Takara Bio USA

Seeker™ 10x10 Bundle User Manual

Cat. No. SK003
(050525)

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I. Introduction

This user manual describes the Seeker 10x10 Spatial Mapping Kit protocol, hereby referred to as the Seeker workflow, to generate high-quality, Illumina® sequencing-ready libraries from fresh-frozen tissues to obtain high-resolution spatial transcriptomic information of a sample. Once tissues are sectioned and placed onto the Seeker tile (hereby referred to as "tile") (Figure 1), a glass substrate containing a monolayer of uniquely DNA-barcoded microparticles (referred to as "beads" in this document), the rest of the workflow can be completed in under eight hours (Figure 2), with multiple safe stopping points. The Seeker workflow (Figure 3) starts with the hybridization of RNA to the beads on the tile, followed by reverse transcription. A tissue clearing step is performed to digest the tissue and release the beads from the glass into solution. Next, second-strand synthesis is performed by semi-random priming followed by cDNA amplification. Finally, the Nextera® XT DNA Sample Preparation Kit is used to generate Illumina sequencing platform-compatible libraries.

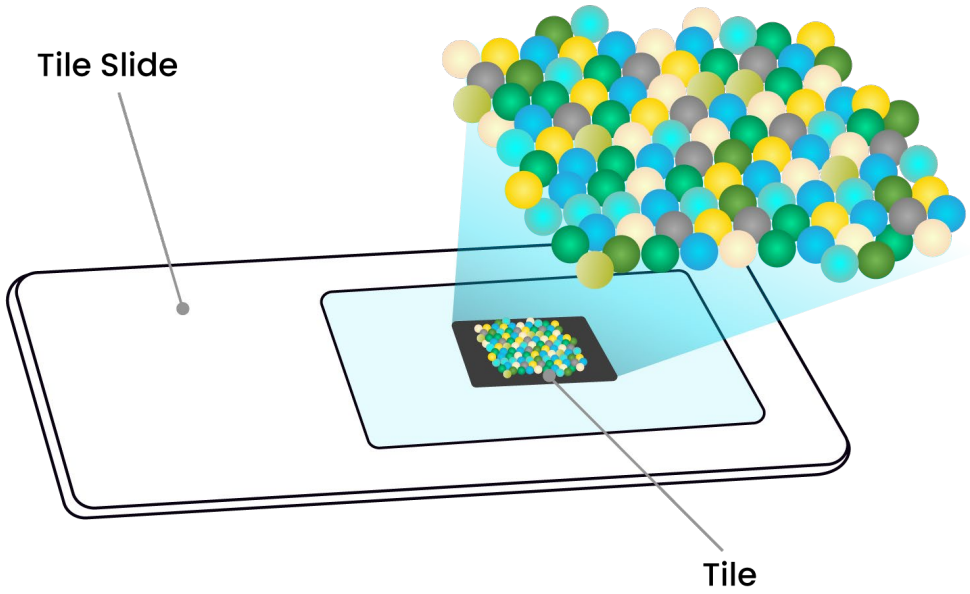

Figure 1. Seeker tile

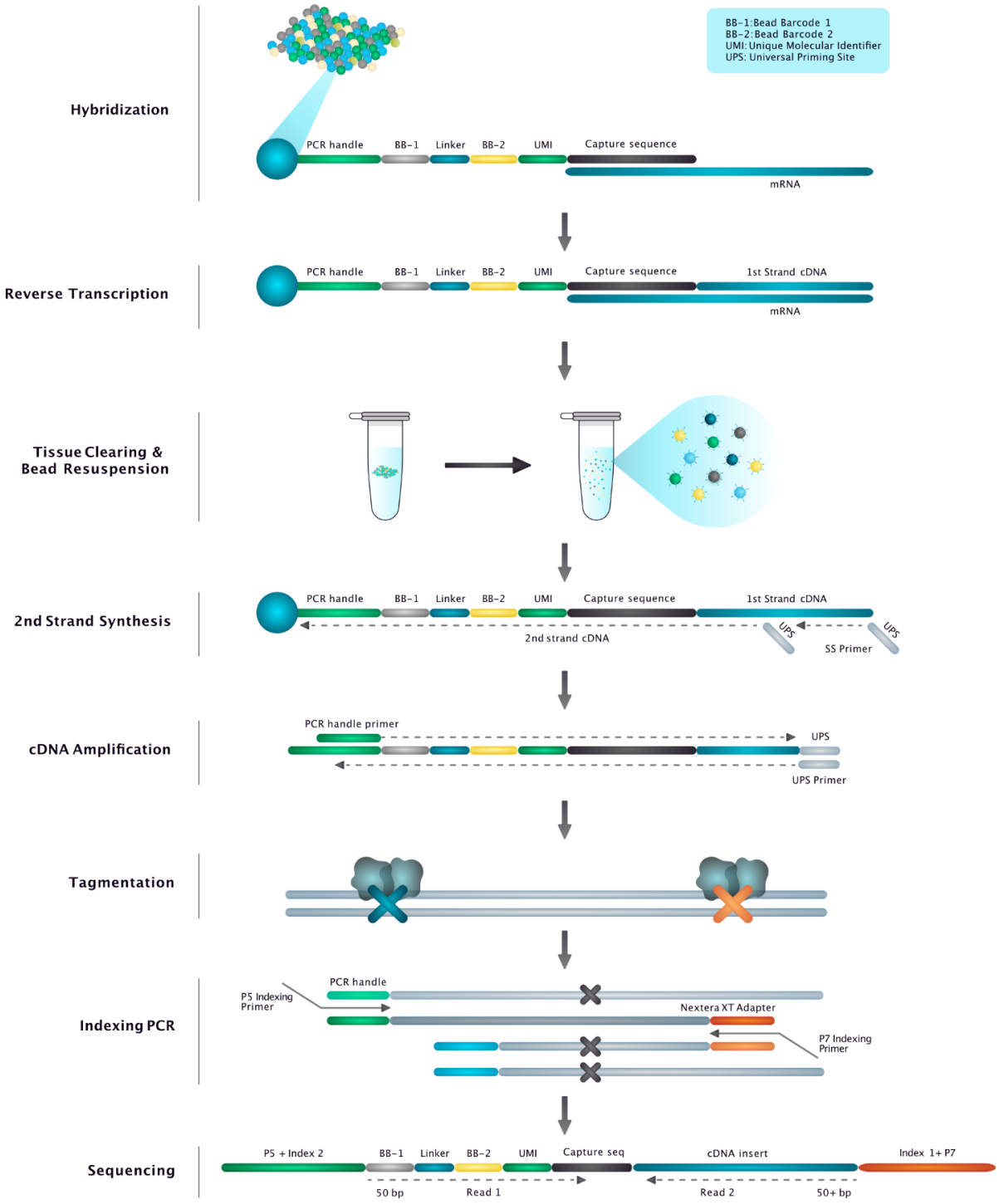

Figure 2. Seeker workflow. Please refer to the supplementary sections at the end of this protocol for more detailed sequence information.

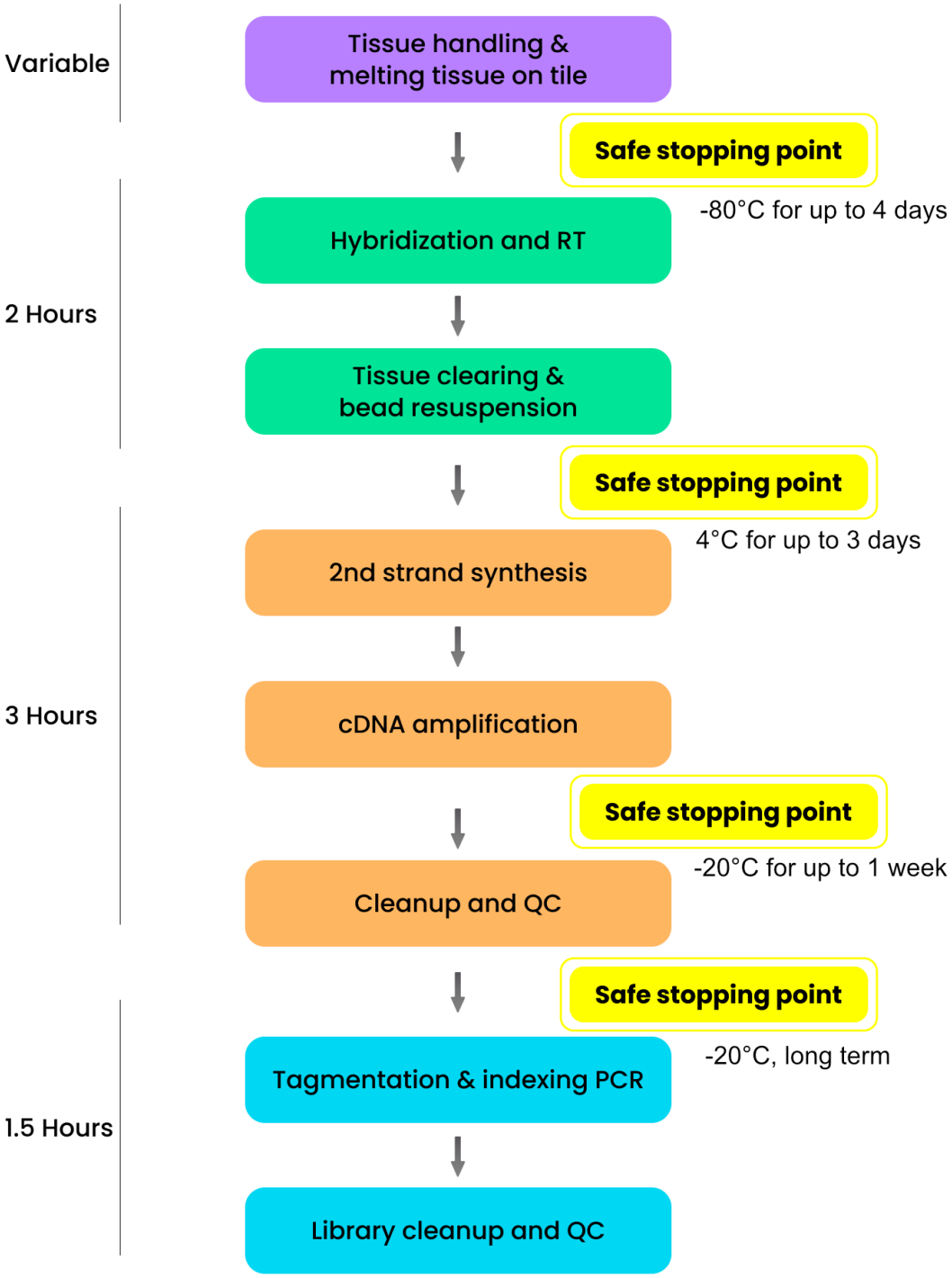

Figure 3. Estimated workflow timing

II. List of Components

Table 1. Seeker 10x10 Bundle components.

Seeker 10x10 Bundle (Cat. No. SK003; 4 rxns)	Cap Color
Seeker 10x10 Tiles (Cat. No. LTB001; Store at 4°C)*	
Seeker 10x10 Tile	–
Seeker Reagent Box 1 (Cat. No. K001; Store at Room Temperature)*	
Hyb Buffer	–
TC Buffer	Red
Nuclease-free Water	–
Bead Wash Buffer	–
Seeker Reagent Box 2 (Cat. No. K002; Store at –20°C)*	
RNase Inhibitor	–
RT/SS Buffer	–
dNTP	–
RT Enzyme	–
TC Enzyme	Red
SS Primer	Yellow
SS Enzyme	Yellow
cDNA Amp Buffer	Blue
cDNA Amp Primer Mix	Blue
cDNA Amp Enzyme	Blue
TE	–
CryoCube (Cat. No. JW001; Store at –80°C)*	
Seeker 10x10 Reaction Chamber Pouch (Cat. No. RC001; Store at Room Temperature)*	
Seeker Reaction Chamber Adapter (Cat. No. TJ002; Store at Room Temperature)	
Seeker Dual Indexing Primer Kit v2 (Cat. No. K006; Store at –20°C)	
Index Primer F1	–
Index Primer F2	–
Index Primer F3	–
Index Primer F4	–
Index Primer F5	–
Index Primer R1	–
Index Primer R2	–
Index Primer R3	–
Index Primer R4	–
Index Primer R5	–

*Not sold separately

III. Additional Materials Required (Not Provided)

Reagents:

- Ethyl Alcohol (Sigma Aldrich, Cat. No. 459844-1L)
- SPRIselect Reagent (Beckman Coulter, Cat. No. B23318) or sparQ PureMag beads (Quanta Bio, Cat. No. 95196-005)
- Nextera XT Library Prep Kit (Illumina, Cat. No. FC-131-1024)

  NOTE: Do not substitute with any other library prep kits.
- Bioanalyzer High Sensitivity DNA kit (Agilent Technologies, Cat. No. 5067-4626) -or- TapeStation High Sensitivity DNA D5000 ScreenTape (Agilent Technologies, Cat. No. 5067-5592) and TapeStation High Sensitivity DNA D5000 Reagents (Agilent Technologies, Cat. No. 5067-5593)
- Qubit 1X dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q33230)

Equipment:

- Cryostat (Leica, Cat. No. CM3050S)
- Single-channel pipette: 10 µl, 20 µl, 200 µl, and 1,000 µl (Rainin, Cat. No. 17014388, 17014392, 17014391 & 17014382)
- Eight-channel or 12-channel pipette: 20 µl and 200 µl (Rainin, Cat. No. 17013803 & 17013805)
- Mini centrifuge for 1.5 ml tubes
- Mini centrifuge for 0.2 ml tubes
- Eppendorf Centrifuge 5415 D (Eppendorf, Cat. No. 5425-55001) or equivalent
- 2 heat blocks for 1.5 ml tubes
- 96-well PCR chiller rack (MIDSCI, Cat. No. 5640-T4) -or- 96-well aluminum block (Light Labs, Cat. No. A-7079)
- C1000 Touch Thermal Cycler (Bio-Rad, Cat. No. 1851148)

  NOTE: Although we do not expect significant differences in results between different models of thermal cyclers, the protocol has been developed with C1000 Touch with a ramp rate of 3°C/sec with a 96-well block.
- Vortexer
- Tweezers (Ted Pella, Cat. No. 58083-NM)
- 2100 Bioanalyzer (Agilent Technologies, Cat. No. G2939BA/G2953CA) -or- 4200 TapeStation (Agilent Technologies, Cat. No. G2991AA)
- DynaMag-2 Magnet (1.5 ml) (Invitrogen , Cat. No. 12321D)
- 12-Tube Magnetic Separation Rack (PCR tube) (NEB, Cat. No. S1515S)
- Qubit 4 Fluorometer (Thermo Fisher, Cat. No. Q33238)

Consumables:

- Filter pipette tips: 20 µl, 200 µl, and 1,000 µl (Rainin, Cat. No. 30389226, 30389240 & 30389213)
- DNA LoBind 1.5 mL tubes (Eppendorf, Cat. No. 22431021), DNA LoBind 2 mL tubes (Eppendorf, Cat. No. 022431048), DNA LoBind 5 mL tubes (Eppendorf, Cat. No. 0030108310)
- 0.2 ml PCR strip tubes (USA Scientific, Cat. No. 1402-4700)
- Optimal Cutting Temperature compound (OCT compound)
- Qubit assay tubes (Thermo Fisher, Cat. No. Q32856)

IV. General Considerations

A. Tissue Requirements and Recommendations for Assessing Tissue Quality

- Fresh-frozen tissue
- Assess RNA quality of your tissue by collecting five 10 µm sections and isolating RNA with the RNeasy Mini Kit (Qiagen, P/N 74104) or equivalent. Analyze the RNA from your sections on an Agilent Bioanalyzer or TapeStation to derive an RNA integrity number (RIN). Good quality RNA should have a RIN value of at least 7. Although you may use samples with lower RIN scores at your own risk, it may result in lower data quality.
- Assess tissue quality by performing H&E staining on an adjacent section to the one used for the Seeker workflow to provide information on tissue structural context and sectioning quality.
- Run a pilot Seeker tile experiment on new tissue samples and perform shallow sequencing of 2.0×10^8 reads to assess library quality.

B. Tips and Techniques

- Use Eppendorf LoBind 1.5 ml tubes (Eppendorf, Cat. No. 022431021) in all steps where 1.5 ml tubes are indicated.
- When using tweezers or forceps to transfer the tile, avoid direct contact with the beads. Instead, grip the glass slide from the beveled corner as shown in Figure 4.

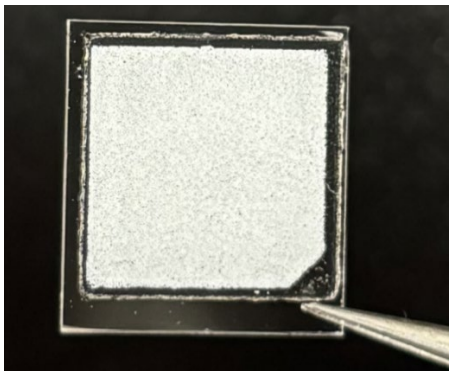

Figure 4. Correct tweezer placement when transferring the Trekker tile. Grip near the chamfered corner. Take care to avoid direct contact with the beads.

- When working with multiple samples, clean tweezers or forceps between each sample by dipping the tips in 100% ethyl alcohol and wiping with Kimwipe to prevent cross-contamination.
- Keep all enzymes on ice when preparing reaction mixes.
- Prepare all master mixes in the order listed in the tables.
- If you are experiencing difficulties due to static charge during tissue sectioning, please follow these steps:
  1. Ground yourself before sectioning.
  2. Change to a new pair of gloves.
  3. Wipe the back of the blue adhesive with 100% ethanol. Allow 15-20 sec to dry before placing it in the cryostat.
  4. Regularly clean the entire cryostat chamber with 100% ethanol to reduce charge.
  5. Place a small piece of a dryer sheet in the corner of the cryostat.

V. Protocol

A. Preparation Before Starting

1. Thaw the following reagents at room temperature, vortex, briefly centrifuge, and then keep on ice:
   a. RT/SS Buffer
      i. If precipitation is observed, heat the RT/SS buffer at 37°C for 5 min and briefly vortex before use.
   b. dNTP
2. Place the reaction chamber adapter into the thermal cycler and set the thermal cycler to 52°C, keeping the lid completely open.

Figure 5. The Seeker Reaction Chamber Adapter placed into the thermal cycler.

B. Tissue Sectioning and Hybridization to the Seeker Tile

1. Equilibrate the fresh-frozen tissue and CryoCube to –18°C in a cryostat (such as Leica CM3050S) for at least 20 min prior to sectioning. The optimal temperature for sectioning may vary depending on the tissue type.
2. Mount the tissue block and the CryoCube onto cutting blocks with Optimal Cutting Temperature compound (OCT compound).

Figure 6. Example Seeker Tile label to identify the tile ID. The tile ID, B0050_004, is indicated by the blue box.

IMPORTANT: Each Seeker tile is unique. The tile ID is required to retrieve the correct file for spatial barcode mapping of the sequencing data.

3. Section a 10 µm section of tissue.
4. Melt the section onto the tile in one of the two ways described below:
   a. OPTION 1: For precise placement of the region of interest.
      i. Place the Seeker tile slide in the cryostat to chill for 1 min.

ii. Place the chilled Seeker tile slide on the cutting stage and arrange the tissue section on top of the tile using a brush. Make sure that the region of interest is positioned directly over the tile.

iii. With the tile and tissue section facing up, melt the tissue section onto the tile by moving the tile off the cryostat stage, and GENTLY placing a finger on the bottom of the slide glass, as shown in the example below. To avoid curling of the tissue, start from one side and slowly move your finger across the region rather than warming it from the center. A small brush can be used to hold the other end of the tissue flat during the initial melting from one end.

Figure 7. Visual example of melting the tissue onto the Seeker tile with precise placement.

b. OPTION 2: For quick placement of the region of interest.

i. Hold a **room temperature** tile in the tile holder with the tile facing down. Hover the tile over the region of interest. Keeping the tile horizontal, gently lower the tile to bring it into contact with the tissue section. The tissue section should melt onto the tile immediately.

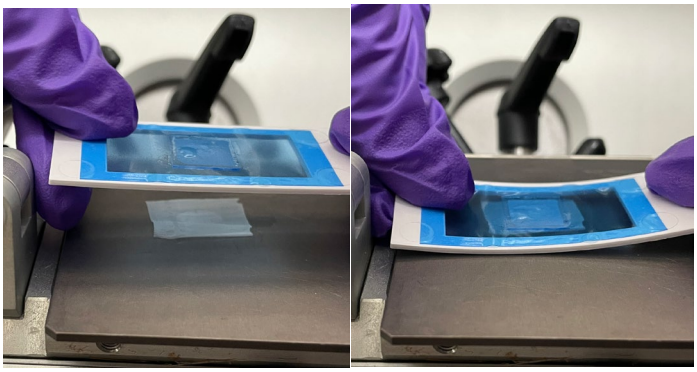

Figure 8. Visual example of the tissue quick placement method.

5. Place the tile with the melted tissue section back into the cryostat.
6. (OPTIONAL) Section a 10 µm adjacent section for H&E staining. Melt the tissue on a microscope slide and store in the cryostat until ready to process.
7. Remove the tissue block and replace it with the CryoCube.
8. Section a 30 µm section of the CryoCube. Alternatively, 60 µm can be used for tissues with higher expression levels.
9. Melt the CryoCube section onto the tile by placing a finger under the tile and moving it across the tile until the entire CryoCube section and tissue section are melted. Alternatively, briefly warm the tile with the tissue section by placing a finger underneath the tile for a few seconds and melt the CryoCube section onto the tile with the stamping method described in step 5b.

10. Place the tile back in the Cryostat or in the tile holder on dry ice while you prepare the hybridization reaction mix in the next step, or store at –80°C for up to four days.

SAFE STOPPING POINT: Tiles can be stored at –80°C for up to 4 days in a sealed container.

11. Prepare the Hybridization Reaction Mix following the table below, plus 5% of the total volume for overage. Mix by pipetting 10X and keep at room temperature. Use within 15 min.

NOTE: When working with tissues with high RNase content, additional (up to 1.8X) RNase inhibitor may be added to the Hybridization reaction mix.

Hybridization Reaction Mix:

380 µl	Hyb Buffer
20 µl	RNAse Inhibitor
400 µl	**Total volume per reaction**

12. Write the sample name and tile ID on the side of a new reaction chamber.

Figure 9. Reaction chamber.

13. Add 400 µl of Hybridization Reaction Mix to Chamber 1.
14. Remove the tile from the cryostat, dry ice or –80°C, and warm up the tile by placing a finger under the tile until the tissue has re-melted.
15. Carefully remove the tile from the blue adhesive with tweezers or forceps and place it in Chamber 1 containing 400 µl of Hybridization Reaction Mix. Place the tile straight up and down in the middle of Chamber 1.

NOTE: There should be no resistance while placing the tile in the chamber. If you encounter resistance, lightly tap the edge of the tile with the tweezer. Make sure the tile is completely submerged.

16. Seal the chambers with a chamber seal with a straight flat edge.
17. Incubate for 30 min at room temperature.
18. Remove the remaining block of tissue and the CryoCube from the cryostat and store it at –80°C.

NOTE: Cover the exposed tissue of the tissue block with a drop of OCT compound and freeze prior to storage to prevent desiccation of the tissue.

C. Reverse Transcription

1. Prepare the 1X RT Wash Buffer in a 1.5 ml tube following the table below, plus 5% of the total volume for overage, for washing the tile prior to RT. Mix by vortexing, centrifuge briefly, and keep at room temperature:

80 µl	RT/SS Buffer
320 µl	Nuclease-Free Water
400 µl	**Total volume per reaction**

2. Prepare the RT Reaction Mix in a 1.5 ml tube following the table below, plus 5% of the total volume for overage. Mix by pipetting 10X and keep on ice:

RT Reaction Mix:

80 µl	RT/SS Buffer
40 µl	dNTP
10 µl	RNase Inhibitor
20 µl	RT Enzyme
250 µl	Nuclease-Free Water
400 µl	**Total volume per reaction**

3. Carefully remove the chamber seal from the reaction chamber.
4. Add 400 µl of 1X RT Wash Buffer to Chamber 2.
5. Add 400 µl of RT Reaction Mix to Chamber 3.
6. Using a pair of clean tweezers or forceps, remove the tile from the Hybridization Reaction Mix in Chamber 1 and dip it in the 1X RT Wash Buffer in chamber 2 for 5 sec.
7. Transfer the tile to Chamber 3 with 400 µl of RT Reaction Mix. Make sure the tile is completely submerged.
8. Remove the liquid from Chambers 1 and 2 and discard.
9. Seal the reaction chamber with a chamber seal with a straight flat edge.
10. Incubate at room temperature for 10 min.
11. Place the reaction chamber onto the reaction chamber adapter in the thermal cycler that was preheated to 52°C.

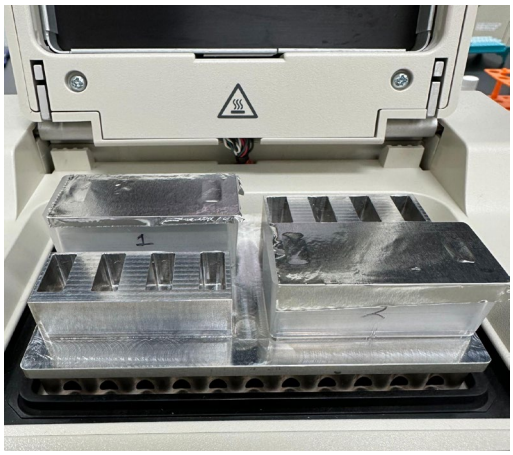

Figure 10. Placement of the reaction chamber adaptor in the thermal cycler.

12. Incubate for 30 min with the thermal cycler lid open.

D. Tissue Clearing and Seeker Bead Resuspension

1. If precipitation is observed, heat the TC buffer at 37°C for 5 min and briefly vortex before use.
2. If the room temperature in the lab is <20°C, warm the bead wash buffer to 37°C to prevent precipitation during the bead wash steps.
3. Make the Tissue Clearing Reaction Mix in a 1.5 ml tube following the table below, plus 5% of the total volume for overage. Mix by pipetting 10x and keep at room temperature:

Tissue Clearing Reaction Mix:

392 µl	TC Buffer
8 µl	TC Enzyme
400 µl	**Total volume per reaction**

4. Take the reaction chambers out of the thermal cycler.
5. Set the thermal cycler to 37°C.
6. Carefully remove the chamber seal from the reaction chamber.
7. Add 400 µl of TC Clearing Reaction Mix into chamber 4.
8. Move the tile from chamber 3 to chamber 4.
9. Remove the liquid from chamber 3 and discard.
10. Seal the reaction chamber with a chamber seal with a straight flat edge.
11. Place the reaction chamber back onto the reaction chamber adapter in the thermal cycler that is set at 37°C.
12. Incubate for 30 min with the thermal cycler lid open.

TIP: If your tissue is difficult to digest or contains plant cell walls, you may increase tissue clearing incubation from 30 min to one hour. Contact technical support if experiencing incomplete tissue digestion.

13. After the incubation, carefully remove the chamber seal.
14. Transfer the tile to a 5 ml Lo-bind tube.
15. Carefully transfer the liquid from chamber 4 to the 5 ml Lo-bind tube.
16. Rinse chamber 4 with 400 µl of Bead Wash Buffer and transfer the contents to the 5 ml tube containing the tile.
17. Add an additional 500 µl of Bead Wash Buffer to the 5 ml tube containing the tile.
18. Dissociate beads from the glass slide by pipetting the wash buffer mixture directly onto the beads. Be careful not to create excess bubbles as it will make it difficult to see the tile during bead dissociation.

IMPORTANT: It is important to completely dissociate the beads from the glass slide for the region of the tile that was covered by your tissue section. You may see a membrane-like layer remaining on the tile surface after you remove the beads. DO NOT attempt to remove this layer as it will inhibit downstream reactions and prevent you from fully recovering beads in subsequent steps.

NOTES:

– Aim the pipette tip at the bead patch when dissociating the beads from the glass slide. You should see patches of beads detach from the glass slide as you pipette. Occasionally the residual tissue

and beads may still be loosely associated after detachment from the glass slide. Pipet up and down until the cloud of tissue and beads are fully dissociated.

– Areas of the tile that are not covered by tissue may be harder to dissociate. For areas that are harder to dissociate, mechanically dislodge the beads from the tile by gently brushing the tip against the tile sideways while the tip is parallel to the tile and pipetting across the tile where beads remain. Incomplete dissociation of beads not covered by tissue will NOT affect data quality and performance.

19. Remove the glass from the tube with tweezers or forceps and discard.
20. Transfer the bead suspension to a new 1.5 ml tube.
21. Pellet the beads for 3 mins at 3,000*g* at **room temperature**. A white bead pellet should be visible to the eye.

Figure 11. Example of the bead pellet after dissociation and centrifugation.

22. Remove any bubbles from the top of the supernatant.
23. Carefully remove and discard the supernatant without disturbing the bead pellet as shown in Figure 12.

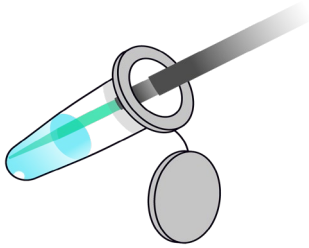

Figure 12. Example of pipette placement for supernatant removal from around the bead pellet.

IMPORTANT: Remove the supernatant immediately after centrifugation as the pellet may slide to the bottom of the tube after some time and become harder to visualize.

– When removing the supernatant, angle the tip away from the pellet and aspirate slowly to not disturb the pellet. Take care to prevent bead loss while removing the supernatant.

– If the pellet begins to slide, spin for an additional 30 sec at 3,000*g*.

– When processing multiple samples, repeat the 30 sec centrifugation immediately before aspirating the supernatant.

THIS APPLIES TO ALL BEAD WASH STEPS.

TIP: To retain as many beads as possible, it is not necessary to remove all the supernatant from the initial washes in step. You may leave 15–20 µl. But You should leave <10 µl of bead wash buffer before adding a reaction mix to the beads. THIS APPLIES TO ALL BEAD WASH STEPS.

24. Resuspend the bead pellet in 800 µl of Bead Wash Buffer and pellet the beads by centrifuging for 3 mins at 3,000*g* at **room temperature**.
25. *Immediately* remove and discard the supernatant. You may leave <10 µl of Bead Wash Buffer to preserve the bead pellet.
26. Resuspend the bead pellet 400 µl of Bead Wash Buffer.

SAFE STOPPING POINT: Beads can be stored at 4°C for up to 3 days.

E. Second-Strand Synthesis

1. Set one heat block to 95°C and another to 37°C.
2. Thaw the following reagents at room temperature, vortex, briefly centrifuge, and then keep on ice:
   a. RT/SS Buffer
   b. dNTP
   c. SS Primer
3. Keep SS Enzyme on ice.
4. Gently pipette mix the beads from the previous step 5 times.
5. Incubate the beads at 95°C for 5 min.
6. Prepare the Second-Strand Mix in a 1.5 ml tube, plus 5% of the total volume for overage. Mix by pipetting 10X and keep at room temperature.

Second-Strand Mix:

80 µl	RT/SS Buffer
40 µl	dNTP
4 µl	SS Primer
10 µl	SS Enzyme
266 µl	Nuclease-Free Water
400 µl	**Total volume per reaction**

TIP: If you are processing multiple samples, please leave samples at 95°C until you are ready to process them, for up to 10 additional minutes.

7. After 5 min of incubation at 95°C, immediately spin beads down for 30 sec at 3,000*g* and carefully remove and discard the supernatant.

IMPORTANT: Remove supernatant immediately after centrifugation. THIS APPLIES TO ALL BEAD WASH STEPS.

8. Immediately resuspend the beads in 400 µl of Second Strand Mix.
9. Incubate at 37°C for 1 hour.
10. Add 400 µl of Bead Wash Buffer.

11. Spin the beads down for 3 min at 3,000*g* at room temperature and *immediately* remove and discard the supernatant.
12. Resuspend the beads in 400 µl of Bead Wash Buffer.

F. cDNA Amplification

1. Thaw the following reagents at room temperature, vortex, briefly centrifuge, and then keep on ice:
   a. cDNA Amp Buffer
   b. cDNA Amp Primer Mix
2. Keep cDNA Amp Enzyme on ice.
3. Preheat a thermocycler to 98°C and hold until ready to proceed with amplification. Heat the lid to 105°C and set the volume to 50 µl.
4. Prepare the cDNA Amplification Mix in a 1.5 ml tube following the table below, plus 5% of the total volume for overage. Mix by pipetting 15X and keep on ice:

cDNA Amplification Mix:

200 µl	cDNA Amp Buffer
16 µl	cDNA Amp Primer Mix
8 µl	cDNA Amp Enzyme
176 µl	Nuclease-Free Water
400 µl	**Total volume per reaction**

5. Spin the beads down for 2 min at 3,000*g* at room temperature and *immediately* remove the supernatant.
6. Add 400 µl of the cDNA Amplification Mix to beads.
7. Split the reaction mix into 8 PCR tubes (50 µl each).
8. Pipette-mix each PCR tube before placing the tubes into the preheated thermal cycler.
9. Immediately run the cDNA amplification program on the thermal cycler according to the table below (~1 hr total run time).

98°C	2 min	
4 cycles:		
98°C	20 sec	Phase 1
65°C	45 sec	
72°C	3 min	
9* cycles:		
98°C	20 sec	Phase 2
67°C	20 sec	
72°C	3 min	
72°C	5 min	
4°C	Hold	

*If starting with a tissue section that does not cover the entire tile or has low cellular content, additional cycles for Phase 2 may be required. For example, if your tissue only covers 50% of the tile, increase the cycle number by 1–2. Add 2–3 cycles for tissues with low RNA abundance. Refer to Table 2 for guidelines.

Table 2. Number of cycles in the cDNA amplification program based on tile coverage by the tissue.

Fraction of tile covered by tissue	Recommended # of Phase 2 cycles
>2/3 but not completely covered	10–11
2/3	11–12
1/3	12–13

G. Purification and Quantification

NOTE: sparQ PureMag beads can be used for all steps where SPRI beads are indicated. Equilibrate PureMag beads to room temperature for 30 min prior to use.

1. First 0.6X Bead Purification

1. Prepare fresh 80% ethyl alcohol.
2. Combine the 8 reaction mixtures into one new 1.5 ml tube.
3. Spin the Seeker beads down for 2 min at 3,000*g* at room temperature and **immediately** transfer the supernatant to a new 1.5 ml tube.
4. Resuspend the Seeker beads in 200 µl of TE and store at 4°C.

   NOTE: Reamplification may be performed on the recovered beads. However, performance is not guaranteed. Contact technical support for further guidance on how to perform bead re-amplification.

5. Measure the total volume of the supernatant and calculate the volume of SPRI beads needed:

   [*Total volume*] x [0.6]

   Example: If the total volume is 400 µl, you will need 240 µl (400 µl x 0.6) of SPRI beads.
6. Vortex the SPRI beads at high speed for 30 sec. The beads should appear homogeneous and uniform in color.
7. Add the volume of SPRI beads calculated in step 3 to the tube of the combined reaction mixture.
8. Vortex to mix for 10–15 sec.
9. Incubate at room temperature for 5 min.
10. Briefly centrifuge the tube.
11. Place the tube on the magnetic rack. Once the solution is clear, carefully aspirate, and discard the supernatant.
12. Keeping the tube on the magnetic stand, add 500 µl of 80% ethanol.
13. Wait 30 sec and remove the supernatant.
14. Add 500 µl of 80% ethanol.
15. Wait 30 sec and remove the supernatant.
16. Briefly spin the tube to collect the remaining ethanol at the bottom of the tube.
17. Place the tube back on the magnetic rack and remove the remaining ethanol.
18. Let the SPRI beads dry at room temperature until the beads appear matte (~10 min).
19. Remove the tube from the magnetic rack and add 50 µl of nuclease-free water to the tube and pipette the beads to mix well.

20. Incubate at room temperature for 1 minute.
21. Place the tube back on the magnetic rack.
22. Once the solution is clear, transfer the supernatant to a new 0.2 ml PCR tube. Discard the used beads.

2. Second 0.6X Bead Purification

23. Vortex the SPRI beads and add 30 µl to the tube (0.6X volume of amplification volume).
24. Vortex to mix for 10–15 sec.
25. Incubate at room temperature for 5 min.
26. Briefly centrifuge the tube
27. Place the tube on the magnetic rack. Once the solution is clear, carefully aspirate, and discard the supernatant.
28. Keeping the tube on the magnetic stand, add 200 µl of 80% ethanol.
29. Wait 30 sec and remove the supernatant.
30. Add 200 µl of 80% ethanol.
31. Wait 30 sec and remove the supernatant.
32. Briefly spin the tube to collect the remaining ethanol at the bottom of the tube.
33. Place the tube back on the magnetic rack and remove the remaining ethanol.
34. Let the SPRI beads dry at room temperature until the beads appear matte (30 sec–2 min).
35. Remove the tube from the magnetic rack and add 20 µl of nuclease-free water to the tube to elute. Pipette the beads to mix well and incubate at room temperature for 1 minute.
36. Place the tube on a magnetic rack and incubate for 1 minute.
37. Transfer the supernatant to a new 0.2 ml PCR tube.
38. Quantify the cDNA products using Qubit (1X dsDNA HS assay kit) and a Bioanalyzer or TapeStation following the manufacturer's guidelines (Bioanalyzer High Sensitivity DNA assay, TapeStation High Sensitivity D5000 assay or TapeStation D5000 assay). Concentrations in the range of **1 ng/µl** and above are acceptable. If a significant amount of primer dimer is present, you may repeat one extra round of bead purification by bringing the total volume to 50 µl with water and following steps 23–37.

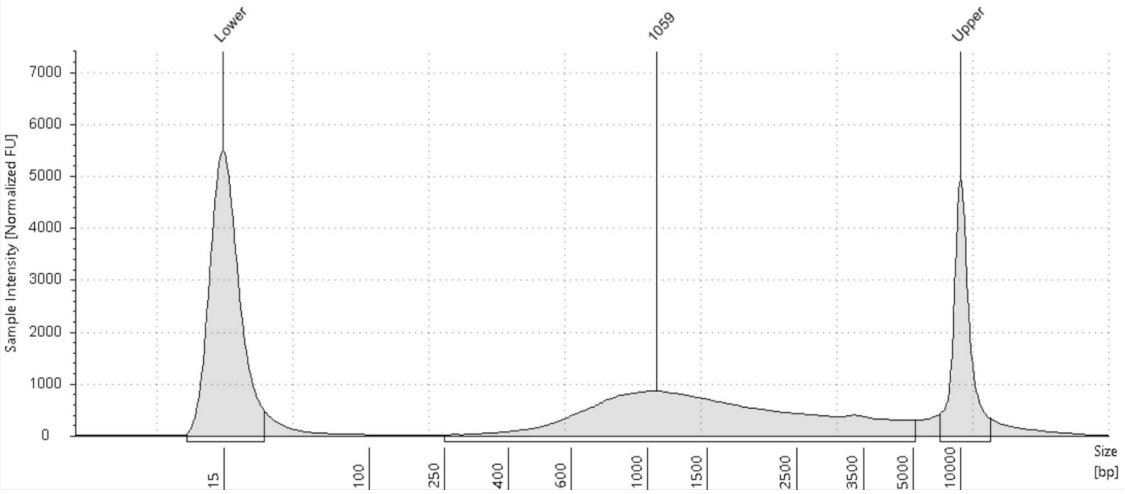

Figure 13. Example TapeStation D5000 electropherogram results after cDNA amplification and purification.

SAFE STOPPING POINT: cDNA samples can be stored at –20°C for one week before proceeding to the next step.

H. Tagmentation (with Nextera XT Library Prep Kit)

1. Preheat a thermal cycler to 55°C and heat the lid to 105°C. Set the volume to 50 µl.
2. Thaw the following reagents at room temperature and then keep on ice:
   a. Dual Indexing Primers
   b. Nextera TD buffer
3. Keep ATM and Nextera PCR mix (NPM) on ice.
4. In a new 1.5 ml tube, add 4.8 ng of cDNA and top off to a total volume of 40 µl with nuclease-free water. For example, if your cDNA amplified product has a concentration of 2 ng/µl, use 2.4 µl. If the calculated volume is <1 µl, dilute the products to avoid pipetting low volumes.
5. Add 80 µl of the Nextera TD buffer.
6. Add 40 µl of ATM.
7. Pipette to mix.
8. Split the reaction mix into 8 PCR tubes (20 µl each).
9. Briefly centrifuge the tubes.
10. Incubate at 55°C for 5 min.
11. After 5 min of incubation, **immediately** add 5 µl of Neutralization Tagment Buffer (NT) to each tube. Mix by pipette-mixing and spin down.
12. Incubate at room temperature for 5 min.
13. Add 15 µl of Nextera PCR Mix to each tube.

IMPORTANT:
– In step 14, please ensure that each sample that will be sequenced together has a unique combination of F and R primers. One F and R primer set will be sufficient for the 8 partitions of a single tile.

– If you plan to pool 2 or 3 samples on a sequencing run using Illumina XLEAP-SBS chemistry on the NextSeq® 2000 or NovaSeq™ X/X Pro, use the recommended combinations below to ensure sufficient color balance:
  - **2 samples:** F1+F3 or F2+F3; R1+R3 or R1+R4
  - **3 samples:** F1+F3+any or F2+F3+any; R1+R3+any or R1+R4+any

14. Add 5 µl of Index primer F and 5 µl of Index Primer R from the **Seeker Dual Indexing Primer Kit** (Cat. No. K006) to each tube. Use the same index primers for each of the 8 partitions from the same sample.
15. Pipette to mix and briefly centrifuge.
16. Run the indexing PCR program according to the table below (~30 min total run time).

72°C	3 min
95°C	30 sec
10 cycles:	
95°C	10 sec
55°C	30 sec
72°C	30 sec
72°C	5 min
4°C	Hold

I. Library Cleanup and Quantification

NOTE: sparQ PureMag beads can be used for all steps where SPRI beads are indicated. Equilibrate PureMag beads to room temperature for 30 min prior to use.

1. First 0.6X Bead Purification

1. Prepare fresh 80% ethyl alcohol.
2. Remove the PCR tubes from the thermal cycler.
3. Combine the reaction mixture into one new 1.5 ml tube.
4. Measure the total volume of the combined reaction mixture.
5. Calculate the volume of SPRI beads needed by multiplying the total reaction mixture volume by 0.6.

   Example: If the total volume is 400 µl, you will need 240 µl of SPRI beads.
6. Vortex the SPRIselect reagent at high speed for 30 sec. The beads should appear homogeneous and uniform in color.
7. Add the volume of SPRI beads calculated in step 4 to the tube of amplified cDNA. Pipette Mix.
8. Vortex to mix for 10–15 sec.
9. Incubate at room temperature for 5 min.
10. Briefly centrifuge the tube.
11. Place the tube on the magnetic rack. Once the solution is clear, carefully aspirate, and discard the supernatant.
12. Keeping the tube on the magnetic stand, add 500 µl of 80% ethanol.

13. Wait 30 sec and remove the supernatant.
14. Add 500 µl of 80% ethanol.
15. Wait 30 sec and remove the supernatant.
16. Briefly spin the tube to collect the remaining ethanol at the bottom of the tube.
17. Place the tube back on the magnetic rack and remove the remaining ethanol.
18. Let the SPRI beads dry at room temperature until the beads appear matte (3–5 min).
19. Remove the tube from the magnetic rack and add 50 µl of TE to the tube and pipette the beads to mix well.
20. Incubate at room temperature for 1 minute.
21. Place the tubes on a magnetic rack and incubate for 1 minute.
22. Transfer the supernatant to a new 0.2 ml PCR tube.

2. Second 0.8X Bead Purification

23. Vortex the SPRIselect reagent at high speed for 30 sec.
24. Add 40 µl of SPRI beads to the tube (0.8X of the eluted volume).
25. Vortex to mix for 10–15 sec.
26. Incubate at room temperature for 5 min.
27. Briefly centrifuge the tube.
28. Place the tube on the magnetic rack. Once the solution is clear, carefully aspirate, and discard the supernatant.
29. Keeping the tube on the magnetic stand, add 200 µl of 80% ethanol.
30. Wait 30 sec and remove the supernatant.
31. Add 200 µl of 80% ethanol.
32. Wait 30 sec and remove the supernatant.
33. Briefly spin the tube to collect the remaining ethanol at the bottom of the tube.
34. Place the tube back on the magnetic rack and remove the remaining ethanol.
35. Let the SPRI beads dry at room temperature until the beads appear matte (30 sec–2 min).
36. Remove the tube from the magnetic rack and add 10 µl of nuclease-free water to the tube to elute. Pipette the beads to mix well and incubate at room temperature for 1 minute.
37. Place the tube on a magnetic rack and incubate for 1 minute.
38. Transfer the supernatant to a new 0.2 ml PCR tube.
39. Quantify the library products using Qubit (1X dsDNA HS assay kit) and a Bioanalyzer or TapeStation following the manufacturer's guidelines (Bioanalyzer High Sensitivity DNA assay, TapeStation High Sensitivity D5000 assay or TapeStation D5000 assay). Concentrations should be **>1 ng/µl**. See an example library trace in Figure 14.

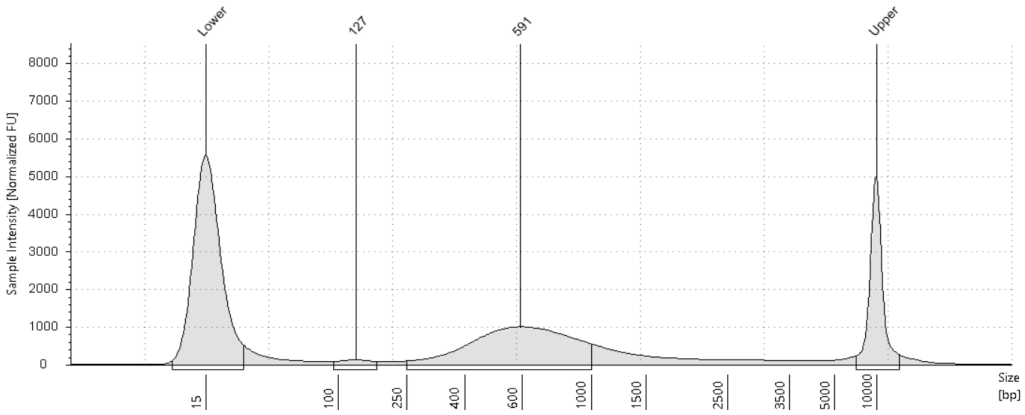

Figure 14. Example TapeStation D5000 electropherogram results after library clean-up.

SAFE STOPPING POINT: Libraries can be stored at –20°C before proceeding to the next step or for long-term storage.

J. Sequencing

- For shallow sequencing to check library quality and preview spatial expression, ~2.0 x 10^8 reads per tile is recommended.
- For deep sequencing, 1–3 x 10^9 reads per tile is recommended depending on the specific tissue type and tile coverage.

1. Read Lengths Required

- Read 1: 50 bp
- Index 1: 8 bp
- Index 2: 8 bp
- Read 2: minimum 50 bp

IMPORTANT: DO NOT PERFORM ADAPTER TRIMMING.

2. Loading Concentration Recommendations

- NextSeq 1000/2000:
  - Start at 750 pM final loading concentration and adjust based on sequencing quality
- NextSeq 500/550:
  - Start at 1.8 pM final loading concentration and adjust based on sequencing quality
- NovaSeq 6000:
  - Start at 250–500 pM final loading concentration. Adjust based on sequencing quality
- NovaSeq X:
  - Start at 180 pM final loading concentration. Adjust based on sequencing quality

3. PhiX Spike-in Recommendations

- NextSeq 1000/2000: 5% PhiX spike-in
- NextSeq 500/550: 5% PhiX spike-in
- NovaSeq 6000 and NovaSeq X:
  - 5% PhiX spike-in when pooling with non-Seeker libraries
  - 10% PhiX spike-in when pooling with only Seeker libraries

4. Index Sequences

Table 3. Index 1 primer sequences.

Index 1 primers	i7 bases for Illumina sample sheet
Index Primer F1	TAAGGCGA
Index Primer F2	CGTACTAG
Index Primer F3	AGGCAGAA
Index Primer F4	TCCTGAGC
Index Primer F5	GGACTCCT

Table 4. Index 2 primer sequences.

Index 2 primers	i5 bases for Illumina Sample Sheet (NovaSeq 6000 with v1.0 reagent kits, MiSeq®, HiSeq® 2000/2500, NextSeq 2000 (Sample Sheet v2))
Index Primer R1	TATCCTCT
Index Primer R2	AGAGTAGA
Index Primer R3	GTAAGGAG
Index Primer R4	ACTGCATA
Index Primer R5	AAGGAGTA

5. Library Structure

Figure 15. Seeker 10x10 library structure.

6. Oligonucleotide Sequences

Table 5. Oligonucleotide primer sequences.

Primers	Sequence
SS Primer	AAGCAGTGGTATCAACGCAGAGTGANNNGGNNNB
cDNA Amp Primer Mix	PCR handle primer: CTACACGACGCTCTTCCGATCT UPS primer: AAGCAGTGGTATCAACGCAGAGT
Index primer F	CAAGCAGAAGACGGCATACGAGAT-N8-GTCTCGTGGGCTCGG
Index primer R	AATGATACGGCGACCACCGAGATCTACAC-N8-ACACTCTTTCCCTACACGACGCTCTTCCGATCT

VI. Bioinformatics

FASTQ files generated by sequencing of the Seeker libraries can be analyzed by the Seeker bioinformatics pipeline. There are two options to access the pipeline:

1. Analyze your data on our cloud-based analysis platform
2. Install the pipeline locally at your institution.

To request either option, please contact technical_support@takarabio.com.

Download your barcode file(s) by Tile ID on our website.

Appendix. Troubleshooting Guide

Table 6. Troubleshooting guide

Problem	Possible Explanation	Solution
Tissue section curling during melting step	The tissue section is not flat	If the tissue section is curling upwards, it may help to flip the section and flatten it with a brush to prevent further curling prior to placing the section onto the tile. Use a small brush to flatten the tissue section prior to melting it onto the tile. For melting, start from one edge of the tissue and slowly move across the entire tissue until the entire section has been melted onto the tile. Use a brush to hold the section flat if the section begins to curl during melting.
cDNA amplification product yield is low (<1ng/µl)	Poor tissue or RNA quality	Ensure the tissue block was processed following best practices for your specific tissue type. Assess the RNA quality of the tissue block by extracting RNA from 5–10 µm thick sections using the Qiagen RNeasy mini kit. Add additional PCR cycles for cDNA amplification if the tissue is of lower quality.
	Bead loss during wash steps	Ensure beads are completely dissociated from the glass slide during the tissue dissociation step. Check that centrifugation steps are conducted with the correct speed and length. Remove supernatant immediately after spin completes. Take care to not pipette any beads when removing the supernatant after the centrifugation steps.
	The tissue section did not cover the entire tile	Additional PCR cycles may be needed if the tissue section only partially covers the tile. Start by increasing the number of cycles by 2–3 cycles and adjust based on yield and library trace.
	The tissue section is too thick	Ensure the tissue section is 10 µm thick
	Buffer precipitation occurred during bead wash steps after tissue clearing	Ensure the bead wash buffer and centrifuge are >20°C. If working in a lab space that is cooler, warm up the buffer to 37°C in a heat block prior to use.
	Too much bead wash buffer was left behind prior to adding the cDNA amp reaction mix to the beads	Ensure <10 µl of bead wash buffer was left behind prior to resuspending beads in the cDNA amp reaction mix.
	An incorrect PCR program was used or entered	Ensure the correct PCR program is used

Problem	Possible Explanation	Solution
The final library yield is too low	Input for the Nextera XT DNA prep workflow was too low	Ensure proper quantification was performed on the cDNA amplification product
	An incorrect PCR program was used or entered	Ensure the correct PCR program is used
Difficulty dissociating beads	The tile was not stored at the correct temperature (4°C)	Areas of the tile that are not covered by tissue may be harder to dissociate. For areas that are harder to dissociate, mechanically dislodge the beads from the tile by gently brushing the tip sideways while pipetting across the tile where beads remain. Incomplete dissociation of beads not covered by tissue will not affect data quality and performance.

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