate bar magnet
- Ethanol, absolute (EtOH)
- Pipets & tips
- Reagent reservoir (optional: for multichannel pipet)
- 1.5 mL microfuge tube, x2
- PCR Plate or strip tube

1. Make Fresh 80% EtOH, ~200 µL per sample
2. Pool 25 µL from each well of the WTA Reaction PCR plate into a new full height PCR plate; avoid touching the bead pellet. Label this plate Purified WTA Products (4 total wells per HIVE, 100uL of supernatant in 1 well)

! **IMPORTANT:** Save remaining WTA product at -20 for future experiments or troubleshooting.

3. Resuspend SPRI beads by vortexing. Add 90 µL to each well, pipet thoroughly to mix
4. Incubate for three minutes at room temperature
5. Place the PCR plate on the magnet for two minutes for magnetic beads to bind; the liquid will turn clear
6. Remove the supernatant and discard
7. Remove the plate from the magnet and add 50 µL of water to each well. Pipet to mix
8. Incubate for three minutes at room temperature
9. Add 45 µL of fresh SPRI beads to each well (beads from the first binding are still present in the well).
10. Incubate for three minutes at room temperature
11. Place the plate back on the magnet for 2 minutes for magnetic beads to bind; the liquid will turn clear
12. Remove and discard supernatant, avoid touching the pellet
13. Add 100 µL of 80% ethanol to all active wells
14. Mix by moving the entire plate across the magnet bars so that beads migrate through the EtOH solution ~6 times, then place the plate back down on the magnet
15. Remove and discard EtOH without touching the bead
16. Repeat steps 13-15
17. Remove residual EtOH using 10 µL pipet, leave plate on the magnet, and air-dry for 10 minutes
18. Add 50 µL of Water to each well
19. Remove plate from magnet, mix by pipetting up and down ~10 times, and incubate for three minutes

STEP H: Index PCR

10 MINS | TOTAL 40 MINS

Materials

- Orange cap - PCR Enzyme
- Index PCR Input Control (Molecular Controls)
- Water
- Index Plate
- Clear PCR plate seal (Plate Kit)

User Provided

- Pipets & tips
- 1.5 mL microfuge tube
- 96- well PCR Plate
- Ice
- Thermocycler

! **IMPORTANT:** Do not disturb beads at bottom of WTA Reaction plate when recombining reactions

! **IMPORTANT:** Keep track of sample position and corresponding Index ID for multiplexing

1. Thaw PCR Enzyme, **Index PCR Input Control** , and **Index Plate** at room temperature, ~15 minutes, transfer to ice until use
2. Set-up Thermocycler program for Index PCR, with heated lid

Step	Temp	Time	# of Cycles
1	95°C	5 minutes	1
2	95°C	30 seconds	7
3	60°C	30 seconds	
4	72°C	1 minute	
5	72°C	3 minutes	1
6	4°C	hold	

Note: For 1,000-2500 cell input, increase the Index PCR to 8 cycles.
For 500-1,000 cell input, increase the Index PCR to 9 cycles.

3. Add 25 ul PCR Enzyme per sample to a new PCR plate. Label this plate Index PCR Reaction Plate. Treat **Index PCR Input Control** as an additional sample.
4. Keeping the purified WTA product plate on the magnet, remove 15 ul of supernatant and add to the wells containing the PCR Enzyme on the Index Reaction plate. Add 2 ul of the Index PCR Input Control and 13 ul of water to the remaining well.

! **IMPORTANT:** Save remaining purified WTA product at -20 for future experiments or troubleshooting.

5. Select and record Index IDs to be used for each sample (see Index Plate Layout Appendix 3). Use one unique Index per sample to multiplex libraries for sequencing.
6. Pierce foil seal on Index Plate with pipet tip and add 10 µL of designated Index to each well
7. Seal Index PCR Reaction Plate firmly with clear seal
8. Run on thermocycler using Index PCR program (~30 minutes)
9. Bring reagents to room temperature and prepare solutions for Index SPRI Clean-Up (STEP I)
   STOPPING POINT: Store overnight at 4°C , or long-term at -20°C

STEP I: Index SPRI Clean-Up

Materials

- SPRI Beads
- Wash A

User Provided

- 96-well plate bar magnet
- Ethanol, absolute (EtOH)
- Pipets & tips
- Reagent reservoir (optional: for multichannel pipet)
- 1.5 mL microfuge tube, x2
- PCR Plate or strip tube

1. Make Fresh 80% EtOH, ~200 µL per sample
2. Resuspend SPRI beads by vortexing. Add 40 µL to each well, pipet thoroughly to mix
3. Incubate for three minutes
4. Place the PCR plate on the magnet for two minutes for magnetic beads to bind; the liquid will turn clear
5. Remove the supernatant and discard
6. Remove the plate from the magnet and add 50 µL of water to each well. Pipet to mix
7. Incubate for three minutes to elute DNA from beads
8. Add 40 µL of fresh SPRI beads to each well (beads from the first binding are still present in the well).
9. Incubate for three minutes to rebind DNA to beads
10. Place the plate back on the magnet for 2 minutes for magnetic beads to bind; the liquid will turn clear
11. Remove and discard supernatant, avoid touching the pellet
12. Add 100 µL of 80% ethanol to all active wells to wash beads
13. Mix by moving the entire plate across the magnet bars so that beads migrate through the EtOH solution ~6 times, then place the plate back down on the magnet
14. Remove and discard EtOH without touching the bead
15. Repeat steps 12-14
16. Remove residual EtOH using 10 µL pipet, leave plate on the magnet, and air-dry for 10 minutes
17. Add 30 µL of Water to each well
18. Remove plate from magnet, mix by pipetting up and down ~10 times, and incubate for three minutes
19. Place the plate on the magnet for one minute
20. Transfer 25 µL of each elution to new wells, or PCR strip tube with lid

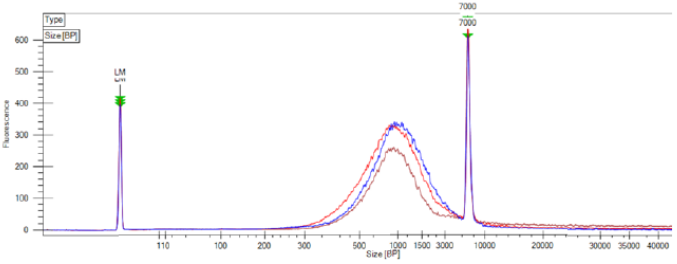
Appendix 1

LIBRARY QC

We recommend Qubit™ for quantification of HIVE library concentrations. Yield may vary depending on the sample type and number of cells loaded. The quantification results from Qubit™ are recommended to be used for pooling normalization. **The expected yield of the library is 1-50 ng/µL.**

We recommend using Tapestation™, Bioanalyzer™ or LabChip GX Touch™ to evaluate library size distribution and quality. Corresponding kits capable of detecting >1,000bp fragments required. **The expected library is a broad smear with a peak that can range from 500-1200 bp.**

Example plot of HIVE libaries with LabChip



LIBRARY POOLING

If you are sequencing multiple libraries, pooling will be required. Sequencing facilities will have minimum library volume and concentration submission requirements , check with your core facility or sequencing provider before pooling.

We recommend using Illumina's instructions for manual normalization and pooling of libraries[1] and how to convert mass concentration (ng/µL) to molar concentration (nM)[2] . We recommend using the universal fragment size of **750 bp** for HIVE libraries for the conversion.

We **highly recommend** quantifying the final pooled samples prior to sequencing with qPCR, using the KAPA Library Quantification Kit™, to ensure accurate loading into the sequencer for optimal clustering density[3].

Other Considerations When Pooling

Samples of the same cell type can be pooled together. We recommend allocating each library to reflect the cell number of each sample. For examples if three samples containing 10,000, 5,000, and 5,000 cells are pooled for sequencing, their proportions should be 50%, 25%, and 25%, respectively, to obtain an even read depth per cell.

We do not recommend pooling two different cell types with different transcript levels, for example a cell line sample and a primary cell sample. If this is necessary, you may need to empirically adjust the allocation to get the desired coverage and read depth per cell.

REFERENCES

1. **Best practices for manually normalizing library concentrations**
2. **Converting mass concentration to molar concentration**
3. **Optimizing Cluster Density on Illumina® Sequencing Systems**

Appendix 2

SEQUENCING SET-UP

HIVE libraries requires paired-end dual-indexed sequencing (we do not recommend spike-in PhiX)

	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
Primers	HIVE Read 1 Seq Primer	HIVE Index 1 Seq Primer	HIVE Index 2 Seq Primer	HIVE Read 2 Seq Primer
Target	Cell Barcode	i7 index	i5 index	insert
Cycles	25	8	8	50

COMPATIBLE SEQUENCERS

Illumina® NextSeq™ 500/550/2000
Illumina® NovaSeq™ 6000

CUSTOM PRIMERS

Custom sequencing primers are provided at **100 µM concentration** (Library Prep Reagents box, red cap). Dual-indexing is required for the sequencing. Illumina® NextSeq™ v2.0 (or later) and Illumina® NovaSeq™ v1.5 kits are compatible with custom primers. Custom primers should be used alone, we do not recommend spike-in with Illumina® standard sequencing primers. Please refer to Illumina's instructions and/or consult sequencing facilities for Custom Primer use[1].

READS PER CELL

We recommend at range of 25,000-50,000 reads per cell. For example, libraries from 3 HIVEs loaded with 15,000 cell each, and an average recovery of 3,000 cells per HIVE, could be pooled and sequenced on one high output NextSeq™ lane (400 millions reads), resulting in ~45,000 reads/cells.

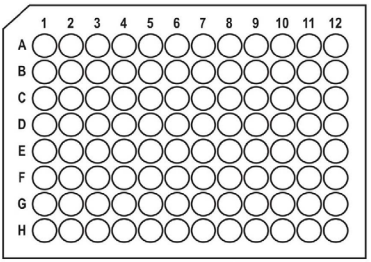
REFERENCES

1. **NovaSeq Custom Primers Guide**

Appendix 3

INDEX PLATE LAYOUT

This is a Combined Dual Index Plate. Each well is an i7+i5 index oligo combination index.



i7 INDEX IDs

Row	Forward Primer	Reverse Complement
A	TCGCCTTA	TAAGGCGA
B	CTAGTACG	CGTACTAG
C	TTCTGCCT	AGGCAGAA
D	GCTCAGGA	TCCTGAGC
E	AGGAGTCC	GGACTCCT
F	CATGCCTA	TAGGCATG
G	GTAGAGAG	CTCTCTAC
H	CCTCTCTG	CAGAGAGG

i5 INDEX IDs

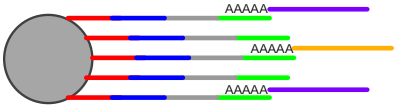
Row	Forward Primer	Reverse Complement
1	TAGATCGC	GCGATCTA
2	CTCTCTAT	ATAGAGAG
3	TATCCTCT	AGAGGATA
4	AGAGTAGA	TCTACTCT
5	GTAAGGAG	CTCCTTAC
6	ACTGCATA	TATGCAGT
7	AAGGAGTA	TACTCCTT
8	CTAAGCCT	AGGCTTAG
9	CGTCTAAT	ATTAGACG
10	TCTCTCCG	CGGAGAGA
11	TCGACTAG	CTAGTCGA
12	TTCTAGCT	AGCTAGAA

Appendix 4

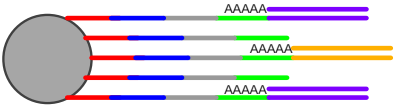
FOLLOW THE MOLECULE

Bead Oligos
Universal Primer Sequence (UPS)
Cell Barcode
Random Linker Sequence

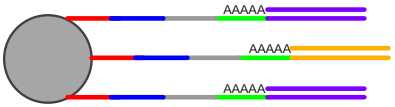
1. Hybridization: Capture poly-A transcripts



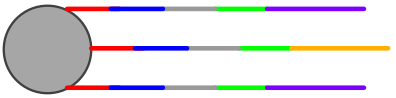
2. 1st Strand Synthesis: Bead oligos acts as primer for making 1st-strand cDNA



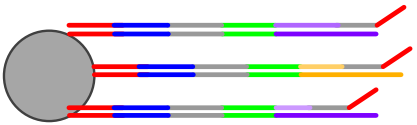
3. Bead Clean-Up: Remove any bead oligos without 1st strand cDNA



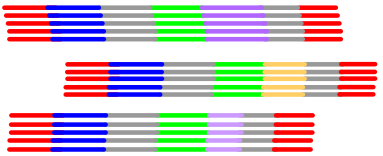
4. NaOH denaturation: Makes 1st strand cDNA single-stranded



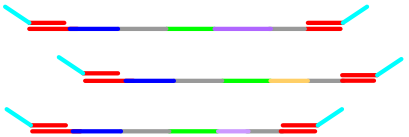
5. 2nd Strand Synthesis: Randomly prime synthesis of 2nd strand cDNA



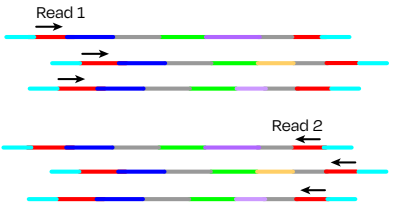
6. WTA: Amplify 2nd strand cDNA with UPS primers



7. Index PCR: Add P5+ i5 and P7+i7 to WTA product with UPS primers , for library multiplexing and Illumina® sequencing



8. Sequencing: Read 1 for cell barcode, and Read 2 for transcript identity



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