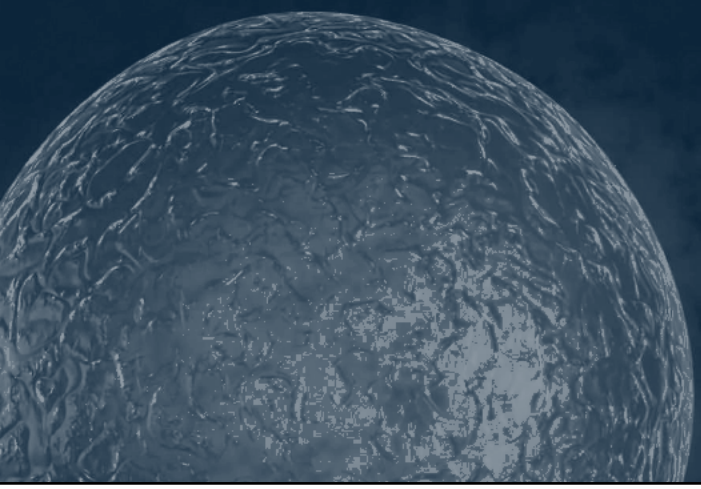

HONEYCOMB

HIVE CLX™ scRNAseq
Transcriptome Recovery & Library Preparation

User Protocol

Version 1.0 | May 2023

Any cell. Any where. Any time.™

HONEYCOMB

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

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Revision History

Version	Date	Description
Version 1.0	May 2023	CLX Product Launch

HIVE CLX™ scRNAseq Transcriptome Recovery and Library Preparation

GENERAL INFORMATION

CLX TRANSCRIPTOME RECOVERY PROTOCOL

CLX LIBRARY PREP PROTOCOL

HIVE CLX™ scRNAseq Transcriptome Recovery and Library Preparation

APPENDICES

GENERAL INFORMATION

PRODUCT OVERVIEW

NOTE

New users require a HIVE UDI Plate and the HIVE CLX Starter Bundle, which includes:

//// Demo Parts
\\\\ Assembled Vacuum
//// HIVE Plates and Seals
\\\\ Lysis Boxes
//// HIVE Accessories

HIVE CLX scRNAseq is a complete solution to create NGS libraries from single cells. Use the HIVE CLX Sample Capture protocol to capture single cells with barcoded capture beads in HIVE Collectors. Use this Transcriptome Recovery and Library Preparation protocol to convert loaded HIVE Collectors into sequencing libraries for scRNAseq.

The HIVE CLX scRNAseq Transcriptome Recovery product contains Spin Parts and HIVE Parts & Reagents for 8 samples. The Library Preparation reagents are included in the HIVE CLX scRNAseq Library Preparation product.

TRANSCRIPTOME RECOVERY PARTS & REAGENTS

NOTE

Store all HIVE Transcriptome Recovery Reagents at 10–35°C (ambient).

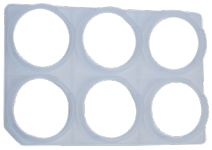
//// HIVE Top with Protective Cover (×8)
\\\\ Bead Collector (×8)
//// Drying Cap (×8)
\\\\ Spin Plate (×2), Spin Lid (×2), HIVE Blanks (×8)
//// HIVE Transcriptome Recovery Reagents (×8): CLX Storage Wash Solution, CLX Lysis Stock, CLX Hybridization Buffer, CLX Bead Recovery Solution, CLX Wash A, CLX SPRI Beads, Water

Bead Collector

Drying Cap

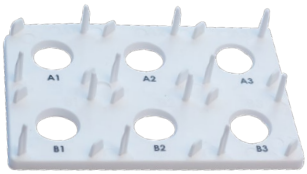
HIVE Blank

Spin Lid



HIVE Top with Protective cover

Red Stopper



Spin Plate

LIBRARY PREP REAGENTS

Reagents (×8 reactions)	Cap Color
CLX Reducing Solution	White
CLX 1st Strand Wash	Pink
CLX 1st Synthesis Buffer	Blue
CLX 1st Strand Enzyme	Blue
CLX Clean-Up Buffer	Green
CLX Clean-Up Enzyme	Green
CLX 1st Strand Control	Clear
10× NaOH (1M)	Clear
CLX 2nd Synthesis Buffer	Violet
CLX 2nd Strand Oligo	Violet
CLX 2nd Strand Enzyme	Violet
CLX WTA Oligo	Orange
CLX PCR Enzyme (×2)	Orange
CLX Read 1 Seq Primer	Red
CLX Read 2 Seq Primer	Red
CLX Index 1 Seq Primer	Red
CLX Index 2 Seq Primer	Red

NOTE

Store all Library Prep reagents at -20°C.

USER-SUPPLIED MATERIALS FOR LIBRARY PREP

//// The Starter Bundle includes one set of plates, sealing films, and foils. Refer to this list to purchase more materials.
\\\\ The Assembled Vacuum includes the 96-well manifold and vacuum trap system. The vacuum pump/line is sold separately.

Material	Recommended Product
96-well filter plates	Millipore MultiScreen™ HTS 96 HV, opaque CAT# MSHVN4B10
96-well deep well storage plate	Abgene™, 0.8 mL, polypropylene CAT# AB0765
96-well PCR plate	Thermo Scientific, semi-skirted, flat deck CAT# AB1400L
Adhesive PCR sealing film	Bio-Rad Microseal™ 'B', optical CAT# MSB1001
Adhesive PCR sealing foil	Excel Scientific eXTReme™ FoilSeal™ CAT# XTR-FOIL-100
96-well vacuum manifold	Millipore Multiscreen™ CAT# MAVM0960R
Vacuum trap system	VWR Vactrap™ 2 HDPE, 2+1 L bottle assembly with tray, 0.2 µm vent filter, not autoclavable CAT# 76207-602
Vacuum pump/line (optional)	Cole-Parmer Air Admiral®, 0.37 cfm CAT# EW-79202-00

NOTE

You may use an existing vacuum pump that pulls ≤15 inches Hg (≤381 mm Hg) and fits tubing with an inner diameter of 0.25 inches (0.63 cm).

IMPORTANT!

If using the Transcriptome Recovery Kit without the Library Prep Kit, you must supply Reducing Solution (1 M DTT).

ADDITIONAL USER-SUPPLIED MATERIALS

//// Molecular biology grade ethanol (absolute)
\\\\ Wescodyne® (bleach alternative)
//// Single-channel P1000 (1,000 µL) pipette & tips
\\\\ Single-channel P200 (200 µL) pipette & tips
//// Single-channel P10 (10 µL) pipette & tips
//// 15 mL centrifuge tubes, RNase-/DNase-free
\\\\ 1.5 mL microfuge tubes, RNase-/DNase-free
\\\\ Absorbent paper towels
\\\\ 8-channel P200 & P10 pipettes (optional)
//// 5–25 mL serological pipettes (optional)
\\\\ 25–50 mL reagent reservoirs (optional), RNase-/DNase-free
//// PCR tube strips (optional), RNase-/DNase-free

NOTE

//// Recommended thermocycler: Eppendorf Mastercycler® X50 (CAT# 2231000923)

\\\\ Recommended bar magnet: Invitrogen DynaMag™-96 Side Skirted (CAT# 12027)

//// Recommended for DNA quantification: Thermo Scientific Qubit™ 4 Fluorometer (CAT# Q33238)

\\\\ Recommended for fragment analysis: LabChip GX Touch™, TapeStation™, or Bioanalyzer™ systems

//// Recommended TapeStation Kit: D5000 ScreenTape Assay and Reagents

REQUIRED EQUIPMENT

//// -20°C freezer, 4°C refrigerator, and ice bucket
\\\\ 37°C oven or Filter Plate Adapter (see Page 8)
//// Thermocycler for 96-well plates
\\\\ Bar magnet for 96-well plates
//// Benchtop vortex mixer
\\\\ Centrifuge
//// Instrument for DNA quantification
\\\\ Instrument for DNA fragment analysis with analysis kit for >1,000 bp DNA smears

NOTE

Example of compatible centrifuge: Eppendorf 5810™ with S-4-104 rotor and MTP/Flex buckets.

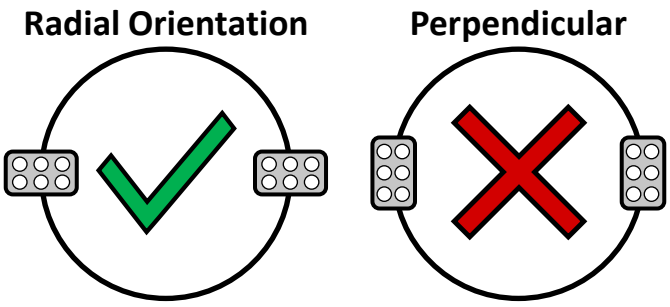
NOTE

The radial plate orientation prevents smearing of the pellet when collecting beads. If laboratory equipment is limited to the perpendicular orientation, then use positions A2 and B2 only on the HIVE Spin Plate.

CENTRIFUGE SPECIFICATIONS

//// Minimum 1800 RCF spin speed
\\\\ Swinging-bucket centrifuge
//// Compatible with deep-well plates
\\\\ Radial plate orientation (see diagram below)

Plates must be loaded into the centrifuge in a radial orientation



HIVE ACCESSORIES

New users will require the HIVE CLX scRNAseq Starter Bundle, which includes HIVE Accessories such as the Closure Tool and Filter Plate Adapter.

Use the Closure Tool to attach the HIVE Top and (optionally) the Bead Collector onto the HIVE Base.

The Filter Plate Adapter can convert thermocycler blocks into 37°C incubators for filter plates. Check lid clearance and fit in the heating block before use.

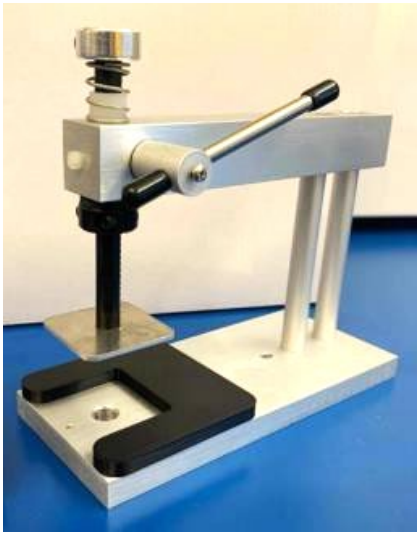
WARNING!

The Closure Tool has a pinch point. Keep hands clear during operation.

NOTE

The Filter Plate Adapter reduces clearance height between the heating block and lid.

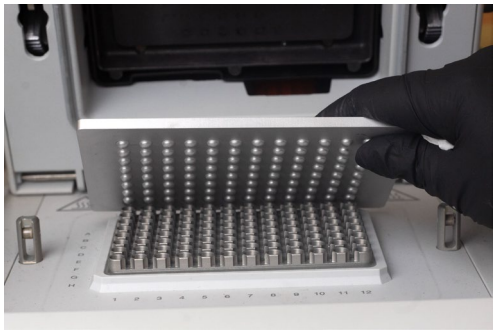
Closure Tool



Filter Plate Adapter

Top (fits Filter Plate)

Bottom (fits thermocycler)



CLX TRANSCRIPTOME RECOVERY PROTOCOL

CLX TRANSCRIPTOME RECOVERY WORKFLOW

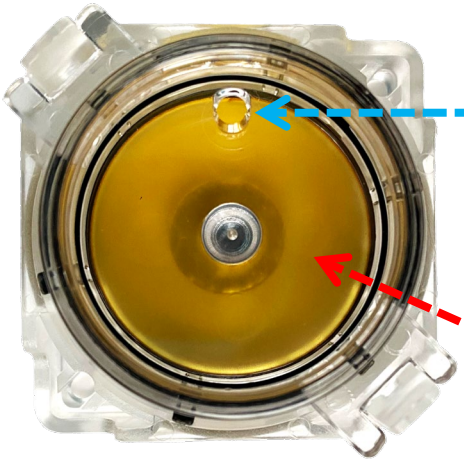
HOW TO HANDLE THE HIVE COLLECTORS

Handle the HIVE Collector along the edges of the device. Keep port at 12 o'clock position.

IMPORTANT!
Pipette along inside of the Cell Loader wall. Avoid pipetting directly onto the yellow HIVE Array.

NOTE
We recommend a vacuum aspirator to easily remove liquids and bubbles that may appear after spinning (see Troubleshooting on Page 62).

HIVE Collector



Port for Pipetting
Insert tip through port and gently pipette at an angle. Dispense along Cell Loader wall.

Yellow HIVE Array
Array of picowells pre-loaded with barcoded beads

IMPORTANT!
Always keep HIVE Collectors flat during transportation and incubation. Only tilt when directed to do so.

NOTE
Keep the port at 12 o'clock with the port edge farthest from you.

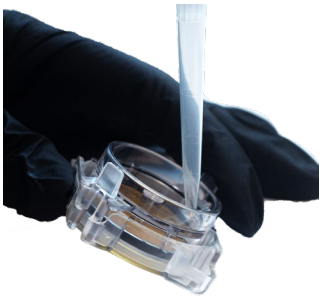
Tilt Towards You

When adding liquid into the HIVE Collector

Keep HIVE Flat

When transporting and incubating

Tilt Away From You



When removing liquid from the HIVE Collector

QUALITY CONTROL FOR SHIPPING & RECEIVING

IMPORTANT!
For quality control, photograph the frozen HIVE Collectors before and after shipping.

Good shipping

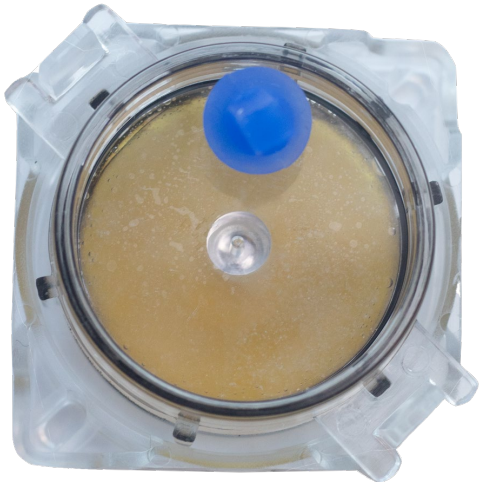
Frozen liquid is white and completely covers the HIVE Array.

Blue Stopper remains in place.

Bad shipping

Frozen liquid is white, but the liquid does not cover the right side (the yellow HIVE Array is visible). The HIVE was tilted during the freezing process.

Blue Stopper is missing.



Bad shipping

Frozen liquid is clear, which indicates CLX Cell Preservation Solution was not used.

Non-frozen liquid indicates that sample thawed during shipping.

Video Protocol

STEP A: SEAL CELL-LOADED HIVE

Materials required

- Loaded HIVE Collectors
- HIVE Tops with Protective Covers
- Drying Caps
- Spin Plate and HIVE Blanks

1. Prepare the **Closure Tool** workstation with **HIVE Tops with Protective Covers** and **Drying Caps.**
2. Remove **HIVE Collectors** from freezer.
3. Fully thaw HIVE Collectors at **37°C for ~15 minutes** or at room temperature for ~60 minutes.
4. Remove **Blue Stopper** from ports. Discard.
5. Wash each HIVE by performing the following steps, **one HIVE at a time.**
   a. **Tilt the HIVE Collector away from you.** Remove all liquid (~2 mL) by aspiration or pipetting.
   b. **Tilt the HIVE Collector towards you.** Dispense 2 mL of **CLX Storage Wash Solution** through the port.
   c. **Place the HIVE Collector flat.** Gently swirl the HIVE Collector to cover the entire HIVE Array.
   d. Repeat Step 5a-c for each remaining HIVE, **one at a time.**

IMPORTANT!
Always keep HIVE Collectors flat. Only tilt when directed to do so.

IMPORTANT!
For CLX Cell Preservation Solution and CLX Storage Wash Solution, **DO NOT MIX WITH BLEACH!**

6. At **Closure Tool** workstation, squeeze **HIVE Tops** and **Protective Covers** together until the assembly fully clicks into place.

HIVE Top With Protective Cover

Squeeze assembly. Listen for a *click* as the HIVE Top snaps into place.

7. Bend out the black arms of the **Protective Cover**, lift to remove and discard. Place the **HIVE Top** on the benchtop with membrane facing up.

IMPORTANT!
Keep membrane face up.
Minimize dust exposure.
Do not touch membrane!

Protective Cover
Bend arms out

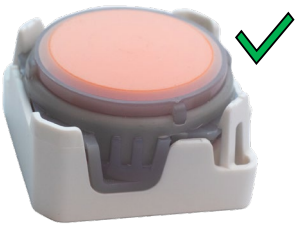
Protective Cover
Remove and discard

DO NOT TOUCH!

HIVE Top
Inner gray frame with membrane

8. Ensure that the **HIVE Top** is fully clicked into place.
   - If assembly was squeezed correctly, the white arms of the HIVE Top will grip the gray membrane frame.
   - If not, press down on edge of gray membrane frame to *click* in the frame.

Inspect HIVE Top



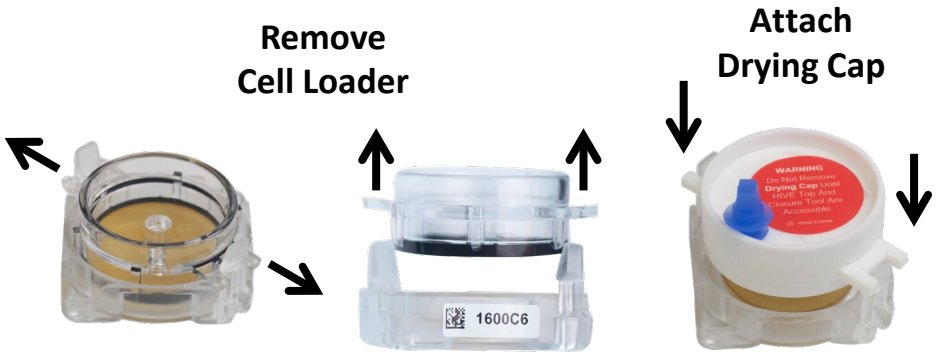
Gray frame is snapped into white housing

White arms do not fully snap over gray frame

IMPORTANT!
Do not dry out the HIVE array! Handle HIVE Collectors one at a time, and **immediately** attach a Drying Cap.

9. **Tilt the HIVE Collectors away from you.** Remove all **CLX Storage Wash Solution** by aspiration or pipetting.

10. Remove the **Cell Loader** from the **HIVE Base** and **immediately attach a Drying Cap** to the Base.
    - To remove the Cell Loader: Push outwards on the HIVE Base tabs while lifting upwards on the wings of the Cell Loader. Discard the Cell Loader.
    - To attach a Drying Cap: Align wings of the Drying Cap to HIVE Base tabs. Snap into the HIVE Base. The red sticker on the Drying Cap should face upwards.



11. Place **HIVE Base with Drying Cap** in the **Spin Plate**. Align open corners in the HIVE Base with the pins of the Spin Plate.
    - Split HIVEs evenly between two spin plates. Balance plates with HIVE Blanks, if needed.

12. Place assembled plates in centrifuge. Set centrifuge to **400 RCF** and spin. Stop the spin once the centrifuge reaches 400 RCF.
    - Set centrifuge to maximum acceleration and maximum braking.

13. Take assembled plates to Closure Tool workstation.

IMPORTANT!
HIVEs should be sealed (Steps 13-17) within 5 minutes of centrifugation.

14. Remove the centrifuged HIVE Base assemblies from the Spin Plate. Remove the **Blue Stopper** from the Drying Cap Port.

15. With the Drying Cap Port at the 6 o'clock position, **tilt the HIVE Base assembly towards you** and gently tap to pool liquid at the Drying Cap Port. Remove remaining liquid by aspiration or pipetting.

Rotate Drying Cap Port to 6 o'clock position

IMPORTANT!
Complete this step one HIVE at a time to avoid drying out the HIVE array! Avoid touching the membrane or array surface.

16. **In less than 5 seconds per HIVE, quickly exchange a Drying Cap with a HIVE Top as follows:**
    a. Remove Drying Cap: Gently push out on HIVE Base tabs and lift up on Drying Cap wings. Dispose of the Drying Cap.
    b. Pick up and flip over the HIVE Top: Membrane will be facing down.
    c. Align open corners of the HIVE Top and Base: Align and assemble HIVE Top and Base but DO NOT squeeze or click closed.
    d. Use Closure Tool: Slide assembly into Closure Tool and use lever. Close Tool slowly and press through the resistance to *click* together. Compress only once.
    e. Repeat 16a-d with remaining HIVEs.

WARNING!
The Closure Tool has a pinch point. Keep hands clear during operation.

17. Incubate the closed HIVE Base and Top assemblies at room temperature for **30 minutes** to seal the membrane onto the HIVE Array.

Video Protocol

STEP B: LYSIS & HYBRIDIZATION

Materials required

- CLX Lysis Stock
- CLX Hybridization Buffer
- CLX Reducing Solution (white cap)
- HIVE Spin Plate
- Lysis boxes from Starter Bundle (optional)

NOTE

If continuing with the Library Prep, prepare 37°C incubation setup to thaw reagents ahead of time (see Step 11).

1. Thaw **CLX Reducing Solution** (white cap) at room temperature. Vortex Solution if white pellet does not dissolve during thaw. Spin down.

2. Prepare **Lysis Solution** by pipetting **CLX Lysis Stock** and **CLX Reducing Solution** into sterile tube. Follow table below.

Lysis Solution Component	1 sample	8 samples	N Samples
CLX Lysis Stock	1,000 µL	8,000 µL	
CLX Reducing Solution	100 µL	800 µL	
Total Volume	**1,100 µL**	**8,800 µL**	
Volume Used in STEP B	1,000 µL	8,800 µL	
Excess Volume	*100 µL*	*800 µL*	

IMPORTANT!

All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

NOTE

HIVE Serial # is on the sticker on the HIVE Base.

Spin Plates are not pre-labeled. Mark Plate with a unique Plate ID.

3. Use the **Sample Tracking Table** (Appendix 1) to record the Serial # and Sample ID of each HIVE. Record the Spin Plate ID and Plate Position of each assembly in the next steps.

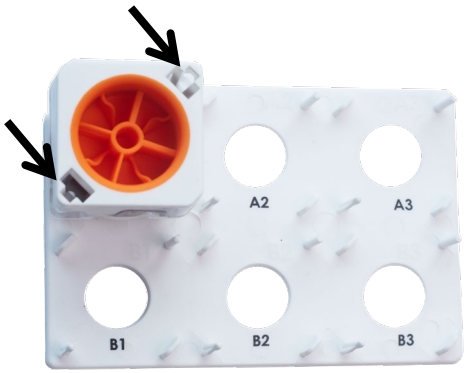
4. Place the sealed **HIVE assemblies** on the **Spin Plate** by aligning the HIVE open corners over Spin Plate pins. Do not push down until the next step.

5. Lyse each HIVE by performing the following steps, **one HIVE at a time.**

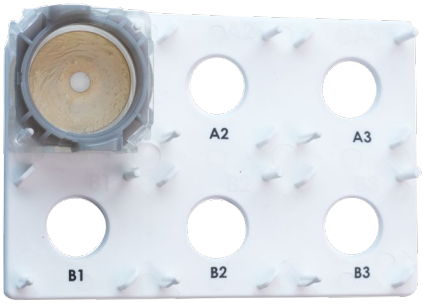
   a. Remove white/orange top: Gently press down on the open corners of a HIVE assembly to pop off the white/orange top. Discard the top.
      - If the white/orange top does not pop off, check that the HIVE is aligned on Spin Plate pins, repeat compression, and lift off the orange top.

   b. Immediately dispense 1 mL **Lysis Solution** and swirl plate: Pipet directly onto the exposed membrane and swirl to cover the array. Do not dry out the HIVE array!

IMPORTANT!
Do not dry out the HIVE array!
Make sure to work one HIVE at a time.



Align assembly onto Spin Plate
Press down on open corners



Remove and discard orange top

IMPORTANT!
For Lysis Solution,
DO NOT MIX WITH BLEACH!

NOTE
To contain the chemical odor, place the Spin Plate in a Lysis Box.

6. Incubate HIVEs on the Spin Plate with **Lysis Solution** at room temperature for **5 minutes**.

7. **Tilt the Spin Plate towards you.** Remove **Lysis Solution** from all membranes by aspiration or pipetting.
   - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.

8. Dispense 1 mL **CLX Hybridization Buffer** onto all membranes. Swirl plate to cover the array.

IMPORTANT!
For CLX Hybridization Buffer, **DO NOT MIX WITH BLEACH!**

9. **Tilt the Spin Plate towards you**. Remove **CLX Hybridization Buffer** from all membranes by aspiration or pipetting.
   - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.
10. Again, dispense 1 mL **CLX Hybridization Buffer** onto all membranes. Swirl plate. Incubate at room temperature for **15 minutes**.
11. If continuing with **Library Preparation**, during the 15-minute incubation in Step 10, prepare for STEP D:
    a. Thaw one tube of **CLX 1st Strand Control** (clear cap) at room temperature.
    b. Thaw **CLX 1st Strand Wash** (pink cap) and **CLX 1st Synthesis Buffer** (blue cap) at **37°C**.
    c. Retrieve **CLX 1st Strand Enzyme** (blue cap) and place on ice.
    d. Vortex reagents and spin down. Keep reagents on ice until use.
    e. Prepare Vacuum setup:
       - Cover a 96-well Filter Plate with a foil seal. Be careful to seal all wells, and DO NOT remove the clear plastic bracket at the bottom of the Filter Plate.
       - Place sealed Filter Plate on vacuum manifold.
       - Plug in pump to an electrical outlet and turn on.
       - Press down firmly on the 96-well Filter Plate and check the vacuum gauge. The vacuum should be between 5–15 inch-Hg (127–381 mmHg).
       - Turn off pump and let vacuum gauge return to 0 before removing Filter Plate.

NOTE
If needed, turn valve clockwise to increase vacuum or counterclockwise to decrease.

Video Protocol

STEP C: BEAD RECOVERY

Materials required

- CLX Bead Recovery Solution
- Bead Collectors
- Red Stoppers
- Filter Plate (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Spin Plate, Spin Lid, and HIVE Blanks

IMPORTANT!
Perform Steps 1–3 one HIVE at a time.
See Page 19 for illustrations.

1. **Tilt the Spin Plate towards you** and remove **CLX Hybridization Buffer** from one membrane.
   - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.

IMPORTANT!
CLX Hybridization Buffer may contain trace lysis buffer, so **DO NOT MIX WITH BLEACH!**

2. Remove the **membrane** from the **HIVE base** by performing the following steps:
   a. Use your right hand to pull the lower right HIVE Base tab towards you.
   b. Use your left hand to gently peel the membrane away from you.
   c. Discard the membrane with the attached gray frame.

IMPORTANT!
If both tabs do not *click* together, the HIVE will leak in the centrifuge.

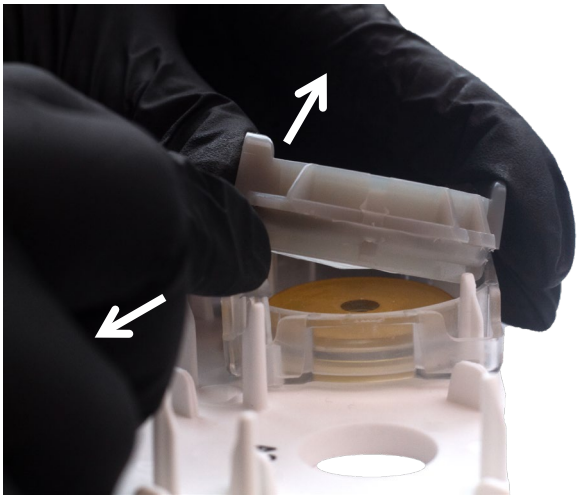
3. Place a **Bead Collector** onto a **HIVE Base** and compress until you hear ***2 CLICKS*** (one per tab).
   - Align Bead Collector wings with the Base tabs.
   - If it is too difficult to compress by hand, put Spin Plate in the Closure Tool and close slowly and firmly until you hear ***2 CLICKS***.

WARNING!
The Closure Tool has a pinch point. Keep hands clear during operation.

4. Repeat Steps 1–3 for each HIVE.

Step 2: Peel off membrane

Place left hand on the grey frame
Use right index finger to pull lower right HIVE Base tab towards you



Use left hand to peel membrane from bottom right to top left, removing membrane completely

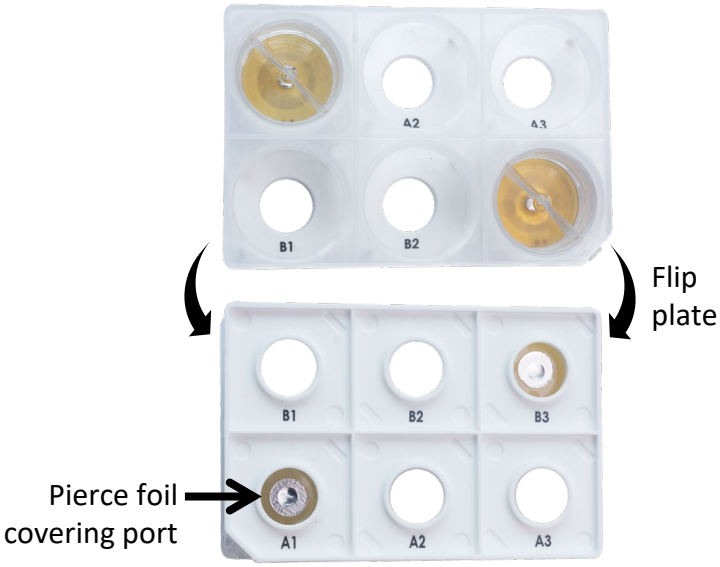
Step 3: Compress Bead Collector

Listen for *2 CLICKS*

IMPORTANT!
If either tab does not *click* together, the HIVE will leak in the centrifuge.

5. Balance **Spin Plate(s)** with **HIVE Blanks**, if needed.

6. Place **Spin Lid** on **Spin Plate**. Flip Spin Plate and Lid assembly upside down. Place on benchtop.
   - Firmly grasp Spin Plate and Lid with both hands as you flip the assembly upside down.



7. Use 1 mL pipette tip to pierce foil and completely open the **HIVE Base Ports** (see picture above).

8. Use pipette to quickly dispense 1 mL **CLX Bead Recovery Solution** into the Port. Repeat 2 more times for a total of 3 mL.
   - Hold the pipette straight up and at the Port opening while dispensing liquid.

9. Insert **Red Stoppers** into the **HIVE Base Ports**.

10. Use pipette or absorbent paper towel to remove any residual solution around the **Red Stopper**.

11. Repeat Steps 5–10 for all **Spin Plates**.

12. Place the **Spin Plate(s)** in centrifuge with the **Red Stoppers** facing upwards. Spin at max speed (≤3,000 RCF) for **5 minutes**.

TROUBLESHOOTING TIP

If converting RPM to g-force (RCF), confirm the radius of the rotor first.

12. Assign an unused well of the Filter Plate for the samples and the **CLX 1st Strand Control** (use the **Sample Tracking Table** in Appendix 1).

13. Remove the foil seals from the assigned wells of the Filter Plate.
    - Do NOT remove the bracket at the bottom of the Filter Plate.

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the Spin Plate position and Filter Plate well for each sample.

14. Set pipette to 300 µL. For each sample, transfer bead pellet to assigned Filter Plate well:
    a. Remove **Red Stopper**. Set aside.
    b. Gently touch the pipette tip to the bottom of the **Bead Collector** and remove bead pellet.
    c. Transfer each bead pellet to the assigned well on the Filter Plate.
    d. Mix beads in well by gently pipetting up and down 5 times to break up any clumps.
    e. Re-insert the **Red Stopper**.
    f. Repeat Step 14 for each sample.

NOTE
You do not need to add any liquid to the Bead Collector after Step 14.

15. Place **Spin Plate(s)** in centrifuge with **Red Stoppers** facing upwards. Spin at max speed (≤3,000 RCF) for **1 minute**.
    - Balance the Spin Plate with HIVE Blanks, if needed.

16. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0 then remove Filter Plate from vacuum manifold.

17. Remove the **Spin Plate(s)** from the centrifuge.
18. Perform second bead pellet transfer by repeating step 14:
    a. Remove **Red Stopper**. Set aside.
    b. Remove bead pellet from the **Bead Collector**.
    c. Transfer each bead pellet to the same assigned well from step 14.
    d. Mix beads in well by gently pipetting up and down 5 times to break up any clumps.
19. Immediately proceed to STEP D if using the **Library Preparation** product.
    - You have now completed Transcriptome Recovery.

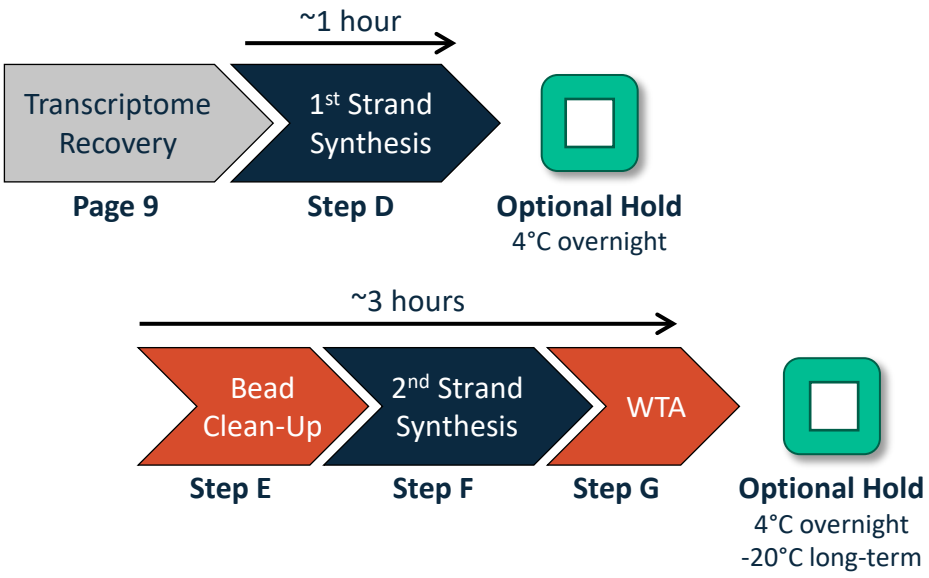
IMPORTANT!

Use the Sample Tracking Table (Appendix 1) to keep track of the Spin Plate position and Filter Plate well for each sample.

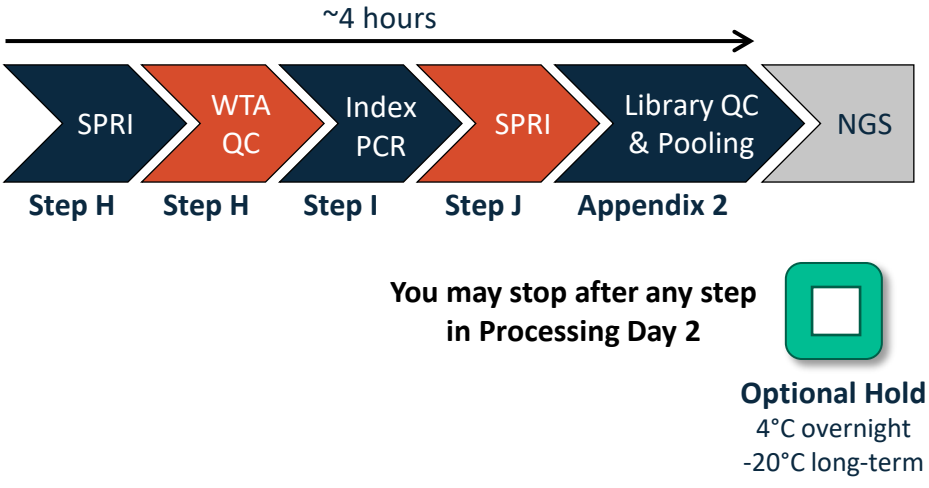
CLX LIBRARY PREP PROTOCOL

CLX LIBRARY PREP WORKFLOW

Processing Day 1



Processing Day 2



Video Protocol

STEP D: 1ST STRAND SYNTHESIS

Materials required

- CLX 1st Strand Wash (pink cap)
- CLX 1st Synthesis Buffer (blue cap)
- CLX 1st Strand Enzyme (blue cap)
- CLX 1st Strand Control (clear cap)
- CLX Bead Recovery Solution
- Clear PCR plate seals (Plate Kit)
- Filter Plate adaptor (optional)
- Vacuum setup (Vacuum Kit)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.
2. Dispense 250 µL of **CLX Bead Recovery Solution** into the thawed tube of **CLX 1st Strand Control**. Pipet to resuspend.
3. Rapidly pipet up-and-down 10 times to mix the **CLX 1st Strand Control** beads, and **immediately** transfer the entire tube contents to the assigned well on the Filter Plate.
4. Prepare **1st Strand Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
   - **Count the CLX 1st Strand Control as a sample!**

1st Strand Reaction Mix Component	1 sample	8 samples	N Samples
CLX 1st Synthesis Buffer	175 µL	1,400 µL	
CLX 1st Strand Enzyme	15 µL	120 µL	
Total Volume	**190 µL**	**1,520 µL**	
Volume Used in STEP D	175 µL	1,400 µL	
Excess Volume	*15 µL*	*120 µL*	

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

5. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
6. Dispense 175 µL **CLX 1st Strand Wash** (pink cap) into each Filter Plate well with sample beads.

IMPORTANT!
DO NOT FLIP OVER FILTER PLATE!

7. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
8. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 5.
9. Pat dry the bottom of the Filter Plate with an absorbent paper towel.

NOTE
Cut the clear adhesive PCR plate film into strips and cover the active wells.

10. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
11. Dispense 175 µL **1st Strand Reaction Mix** (Step 4) into each Filter Plate well with sample beads.
12. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
13. Use scissors to cut a strip of clear adhesive PCR plate film that fits over the Filter Plate wells. Seal the sample wells.
14. Incubate Filter Plate at **37°C for 60 minutes**.
15. If not pausing overnight, proceed to STEP E and prepare the **Clean-Up Reaction Mix**.

IMPORTANT!

Only perform the washes in Step 16 if pausing overnight. If continuing with Library Prep, proceed to STEP E.

16. To pause the protocol and store the Filter Plate overnight, complete the following washes after the 60-minute incubation in Step 14:

    a. Carefully remove the bottom and top films from the Filter Plate.

    b. Use the vacuum manifold to remove liquid from the plate as previously described in Step 5.

    c. Dispense 175 µL **CLX Wash A** into each sample well.

    d. Use the vacuum manifold to remove liquid from the plate as previously described in Step 5.

    e. Pat dry the plate bottom with an absorbent paper towel.

    f. Use a clear adhesive film to seal the plate bottom.

    g. Dispense 175 µL **CLX Wash A** into each sample well.

    h. Seal the top of the sample wells with adhesive film.

    i. Store the sealed Filter Plate overnight at 4°C.

PAUSE POINT

Video Protocol

STEP E: BEAD CLEAN-UP

Materials required

- CLX Clean-Up Buffer (green cap)
- CLX Clean-Up Enzyme (green cap)
- CLX Wash A
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Filter Plate adaptor (optional)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.
2. Thaw **CLX Clean-Up Buffer** (green cap) at **37°C**. Vortex, spin down, and keep on ice until use. Retrieve **CLX Clean-Up Enzyme** (green cap), spin down, and keep on ice until use.
3. Prepare **Clean-Up Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
   - **Count the CLX 1st Strand Control as a sample!**

Clean-Up Reaction Mix Component	1 sample	8 samples	N samples
CLX Clean-Up Buffer	175 µL	1,400 µL	
CLX Clean-Up Enzyme	10 µL	80 µL	
Total Volume	**185 µL**	**1,480 µL**	
Volume Used in STEP E	**175 µL**	**1,400 µL**	
Excess Volume	*10 µL*	*80 µL*	

4. Carefully remove the adhesive film from the bottom, then top of the Filter Plate.

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

NOTE
There may be some liquid on the bottom seal.

5. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
6. Dispense 175 µL **CLX Wash A** into each Filter Plate well with sample beads.
7. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 5.
8. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
9. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
10. Dispense 175 µL **Clean-Up Reaction Mix** (Step 3) into each Filter Plate well with sample beads.
11. Tilt the Filter Plate**.** Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
12. Use a clear adhesive PCR plate film to seal the sample wells.
13. Incubate Filter Plate at **37°C for 45 minutes**.

Video Protocol

STEP F: 2nd STRAND SYNTHESIS

Materials required

- CLX 2nd Synthesis Buffer (violet cap)
- CLX 2nd Strand Oligo (violet cap)
- CLX 2nd Strand Enzyme (violet cap)
- 10× NaOH (clear cap)
- CLX Wash A
- Water
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Filter Plate adaptor (optional)
- Reagent reservoirs (optional)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.

2. Thaw **CLX 2nd Synthesis Buffer** (violet cap), **CLX 2nd Strand Oligo** (violet cap), and **10× NaOH** (clear cap) at room temperature for **15 minutes**. Vortex solutions if pellets do not dissolve during thaw. Spin down and transfer to ice until use.

3. Retrieve **CLX 2nd Strand Enzyme** (violet cap), spin down, and keep on ice until use.

TROUBLESHOOTING TIP

The stock 10× NaOH remains stable for 10 freeze-thaw cycles. Mark the vial to keep record of each freeze-thaw cycle.

4. Dilute **10× NaOH** (clear cap) with **Water** to make **1× NaOH**. Keep at room temperature until use.
   - Prepare fresh **1× NaOH** for each experiment.
   - Immediately return stock **10× NaOH** (clear cap) to the -20°C freezer.
   - **Count the CLX 1st Strand Control as a sample!**

Dilution Component	1 sample	8 samples	N samples
Water	180 µL	1,440 µL	
10× NaOH	20 µL	160 µL	
Total Volume	**200 µL**	**1,600 µL**	
Volume Used in STEP F	175 µL	1,400 µL	
Excess Volume	*25 µL*	*200 µL*	

IMPORTANT!

All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

5. Prepare **2nd Strand Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
   - **Count the CLX 1st Strand Control as a sample!**

2nd Strand Reaction Mix Component	1 sample	8 samples	N samples
CLX 2nd Synthesis Buffer	175 µL	1,400 µL	
CLX 2nd Strand Oligo	10 µL	80 µL	
CLX 2nd Strand Enzyme	5 µL	40 µL	
Total Volume	**190 µL**	**1,520 µL**	
Volume Used in STEP F	175 µL	1,400 µL	
Excess Volume	*15 µL*	*120 µL*	

IMPORTANT!

All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

NOTE

There may be some liquid on the bottom seal.

6. Carefully remove the PCR plate film covering the bottom, then top of the Filter Plate.

7. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
8. Dispense 175 µL **CLX Wash A** into each Filter Plate well with sample beads.
9. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 7.
10. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
11. Place Filter Plate onto the vacuum manifold but **do not turn on pump**.
12. Dispense 175 µL **1× NaOH** (Step 4) into each Filter Plate well with sample beads.
13. Tilt Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
14. Incubate for **exactly 5 minutes**.
15. Turn on pump. Press down on the Filter Plate so liquid flows out. Leave the pump on for Step 16.
16. Wash the Filter Plate with CLX Wash A:
    a. Dispense 175 µL **CLX Wash A** into each sample well of the Filter Plate.
    b. Repeat Step 16a 2 more times.
17. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0. Leave the Filter Plate on the vacuum manifold.

18. Remove the Filter Plate from the vacuum manifold. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
19. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
20. Dispense 175 µL **2nd Strand Reaction Mix** (Step 5) into each Filter Plate well with sample beads.
21. Tilt the Filter Plate**.** Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
22. Use a clear adhesive PCR plate film to seal the sample wells.
23. Incubate Filter Plate at **37°C for 30 minutes**.
24. During the 30-minute incubation, prepare reagents for the WTA Reaction in STEP G.

Video Protocol

STEP G: WHOLE TRANSCRIPTOME AMPLIFICATION (WTA)

Materials required

- CLX PCR Enzyme (orange cap)
- CLX WTA Oligo (orange cap)
- CLX Wash A
- Water
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Full-height PCR Plate
- Deep Well Plate
- Filter Plate adaptor (optional)
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Thaw **CLX PCR Enzyme** (orange cap) and **CLX WTA Oligo** (orange cap) at room temperature. Transfer to ice until use.

2. Set up **WTA Reaction Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	98	20 seconds	20
3	60	45 seconds	
4	70	60 seconds	
5	72	2 minutes	1
6	4	hold	—

3. Prepare **WTA Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
   - **Count the CLX 1st Strand Control as a sample!**

WTA Reaction Mix Component	1 sample	8 samples	N samples
CLX PCR Enzyme	200 µL	1,600 µL	
CLX WTA Oligo	40 µL	320 µL	
Water	180 µL	1,440 µL	
Total Volume	**420 µL**	**3,360 µL**	
Volume Used in STEP G	400 µL	3,200 µL	
Excess Volume	*20 µL*	*160 µL*	

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

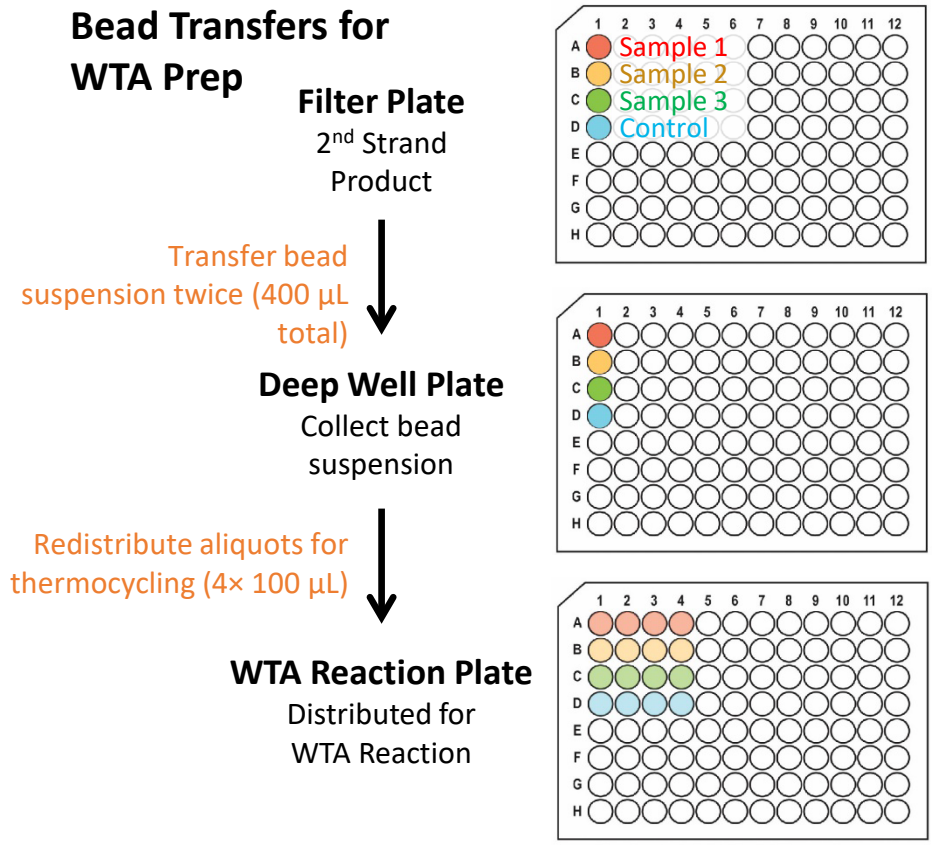
4. Carefully remove the PCR plate film covering the bottom, then top of the Filter Plate.
5. Place Filter Plate on the vacuum manifold. Turn on pump. Press down on the Filter Plate so liquid flows out. Leave the pump on for Step 6.

NOTE
There may be some liquid on the bottom seal.

6. Wash the Filter Plate with **CLX Wash A**:
   a. Dispense 175 µL **CLX Wash A** into each Filter Plate sample well.
   b. Repeat Step 6a 2 more times.
7. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0. Leave the Filter Plate on the vacuum manifold.
8. Remove the Filter Plate. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
9. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.

IMPORTANT!
Ensure all wells are clean and unused.

10. Assign 1 well in the Deep Well Plate and 4 wells in a **WTA Reaction Plate** for each sample using the **Sample Tracking Table** (Appendix 1).



11. Transfer beads from the Filter Plate to the Deep Well Plate, one sample at a time:
    a. Tilt the Filter Plate and dispense 200 µL **WTA Reaction Mix** (Step 3) into each Filter Plate sample well.
    b. Rapidly pipet 10 times to flush sample beads to the right edge of the Filter Plate well.
    c. Immediately transfer the bead suspension to the assigned well on the Deep Well Plate.
12. Repeat Step 11 with a clean pipette tip and another 200 µL **WTA Reaction Mix**. Combine the transfers for a total volume of 400 µL in the assigned well of the Deep Well Plate.

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

13. Set single- or multi-channel pipette to 100 µL. Redistribute beads from Deep Well Plate to the **WTA Reaction Plate**, one sample at a time:
    a. Rapidly pipet 10 times to resuspend beads.
    b. Immediately transfer 100 µL bead suspension to one of the assigned wells on the plate.
    c. Repeat Steps 13a–b 3 more times to transfer remaining beads to 3 additional wells in the plate. This step will result in 4× 100 µL reactions for WTA.
14. Seal the **WTA Reaction Plate** with a clear adhesive PCR film.
15. Place the plate on the thermocycler and run the **WTA Reaction Program** (~1 hour) from Step 2.
16. Store **WTA Reaction Plate** overnight at 4°C or long-term at -20°C.

IMPORTANT!
Beads settle quickly. Fully resuspend beads before each 100 µL transfer.

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

STOP POINT

Video Protocol

STEP H: WTA SPRI CLEAN-UP

Materials required

- CLX SPRI Beads
- CLX Wash A
- Molecular biology grade ethanol (absolute)
- 96-well plate bar magnet
- 1.5 mL microfuge tubes
- PCR Plate or strip tubes
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Dilute absolute ethanol with **Water** to make an **80% Ethanol** solution. Follow table below.
   - **Count the CLX 1st Strand Control as a sample!**

80% Ethanol Component	1 sample	8 samples	N samples
Absolute ethanol	400 µL	3,200 µL	
Water	100 µL	800 µL	
Total Volume	**500 µL**	**4,000 µL**	
Volume Used in STEP H	350 µL	2,800 µL	
Excess Volume	*150 µL*	*1,200 µL*	

2. Label a new PCR Plate as “Purified WTA Product”. Use the **Sample Tracking Table** (Appendix 1) to **assign 2 wells for each sample** in the **Purified WTA Product Plate**.

3. Carefully remove the film sealing the **WTA Reaction Plate** from STEP G.

4. For each sample, combine 25 µL from the 4 wells in the **WTA Reaction Plate** into **1 new well** assigned on the **Purified WTA Product Plate**.

5. Seal the **WTA Reaction Plate** with a clear adhesive PCR film and store at -20°C for future experiments or troubleshooting.

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

NOTE
The Purified WTA Product Plate wells will contain 100 µL after combining the WTA Reaction wells (25 µL each).

6. Vortex the **CLX SPRI Beads** to resuspend. Dispense 90 µL into each well with WTA product. Pipet up and down 15 times to mix thoroughly. Repeat with a fresh pipette tip for each sample.
7. Incubate at room temperature for **3 minutes**.
8. Place the **Purified WTA Product Plate** onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
9. Remove the supernatant and discard.
10. Remove the plate from the magnet.
11. Dispense 50 µL **CLX Wash A** to each well with WTA product. Pipet up and down 15 times to resuspend SPRI beads. Repeat with a fresh pipette tip for each sample.
12. Incubate at room temperature for **3 minutes** to **elute DNA**.
13. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
14. For each sample, transfer the supernatant to the second well assigned in the **Sample Tracking Table** (Appendix 1).
    - **DO NOT DISCARD THE SUPERNATANT!**
15. Remove the plate from the magnet.
16. Vortex the **CLX SPRI Beads** to resuspend. Dispense 45 µL to each well with transferred supernatant. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.

IMPORTANT!
The supernatant in Step 14 contains the WTA product.

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

17. Incubate at room temperature for **3 minutes**.
18. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
19. Remove the supernatant and discard.
20. Wash the SPRI beads with **80% Ethanol**:
    a. Dispense 175 µL of **80% Ethanol** (Step 1) into each well containing WTA product.
    b. Move the entire plate over the bar magnet 6 times to pull the beads through the ethanol solution.
    c. Place the plate onto the magnet for **30 seconds**.
    d. Remove ethanol and discard. Avoid the pellet.
    e. Repeat Steps 20a–d one more time.

NOTE
If using a different style of magnet than the bar type, pipet up and down several times to wash the beads. They will not resuspend in the 80% Ethanol.

IMPORTANT!
Removing the plate from the magnet would cause beads to enter solution and sample loss when pipetting.

22. Use a 10 µL pipette to remove any residual ethanol from the bead pellet. Avoid the pellet.
23. Leave the plate on the magnet for **5 minutes** to air dry.

NOTE
If environment is humid, increase drying time to 10 minutes.

24. Dispense 20 µL **CLX Wash A** to each well with WTA product. Remove the plate from the magnet.
25. Adjust pipette volume to 10 µL and pipet up and down 10 times to mix. Repeat with a fresh pipette tip for each sample.

26. Incubate at room temperature for **3 minutes** to **elute DNA**.

27. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.

    - **DO NOT DISCARD THE SUPERNATANT!**
    - Beads in the Purified WTA Product will not interfere with the downstream application, but you may still choose to transfer product to a clean PCR plate or strip tube. If beads remain in the well, be sure to return the plate to the magnet when withdrawing liquid.

IMPORTANT!
The supernatant in Step 27 contains the purified WTA product.

28. Measure and record the DNA concentration of each sample (and the **CLX 1st Strand Control**) in the **Purified WTA Product Plate**.

NOTE
We recommend using the Qubit™ to quantify DNA concentrations.

NOTE
WTA yield varies with the sample type and the number of cells loaded.

Expect >2 ng/µL WTA product for the CLX 1st Strand control and 1–5 ng/µL for an input of 30,000 PBMCs .

Sample ID	Purified WTA Product Wells	DNA Concentration (ng/µL)
CLX 1st Strand Control		

29. If the purified WTA product is ≥0.2 ng/µL for all samples, proceed to Index PCR in STEP I.

    - If the WTA product is <0.2 ng/µL and the sample contained <2,000 cells, then perform WTA Reamplification (Appendix 5).

IMPORTANT!
If the WTA Product is <0.2 ng/µL and the sample contained ≥2,000 cells, see Troubleshooting on Page 63.

Video Protocol

STEP I: INDEX PCR

Materials required

- CLX PCR Enzyme (orange cap)
- Index Plate
- 96-well PCR Plate
- 96-well plate bar magnet
- Thermocycler

NOTE

- HIVE UDI Plate: clear plate
- HIVE CDI Plate: blue plate

Each well in the Index Plate is a unique i7+i5 oligo index (see Appendix 4). We recommend using the HIVE UDI plate because both i7 (index 1) and i5 (index 2) are unique.

1. Thaw **CLX PCR Enzyme** (orange cap) and **Index Plate** (HIVE UDI Plate or HIVE CDI Plate) at room temperature for **15 minutes**. Transfer to ice until use.
2. Set up **Index PCR Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	95	30 seconds	9
3	60	30 seconds	
4	72	60 seconds	
5	72	2 minutes	1
6	4	hold	—

NOTE

Use one unique Index per sample to multiplex libraries for sequencing.

3. Label a new PCR Plate as “Index Reaction Plate”. Using the **Sample Tracking Table** (Appendix 1), assign an **Index Reaction Plate** well and an Index Plate ID for each sample.

4. Copy DNA Concentrations from Page 40 into the table below. Calculate the volumes of Purified WTA Product needed for 5 ng of DNA for the Index Reaction. Add **Water** (if needed) to bring the volume up to 5 µL.
   - If the WTA Product concentration is <1 ng/µL, then add 5 µL WTA Product to the Index Reaction.
   - If WTA Product concentration is >10 ng/µL, dilute 1 µL of the sample to 1 ng/µL and add 5 µL into the Index Reaction.

Sample ID	DNA Concentration (ng/µL)	Volume Product for up to 5 ng DNA (µL)	Volume Water (µL)
Example	*3.2*	*5/3.2=1.6 µL*	*5–1.6=3.4 µL*
Example	*0.8*	*5 µL*	*0 µL*
CLX 1st Strand Control			

Volume for 5 ng (µL) *= 5 ng / DNA Concentration (ng/µL)*

Volume Water (µL) *= 5 µL – Volume for 5 ng (µL)*

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the sample position and Index ID while preparing the Index Reaction Plate.

5. Dispense 25 µL of **CLX PCR Enzyme** (orange cap) into all assigned wells in the **Index Reaction Plate**.

6. Using the table above, dispense the calculated volume of Purified WTA Product and **Water** into the assigned wells in the **Index Reaction Plate** with **CLX PCR Enzyme**.

NOTE

Each well in the Index Plate is a unique i7+i5 oligo index (see Appendix 4). Record the Index ID!

7. For each sample, use pipette tip to pierce the foil seal on the **Index Plate** well. Dispense 20 µL of Index into the assigned **Index Reaction Plate** well.
8. Seal the **Index Reaction Plate** with a clear adhesive PCR film. Place on thermocycler and run **Index PCR Program** from Step 2.
9. If not pausing overnight, proceed to STEP J and prepare solutions for SPRI Clean-Up.
10. After the Index PCR Reaction, you may leave the **Index Reaction Plate** holding overnight at 4°C in the thermocycler or store long-term at -20°C.

STOP POINT

Video Protocol

STEP J: INDEX SPRI CLEAN-UP

Materials required

- CLX SPRI Beads
- CLX Wash A
- Molecular biology grade ethanol (absolute)
- 96-well plate bar magnet
- 1.5 mL microfuge tubes
- PCR Plate or strip tubes
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Dilute absolute ethanol with **Water** to make an **80% Ethanol** solution. Follow table below.
   - **Count the CLX 1st Strand Control as a sample!**

80% Ethanol Component	1 sample	8 samples	N samples
Absolute ethanol	400 µL	3,200 µL	
Water	100 µL	800 µL	
Total Volume	**500 µL**	**4,000 µL**	
Volume Used in STEP J	350 µL	2,800 µL	
Excess Volume	*150 µL*	*1,200 µL*	

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

2. Assign a well in the same **Index Reaction Plate** from Step I for the Purified Index PCR Product using the **Sample Tracking Table** (Appendix 1).
   - To prevent transfer errors, choose an adjacent column or row and maintain order of the samples.
   - You may also transfer purified product into a strip of PCR tubes (with lid).
3. Carefully remove the film sealing the **Index Reaction Plate** from STEP I.

4. Vortex the **CLX SPRI Beads** to resuspend. Dispense 40 µL to each sample well. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.
    - Pipet the CLX SPRI Beads into the reaction wells, not the wells assigned for purified product.
5. Incubate at room temperature for **3 minutes**.
6. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
7. Remove the supernatant and discard.
8. Remove the plate from the magnet.
9. Dispense 50 µL **CLX Wash A** to each sample well. Pipet up and down 15 times to resuspend SPRI beads. Repeat with a fresh pipette tip for each sample.
10. Incubate at room temperature for **3 minutes** to **elute DNA**.
11. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
12. For each sample, transfer the supernatant to the well assigned in the **Sample Tracking Table** (Appendix 1).
    - **DO NOT DISCARD THE SUPERNATANT!**
13. Remove the plate from the magnet.

IMPORTANT!
The supernatant in Step 12 contains the purified Index PCR product.

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

14. Vortex the **CLX SPRI Beads** to resuspend. Dispense 40 µL to a well with transferred supernatant. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.
15. Incubate at room temperature for **3 minutes**.
16. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
17. Remove the supernatant and discard.
18. Wash the SPRI beads with **80% Ethanol**:
    a. Dispense 175 µL of **80% Ethanol** (Step 1) into every well containing WTA product.
    b. Move the entire plate over the 96-well bar magnet 6 times to pull the beads through the ethanol solution.
    c. Place the plate onto the magnet for **30 seconds**.
    d. Remove ethanol and discard. Avoid the pellet.
    e. Repeat Steps 18a–d one more time.
19. Use a 10 µL pipette to remove any residual ethanol from the bead pellet. Avoid the pellet.

NOTE

If using a different style of magnet than the bar type, pipet up and down several times to wash the beads. They will not resuspend in the 80% Ethanol.

IMPORTANT!

Removing the plate from the magnet would cause beads to enter solution and sample loss when pipetting.

NOTE
If environment is humid, increase drying time to 10 minutes.

21. Leave the plate on the magnet for **5 minutes** to air dry.
22. Remove the plate from the magnet.
23. Dispense 30 µL **CLX Wash A** to a well with Index Reaction product. **Pipet up and down 10 times** to mix. Repeat with a fresh pipette tip for each sample.
24. Incubate at room temperature for **3 minutes** to **elute DNA**.

IMPORTANT!
The supernatant in Step 25 contains the purified Index PCR product.

25. Place the plate onto the magnet for **1 minute**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
    - **DO NOT DISCARD THE SUPERNATANT!**

IMPORTANT!
Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

26. Transfer 25 µL **Purified Index PCR Product** to the assigned well or PCR tube assigned on the **Sample Tracking Table** (Appendix 1).
27. Store **Purified Index PCR Product** at -20°C.
    a. See **Appendix 2** for recommendations for library QC and pooling.
    b. See **Appendix 3** for recommendations for sequencing setup and information on the required custom primers.

IMPORTANT!
CUSTOM PRIMERS ARE REQUIRED (Appendix 3).

STOP POINT

APPENDIX 1: SAMPLE TRACKING TABLE

Sample ID	HIVE Serial #	Spin Plate Position/ID	Filter Plate Well	Deep Well Plate Position	WTA Reaction Wells	Purified WTA Product Wells	Index Reaction Plate Well	Index Plate Type (UDI/CDI)	Index Plate Well ID	Index Plate Purified Product Well
Example	*1437A6*	*A1/Plate-1*	*A1*	*A1*	*A1–A4*	*A1*	*A1*	*UDI*	*A1*	*A4*

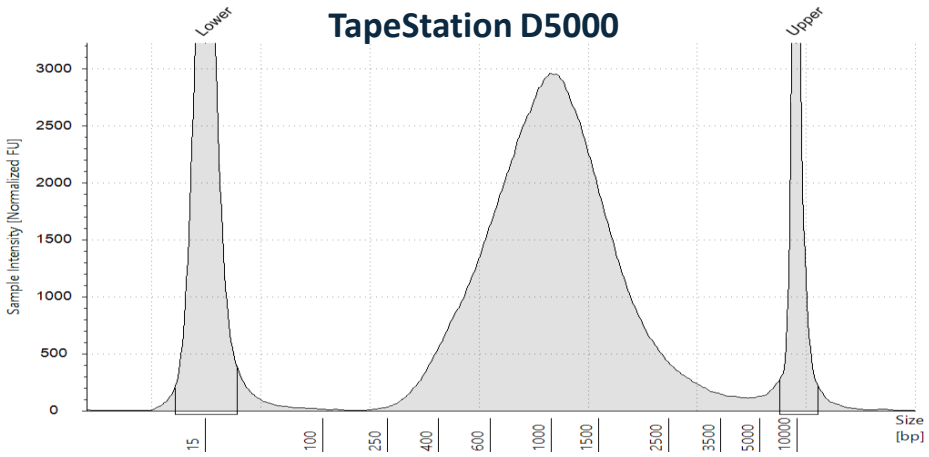
APPENDIX 2: LIBRARY QC & POOLING

LIBRARY QC

We recommend using the Qubit™ to quantify DNA concentrations for QC and for normalization when pooling HIVE libraries. The expected library yield is 1–50 ng/µL, but yield will vary with the sample type and the number of cells loaded.

We recommend using the LabChip GX Touch™, TapeStation™, or Bioanalyzer™ systems and kits suited for sizing >1,000 bp DNA fragments to evaluate library quality and size distributions (recommended TapeStation Kit: D5000 ScreenTape Assay and Reagents). HIVE libraries are expected to have a broad smear of DNA sizes with a peak between 500–1,200 bp.

Example Plot of HIVE Libraries with TapeStation D5000



LIBRARY POOLING

Pooling is required to sequence multiple libraries in a single run. Check with your sequencing provider or core facility before pooling, because each facility will have submission requirements for minimum library volumes and concentrations.

Illumina® provides instructions for normalizing and pooling libraries[1] and for converting mass concentrations (ng/µL) to molar concentrations (nM).[2] For HIVE libraries, we recommend using the universal fragment size (750 bp) for conversion instead of the peak size from the fragment analyzer.

Prior to sequencing, we highly recommend quantifying the final pooled sample with qPCR using the KAPA Library Quantification Kit™ to ensure accurate loading into the sequencer and optimizing cluster density.[3]

APPENDIX 2: LIBRARY QC & POOLING

OTHER CONSIDERATIONS WHEN POOLING

We recommend pooling libraries with proportions that reflect the number of cells in each sample. For example, if 3 samples contained 10,000, 5,000, and 5,000 cells and were pooled for sequencing, we would pool libraries at 50%, 25%, and 25% proportions, respectively. These proportions would allocate an even read depth per cell across all samples.

Samples with the same cell type can be pooled, but we do not recommend pooling two different cell types with different transcript levels. For example, we would not advise pooling a cell line sample and a primary cell sample. If this type of pooling is necessary, you may need to empirically adjust the sample proportions to achieve the desired coverage and read depth per cell.

REFERENCES FOR APPENDIX 2

1. Illumina® Best practices for manually normalizing library concentrations.
   https://support.illumina.com/bulletins/2017/03/best-practices-for-manually-normalizing-library-concentrations.html
2. Illumina® Converting ng/µl to nM when calculating dsDNA library concentration.
   https://support.illumina.com/bulletins/2016/11/converting-ngl-to-nm-when-calculating-dsdna-library-concentration-.html
3. Optimizing cluster density on Illumina® sequencing systems.
   https://assets.illumina.com/content/dam/illumina-marketing/documents/products/other/miseq-overclustering-primer-770-2014-038.pdf

APPENDIX 3: SEQUENCING RECOMMENDATIONS

SEQUENCING SETUP

HIVE libraries require paired-end, dual-indexed sequencing. We do not recommend spiking in PhiX controls.

	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
Primers	CLX Read 1 Seq Primer	CLX Index 1 Seq Primer	CLX Index 2 Seq Primer	CLX Read 2 Seq Primer
Target	Cell Barcode	I7 index	I5 index	Insert
Cycles	25	8	8	50

COMPATIBLE SEQUENCERS

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

CUSTOM PRIMERS

Custom CLX sequencing primers (red caps) are provided at 100 µM in the Library Prep Reagents. HIVE sequencing requires dual-indexing and is compatible with the Illumina® NextSeq™ 500/550 75-cycle kits, NextSeq™ 2000 P1/P2/P3 100-cycle kits, and NovaSeq™ v1.5 100-cycle kits. Custom sequencing primers should be used alone, and we do not recommend spike-in with Illumina® standard sequencing primers. For custom primers, please refer to the Illumina® instructions[1–3] and/or consult with sequencing facility staff.

APPENDIX 3: SEQUENCING RECOMMENDATIONS

READS PER SAMPLE

Consult the table below to determine the optimal number of reads per sample. For example, if we loaded 2 HIVE Collectors with 30,000 cells each, we could assume an average recovery of 12,000 cells per HIVE. We would then pool the 2 HIVE Collectors on a single NovaSeq™SP flow cell using both lanes (~800M reads per flow cell) to expect ~33,000 reads/cell.

Single-Cell Recovery†	Cell Input	Recommended Reads/Sample (million)‡	# of HIVEs per Novaseq SP flowcell*	# of HIVEs per NextSeq 550 HO flowcell^
220	500	7	na	na
850	2,000	27	na	na
3,000	7,500	100	8	4
6,000	15,000	200	4	2
11,000	30,000	400	2	1
17,000	60,000	800	1	na

†Performance metrics estimated from experimental data using human PBMCs.
‡Recommendations for reads/sample balance the amount of biological information gathered with sequencing costs and should achieve 80% recovery of cells, genes, and transcripts. These are recommended starting points for most applications, but you may need to tailor sequencing depths for your specific experiments.
**The nominal NovaSeq SP flow cell offers 800 M reads.*
^The nominal NextSeq 550 HO flow cell offers 400 M reads.

REFERENCES FOR APPENDIX 3

1. Illumina® NovaSeq™ Series Custom Primers Guide.
   https://support.illumina.com/downloads/novaseq-custom-primers-guide-1000000022266.html
2. Illumina® NextSeq™ 500/550 Series Custom Primers Guide.
   https://support.illumina.com/downloads/nextseq-500-custom-primers-guide-15057456.html
3. Illumina® NextSeq™ 2000 Series Custom Primers Guide.
   https://support.illumina.com/downloads/nextseq-1000-2000-custom-primers-guide.html

APPENDIX 4: INDEX PLATE LAYOUT

HIVE UDI INDEX PLATE LAYOUT (PN 10922, clear plate)

Each well of the Unique Dual Index Plate contains a unique i7+i5 index.

	i7 Index 1	i5 Index 2	
Well ID	Sequencing Primer	Forward Strand Workflow	Reverse Complement Workflow
A01	AATCGTTA	AATAACGT	ACGTTATT
B01	AATCGGCG	AATATTGA	TCAATATT
C01	AATCCGTT	AATATGCT	AGCATATT
D01	AAGATACA	AATATCTG	CAGATATT
E01	AAGACGAA	AATAGATT	AATCTATT
F01	AAGTAAGT	AATAGTCC	GGACTATT
G01	AAGTTATC	AATAGCAA	TTGCTATT
H01	AAGTTGGA	AATACAGG	CCTGTATT
A02	GTCTACAT	TTCTTGAA	TTCAAGAA
B02	TTCGCCGA	GTATACCG	CGGTATAC
C02	TGCGTACA	TTCTCATA	TATGAGAA
D02	GTCGCTGT	TTATATCA	TGATATAA
E02	TTATTATG	TTAGCGCA	TGCGCTAA
F02	TGACTGAA	TTAGACGT	ACGTCTAA
G02	GTACAGCT	TGATCGGT	ACCGATCA
H02	GGACAACG	TGATGGCC	GGCCATCA
A03	CGCTGCTC	GGCAGATC	GATCTGCC
B03	CTGGCCTC	GATCCAAC	GTTGGATC
C03	GAATCAAT	TCTGTGAT	ATCACAGA
D03	TCGGATGT	CTGCGGAT	ATCCGCAG
E03	CGCTATTA	GCGGCCGT	ACGGCCGC
F03	AAGACTGT	GTGGACTA	TAGTCCAC
G03	CAACTGCT	AGTAGTAT	ATACTACT
H03	TTCGAACC	TGTCACCT	AGGTGACA
A04	GATCAACA	CTATGTTA	TAACATAG
B04	GAACTTAT	AGATACGC	GCGTATCT
C04	TGAGTCAG	CCGAACTT	AAGTTCGG
D04	CGAGCCGG	GCGGCTTG	CAAGCCGC
E04	TCTATCAG	CAGTAACC	GGTTACTG
F04	CAATGATG	CACGGACG	CGTCCGTG
G04	CATGATGA	GTTAGAGG	CCTCTAAC
H04	CAGACCAC	GCTTCGGC	GCCGAAGC
A05	CGAAGGAC	GTTGACGC	GCGTCAAC
B05	CGTATTGG	GGTATCTT	AAGATACC
C05	GAATGCTC	GTCTAACA	TGTTAGAC
D05	CGATTATC	GAGTTGAT	ATCAACTC
E05	CGGTGGTA	GCCTAGTA	TACTAGGC
F05	CACAGTAA	CACTAGAG	CTCTAGTG
G05	TGACTACT	CCTTACAG	CTGTAAGG
H05	TTCTGGTG	CCAGTGGT	ACCACTGG
A06	GATGCCGG	ATCTACGA	TCGTAGAT
B06	GAAGCACA	CCTCTGGC	GCCAGAGG
C06	GAATATCC	GACGCCAT	ATGGCGTC
D06	TCGAAGCT	GCACTGAG	CTCAGTGC
E06	TCACCAAT	CACGGCGC	GCGCCGTG
F06	TGGTCATT	GCAGATGG	CCATCTGC
G06	CAGAAGAT	GTACATTG	CAATGTAC
H06	CAATCGAA	GCACACGC	GCGTGTGC

	i7 Index 1	i5 Index 2	
Well ID	Sequencing Primer	Forward Strand Chemistry	Reverse Complement Workflow
A07	CTACGAAG	CTCGACAG	CTGTCGAG
B07	CTTAATAC	CCATTGTG	CACAATGG
C07	CTTATGAA	GCCAATGT	ACATTGGC
D07	CTATCATT	GACCACCT	AGGTGGTC
E07	CTGGAAGC	GGTGCAGA	TCTGCACC
F07	CAACCGTG	CTCTCACG	CGTGAGAG
G07	TGAGGCGC	GGAGACCA	TGGTCTCC
H07	AAGTACAG	GTCACGTC	GACGTGAC
A08	GATGCGTC	GAGGCTGC	GCAGCCTC
B08	GAAGTCTT	ACTACGGT	ACCGTAGT
C08	TCGGCACC	CCAACGTC	GACGTTGG
D08	CGCGCCAA	TGGCTAGG	CCTAGCCA
E08	TCCTCGAT	GTAACTGC	GCAGTTAC
F08	TGGTGCAC	GGAATCAC	GTGATTCC
G08	CAGGTTCC	CGAACACC	GGTGTTCG
H08	CCGTGCCA	GCAGCTCC	GGAGCTGC
A09	CTACGGCA	CCTCGTAG	CTACGAGG
B09	GAAGAGGC	AAGTGCTA	TAGCACTT
C09	AAGAAGCG	GTAGATAA	TTATCTAC
D09	CGAACGGA	CCTACCGG	CCGGTAGG
E09	AAGAGAGC	CAGCCAGT	ACTGGCTG
F09	CCACAATG	CGTTGACG	CGTCAACG
G09	TGAACAGG	GAGAACAA	TTGTTCTC
H09	CATTGCAC	CATGCAGC	GCTGCATG
A10	GATTCCTT	CATAGGCA	TGCCTATG
B10	CGGATAAC	GCCGAACG	CGTTCGGC
C10	CTCACGAT	CTTACGGC	GCCGTAAG
D10	CTACTGAC	GGAGGATG	CATCCTCC
E10	TCAACGAG	CGTCAACC	GGTTGACG
F10	TGTGTGCC	CATCAGGT	ACCTGATG
G10	CAGTGTGG	TGTGAATC	GATTCACA
H10	TTACCTGG	ACGATTGC	GCAATCGT
A11	CTACTCGA	AGATGAAC	GTTCATCT
B11	GAATCTGG	TGTCCACG	CGTGGACA
C11	TCGGTCGA	CCAAGTGC	GCACTTGG
D11	TCTTAAGT	CGCTGAAT	ATTCAGCG
E11	TGCGAGAC	GCCGGCGA	TCGCCGGC
F11	CACCACGG	CGTTGTAA	TTACAACG
G11	TTCCACCA	GGTTAAGG	CCTTAACC
H11	CTGCAACG	GACATTCG	CGAATGTC
A12	GATTCGAG	CCGAGTAT	ATACTCGG
B12	CTGATTGA	GACACACT	AGTGTGTC
C12	TCGGTAAG	CTAACTCA	TGAGTTAG
D12	TTAGAGTC	TGTGACGA	TCGTCACA
E12	CCTGGTGT	GCCTCCGG	CCGGAGGC
F12	TGTGTTAA	GGCACGGT	ACCGTGCC
G12	CCGCTGTT	AGACCGCA	TGCGGTCT
H12	TACTGTTA	GCGAATAC	GTATTCGC

Download the HIVE UDI Index Plate Layout

https://honeycombbio.zendesk.com/hc/en-us/articles/14283947472923-HIVE-UDI-Index-Plate

APPENDIX 4: INDEX PLATE LAYOUT

HIVE CDI INDEX PLATE LAYOUT (PN 10271, blue plate)

Each well of the Combined Dual Index Plate contains an i7+i5 combination index.

I7 INDEX IDs

Row	Sequencing Primer
A	TAAGGCGA
B	CGTACTAG
C	AGGCAGAA
D	TCCTGAGC
E	GGACTCCT
F	TAGGCATG
G	CTCTCTAC
H	CAGAGAGG

I5 INDEX IDs

Row	Forward Strand Workflow	Reverse Complement Workflow
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

Download the HIVE CDI Index Plate Layout

https://honeycombbio.zendesk.com/hc/en-us/articles/14284034410779-HIVE-CDI-Index-Plate

APPENDIX 5: WTA REAMPLIFICATION

WTA REAMPLIFICATION USING PURIFIED WTA PRODUCT

1. Thaw **CLX PCR Enzyme** (orange cap) and **CLX WTA Oligo** (orange cap) at room temperature. Transfer to ice until use.

2. Set up **WTA Re-Amp Reaction Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	98	20 seconds	8
3	60	45 seconds	
4	70	60 seconds	
5	72	2 minutes	1
6	4	hold	—

3. Prepare **WTA Re-Amp Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
   - **Count the CLX 1st Strand Control as a sample!**

WTA Reaction Mix Component	1 sample	8 samples	N samples
CLX PCR Enzyme	50 µL	400 µL	
CLX WTA Oligo	10 µL	80 µL	
Nuclease-free Water	40 µL	320 µL	
Total Volume	**100 µL**	**800 µL**	
Volume Used	95 µL	760 µL	
Excess Volume	*5 µL*	*40 µL*	

IMPORTANT!
All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

APPENDIX 5: WTA REAMPLIFICATION

4. Copy information from the **Sample Tracking Table** (Appendix 1) into the table below. Label a new PCR Plate as "**WTA Re-Amp Reaction Plate**". For each sample, assign a Re-Amp Reaction Plate well.

Sample ID	WTA Reaction Wells	WTA Re-Amp Reaction Wells
CLX 1st Strand Control		

5. Aliquot 95 µL of the **WTA Re-Amp Reaction Mix** to the wells in the **WTA Re-Amp Reaction Plate**.

6. Place the **Purified WTA Product Plate** onto the magnet for **1 minute**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.

7. Dispense 5 µL of purified WTA product (STEP H) to the assigned well of the **WTA Re-Amp Reaction Plate**. Pipet up and down to mix. Repeat with a fresh pipette tip for all samples.

8. Seal the **WTA Re-Amp Reaction Plate** with a clear adhesive PCR film. Place on thermocycler and run **WTA Re-Amp Reaction Program** from Step 2.

9. After the WTA Re-Amp Reaction, you can leave the **WTA Re-Amp Reaction Plate** holding overnight at 4°C in the thermocycler or store long-term at -20°C.

10. Again, proceed to STEP H for WTA SPRI Clean-Up.

STOP POINT

APPENDIX 6: QUICK PROTOCOL FOR TRANSCRIPTOME RECOVERY

HIVE CLX scRNAseq Transcriptome Recovery & Library Preparation

Step A: Prepare HIVE Collectors

1. Thaw HIVEs at **37°C for 15 minutes** or at room temperature for 60 minutes.
2. Remove Storage Liquid.
3. Add 2 mL **CLX Storage Wash** Solution.
4. Prepare **HIVE Top**.
5. Remove liquid and immediately add **Drying Cap**.
6. Briefly centrifuge at 400 RCF then stop spin.
7. Remove liquid from HIVE through Drying Cap port.
8. Remove **Drying Cap** and immediately use **Closure Tool** to add **HIVE Top**.
9. Incubate at room temperature for **30 minutes**.

Step B: Lysis & Hybridization

1. Prepare **Lysis Solution** (1 mL **CLX Lysis Stock** + 100 µL **CLX Reducing Solution**).
2. Place **HIVE Collectors** on **Spin Plate**. Push down to remove plungers.
3. Add 1 mL **Lysis Solution** and incubate for **5 minutes**, then remove.
4. Wash with 1 mL **CLX Hybridization Buffer**.
5. Add 1 mL **CLX Hybridization Buffer** and incubate for **15 minutes**.

APPENDIX 6: QUICK PROTOCOL FOR TRANSCRIPTOME RECOVERY

Step C: Bead Recovery

1. Remove liquid.
2. Peel off membrane.
3. Add **Bead Collector**.
4. Add **Spin Lid** and flip assembly upside down.
5. Add 3 mL **CLX Bead Recovery Solution**.
6. Insert **Bead Recovery Stopper**.
7. Spin at max speed for **5 minutes**.
8. Set pipette to 300 µL and remove bead pellet.
9. Transfer bead pellet to **Filter Plate** well.
10. Pipet up and down to break up clumps.
11. Spin **HIVE Collectors** at max speed for **1 minute**.
12. Place **Filter Plate** on the vacuum and remove liquid.
13. Remove bead pellet and transfer to the same well in the **Filter Plate**.
14. Pipet up and down to break up clumps.

APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

Step D: 1st Strand Synthesis

1. Add 250 µL of **CLX Bead Recovery Solution** to the **CLX 1st Strand Control**.
2. Mix control and add to a **Filter Plate** well.
3. Prepare **1st Strand Reaction Mix** (175 µL **CLX 1st Strand Buffer** + 15 µL **CLX 1st Strand Enzyme**).
4. Place **Filter Plate** on the vacuum and remove liquid.
5. Add 175 µL **CLX 1st Strand Wash** and pipet to mix.
6. Use vacuum to remove liquid.
7. Dry bottom of **Filter Plate** and seal.
8. Add 175 µL of **1st Strand Reaction Mix** and pipet to mix.
9. Incubate at **37°C for 60 minutes**.

Step E: Bead Clean-Up

1. Prepare **Clean-Up Reaction Mix** (175 µL **CLX Clean-Up Buffer** + 10 µL **CLX Clean-Up Enzyme**).
2. Place **Filter Plate** on the vacuum and remove liquid.
3. Wash with 175 µL **CLX Wash A**.
4. Use vacuum to remove liquid.
5. Dry bottom of **Filter Plate** and seal.
6. Add 175 µL of **Clean-Up Reaction Mix** and pipet to mix.
7. Incubate at **37°C for 45 minutes**.

APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

Step F: 2nd Strand Synthesis

1. Dilute **10× NaOH** to make **1× NaOH** (180 µL **Water** + 20 µL **10× NaOH**)
2. Prepare **2nd Strand Reaction Mix** (175 µL **CLX 2nd Strand Buffer** + 10 µL **CLX 2nd Strand Oligo** + 5 µL **CLX 2nd Strand Enzyme**).
3. Place **Filter Plate** on the vacuum and remove liquid.
4. Wash with 175 µL **CLX Wash A**.
5. Use vacuum to remove liquid.
6. Dry bottom of **Filter Plate**. Return Filter Plate to manifold but do not turn on.
7. Add 175 µL **1× NaOH** and pipet to mix.
8. Incubate **5 minutes**.
9. Turn pump on to remove liquid.
10. Wash 3 times with 175 µL **CLX Wash A**.
11. Dry bottom of **Filter Plate** and seal.
12. Add 175 µL of **2nd Strand Reaction Mix** and pipet to mix.
13. Incubate at **37°C for 30 minutes**.

Step G: Whole Transcriptome Amplification (WTA)

1. Prepare **WTA Reaction Mix** (200 µL **CLX PCR Enzyme** + 40 µL **CLX WTA Oligo** + 180 µL **Water**).
2. Place **Filter Plate** on the vacuum and remove liquid.
3. Wash 3 times with 175 µL **CLX Wash A**.
4. Dry bottom of **Filter Plate** and seal.
5. Add 200 µL of **WTA Reaction Mix** to the **Filter Plate** and pipet to mix.
6. Transfer beads to the **Deep Well Plate**.
7. Repeat transfer with another 200 µL of **WTA Reaction Mix**.
8. Redistribute WTA bead suspension into 4 reactions (100 µL each) in a PCR plate
9. Run **WTA Reaction Program** on thermocycler (95°C 5 min, [98°C 20 sec, 60°C 45 sec, 70°C 60 sec] for 20 cycles, 72°C 2 min, 4°C hold).

APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

Step H: WTA SPRI Clean-Up

1. Prepare **80% Ethanol**, 400 µL per sample.
2. Combine 25 µL from each well containing the WTA reaction into 1 new well.
3. Add 90 µL **SPRI Beads** to each well. Incubate for **3 minutes**.
4. Place plate on magnet. Incubate for **2 minutes**.
5. Remove and discard supernatant. Remove plate from magnet.
6. Add 50 µL **CLX Wash A** and pipet to mix. Incubate for **3 minutes**.
7. Place plate on magnet. Incubate for **2 minutes**.
8. Transfer supernatant to a new well.
9. Add 45 µL **SPRI Beads** to each well. Incubate for **3 minutes**.
10. Place plate on magnet. Incubate for **2 minutes**.
11. Remove and discard supernatant
12. Add 175 µL **80% Ethanol**.
13. Move plate across bar magnet multiple times to mix.
14. Discard ethanol and repeat wash.
15. Dry pellet on magnet for **5 minutes**.
16. Add 20 µL **CLX Wash A**. Incubate for **3 minutes**.
17. Place plate on magnet and remove 2 µL for quantification.
18. If the purified WTA Product is ≥0.2 ng/µL, proceed to Step I.

APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

Step I: Index PCR

1. Calculate the volume for 5 ng of purified WTA product. Use **Water** to bring volume up to 5 µL.
2. Add 25 µL of **CLX PCR Enzyme** to one well per sample.
3. Add 5 µL (containing 5 ng) of purified WTA product.
4. Add 20 µL **Index**.
5. Run **Index PCR Program** on thermocycler (95°C 5 min, [95°C 30 sec, 60°C 30 sec, 73°C 60 sec] for 9 cycles, 72°C 2 min, 4°C hold).

Step J: Index PCR SPRI Clean-Up

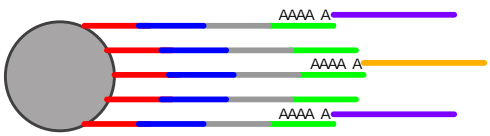
1. Prepare **80% Ethanol** (400 µL per sample).
2. Add 40 µL **SPRI Beads** to each well. Incubate for **3 minutes**.
3. Place plate on magnet. Incubate for **2 minutes**.
4. Remove and discard supernatant. Remove plate from magnet.
5. Add 50 µL **CLX Wash A** and pipet to mix. Incubate **3 minutes**.
6. Place plate on magnet. Incubate for **2 minutes**.
7. Transfer supernatant to a new well.
8. Add 40 µL **SPRI Beads** to each well. Incubate **3 minutes**.
9. Place plate on magnet. Incubate for **2 minutes**.
10. Remove and discard supernatant.
11. Add 175 µL **80% Ethanol**.
12. Move plate across bar magnet multiple times to mix.
13. Discard ethanol and repeat wash.
14. Dry pellet on magnet for **5 minutes**.
15. Add 30 µL **CLX Wash A**. Incubate for **3 minutes**.
16. Place plate on magnet. Incubate for **1 minute**.
17. Move supernatant to a new well.

APPENDIX 8: FOLLOW THE MOLECULE

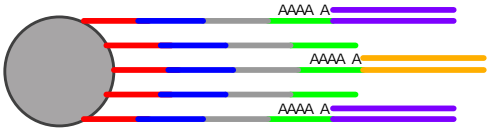
FOLLOW THE MOLECULE

Bead Oligos
Universal Primer Sequence (UPS)
Cell Barcode
Random Linker Sequence
Transcript Capture Sequence - poly(dT)

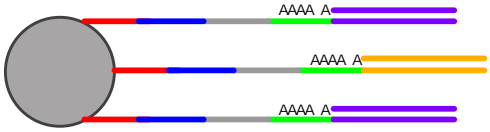
1) Hybridization: Capture poly-A transcripts



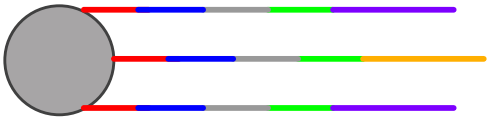
2) 1st Strand Synthesis: Bead oligos acts as primer for making 1st-strand cDNA



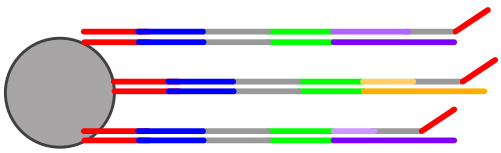
3) Bead Clean-Up: Remove any bead oligos without 1st strand cDNA



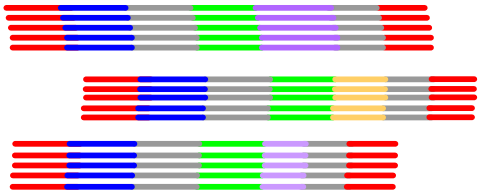
4) NaOH denaturation: Makes 1st strand cDNA single-stranded



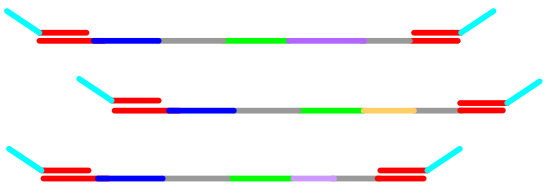
5) 2nd Strand Synthesis: Randomly prime synthesis of 2nd strand cDNA



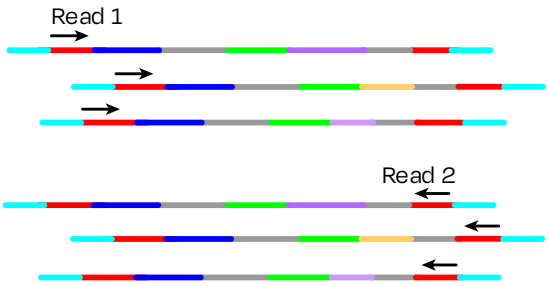
6) WTA: Amplify 2nd strand cDNA with UPS primers



7) Index PCR: Add P5+ i5 and P 7+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 ide ntity



TROUBLESHOOTING

Problem	Possible Causes and Suggested Solutions
WTA Product is low (<0.2 ng/µL) despite loading ≥2,000 cells *Even though there were sufficient cells loaded into the HIVE Collector, the first WTA reaction failed to produce sufficient DNA to proceed.*	**Double-check calculations for sample loading** **Poor sample quality may prohibit analysis** If DNA concentration of the WTA Product is low before reamplification when you loaded sufficient cells, the sample may be compromised. Proceeding with WTA reamplification may not be worthwhile.
WTA Product is low (<0.2 ng/µL) after WTA reamplification *After performing WTA Reamplification in Appendix 5 then SPRI Clean-Up in STEP H, the DNA concentration of the WTA Product remains too low to proceed.*	**Contact Technical Support** Email: **support@honeycomb.bio**

Problem	Possible Causes and Suggested Solutions
Few reads in sequencing data *Even though there was sufficient WTA Product for scRNAseq, the sequencing data contained few reads.*	**HIVE sequencing requires custom primers** Check to make sure you used custom primers for sequencing (see Appendix 3).
Poor sequencing data quality *Despite there being sufficient reads, the scRNAseq data contained fewer cells than expected, fewer genes than expected, and/or high mitochondrial reads.*	**Poor sample quality causes poor data quality** Poor cell viability and/or poor sample purity is the most common reason for poor data quality. If these sample features cannot be improved, measure cell viability and sample purity and increase the number of cells loaded per HIVE to compensate for dead and non-nucleated cells. We highly recommend the Nexcelom Cellometer® K2 Fluorescent Cell Counter for sample QC, which measures cell viability and sample purity faster and more accurately than trypan blue.[1]

REFERENCES FOR TROUBLESHOOTING

1. Honeycomb™ Appnote. Best Practices: The Cellometer® K2 for HIVE scRNAseq
   https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource_id=48ab2f9b45957ab574cf005eb8a76760
2. Honeycomb™ Troubleshooting Guide
   https://honeycombbio.zendesk.com/hc/en-us/articles/14881004938907-HIVE-CLX-Troubleshooting-Guide

NOTES

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MORE INFORMATION

Download protocols & example data: www.honeycomb.bio

Contact HIVE technical support: support@honeycomb.bio

Speak with a HIVE expert: sales@honeycomb.bio

Ready for quotes & ordering: NGS@revvity.com

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