Takara Bio USA

Seeker™ 3x3 Bundle V1.1 User Manual

Cat. Nos. SK004 & K006
(061325)

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I. Introduction

This user manual describes the protocol for the **Seeker 3x3 Bundle V1.1** (Cat. No. SK004), 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 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.

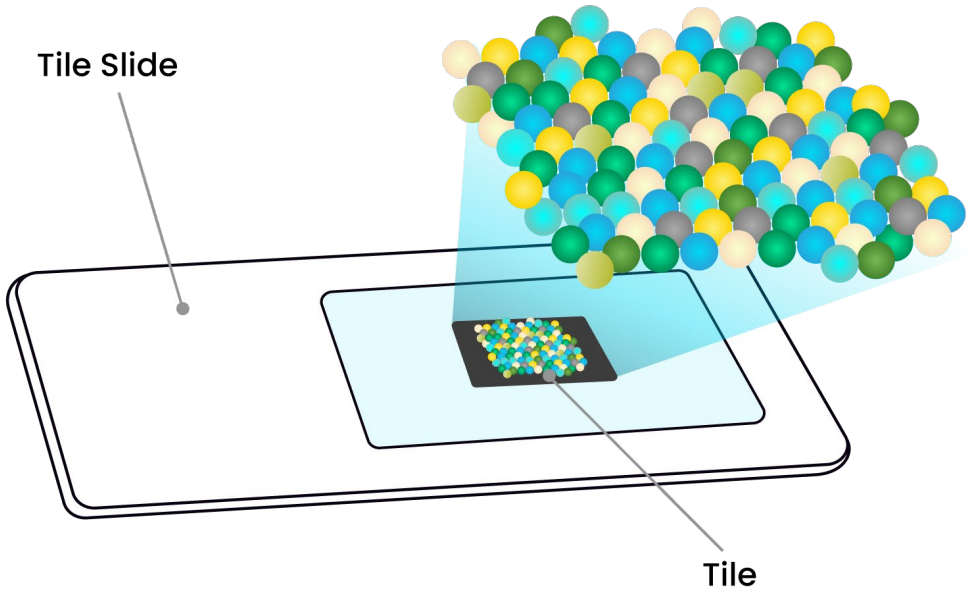

Figure 1. Seeker tile

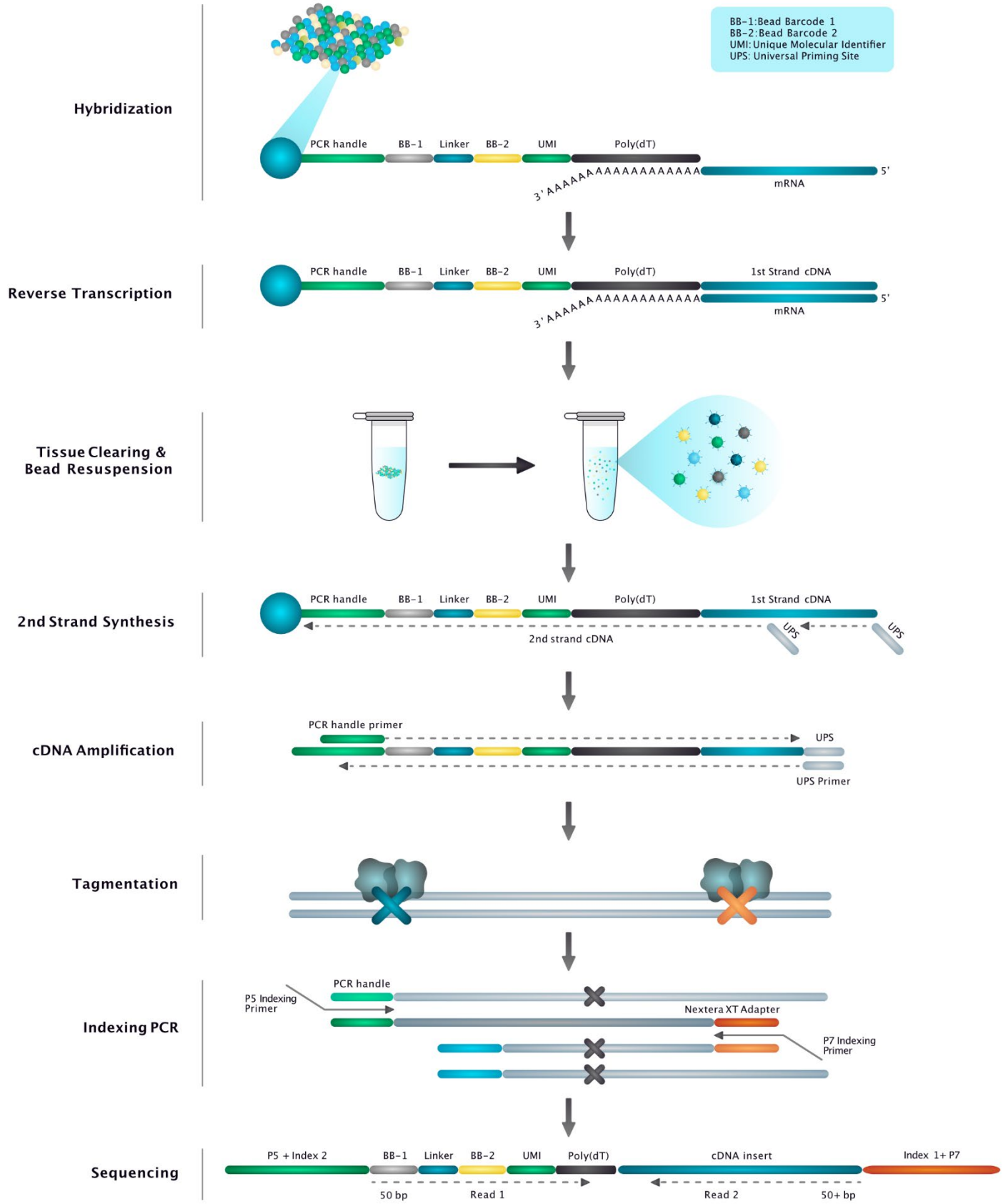

Figure 2. Seeker workflow. Please refer to supplementary sections at the end of this manual for more detailed sequence information.

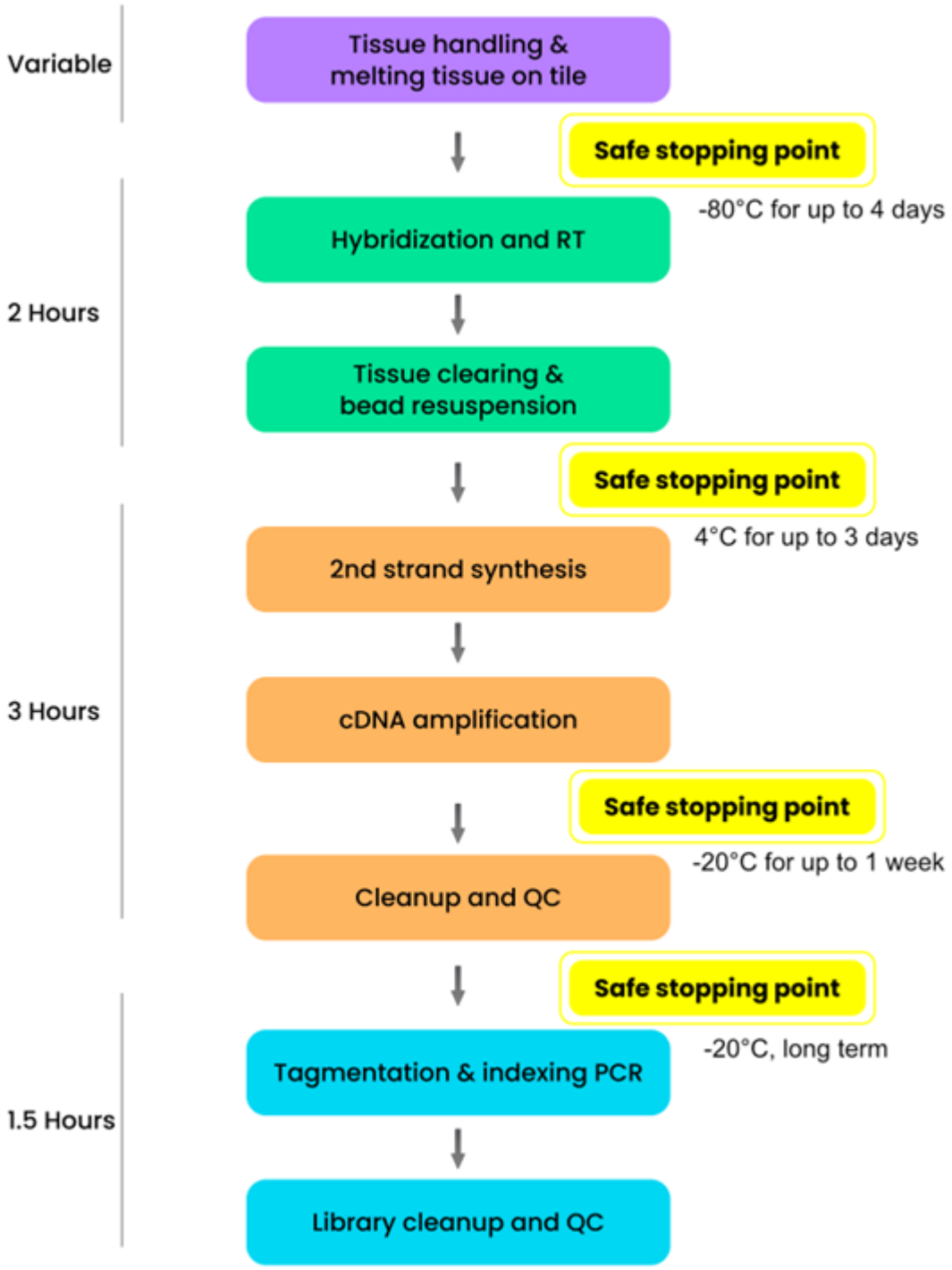

Figure 3. Estimated workflow timing

II. List of Components

Table 1. Seeker 3x3 Bundle V1.1 components.

Seeker 3x3 Bundle V1.1 (Cat. No. SK004; 8 rxns)	Cap Color
Seeker 3x3 Tiles (Cat. No. SQB003; Store at 4°C)*	
Seeker 3x3 Tile v1.1	–
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 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, Cat. No. 5067-4626) -or- TapeStation High Sensitivity DNA D5000 ScreenTape (Agilent, Cat. No. 5067-5592) and TapeStation High Sensitivity DNA D5000 Reagents (Agilent, 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, Cat. No. G2939BA/G2953CA) -or- 4200 TapeStation (Agilent, 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)
- 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

- 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 a 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 30 million 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 on the right.

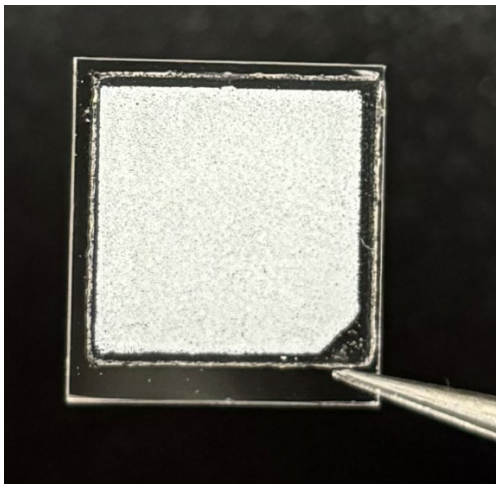

Figure 4. Correct tweezer placement when transferring the Seeker tile. Grip near the chamfered corner. Take care to avoid direct contact with the beads.

- 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 keep them on ice until ready to use:
   a. RT/SS buffer
      i. If precipitate is observed, heat the RT/SS buffer at 37°C for 5 min and briefly vortex before use.
   b. dNTP
2. Set one heat block to 52°C and another to 37°C.

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).
3. Place the Seeker tile slide in the cryostat to chill for at least 1 min.
4. Record the tile ID (e.g., A0084_046) of the Seeker tile as shown in the example in Figure 5.

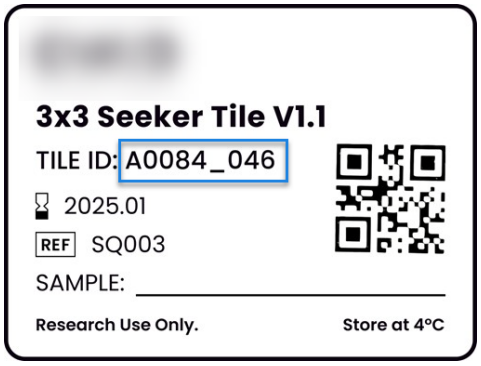

Figure 5. Example Seeker Tile label to identify the tile ID. The tile ID, A0084_046, is indicated by the blue box.

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

5. Section a 10 µm section of tissue.
6. 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 Figure 6.

Figure 6. Example of precise tissue placement on the Seeker tile.

iv. 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.

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.

7. Place the tile with the melted tissue section back into the cryostat.
8. (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.
9. Remove the tissue block and replace it with the CryoCube.
10. Section a 30 µm section of the CryoCube. Alternatively, 60 µm can be used for tissues with higher expression levels.

NOTE: The CryoCube overlay is used to prevent leakage of mRNA signal across the surface of the tissue after cryosectioning to reduce background noise.

11. Move the CryoCube section onto the tile so that it covers the entire tile.
12. 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 6b.
13. 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.
14. Remove the remaining block of tissue and CryoCube from the cryostat and store them at –80°C.

NOTE: It is recommended to cover the exposed tissue of the tissue block with a drop of OCT and freeze prior to storage to prevent desiccation of the tissue.

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

15. 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:

190 µl	Hyb buffer
10 µl	RNAse inhibitor
200 µl	**Total volume**

16. 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.
17. Carefully remove the tile from the blue adhesive with tweezers and place it in a 1.5 ml Eppendorf LoBind tube containing 200 µl of Hybridization Reaction Mix. Make sure the tile is completely submerged as shown in Figure 7.

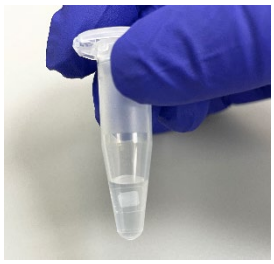

Figure 7. Seeker Tile submerged in Hybridization Reaction Mix.

18. Incubate for 30 min at room temperature.

C. Reverse Transcription

1. Prepare the 1X RT wash buffer following the table below for washing the tile prior to RT, plus 5% of the total volume for overage. Mix by vortexing, centrifuge briefly, and keep at room temperature:

40 µl	RT/SS buffer
160 µl	Nuclease-free water
200 µl	**Total volume per reaction**

2. Prepare the RT Reaction Mix following the table below, plus 5% of the total volume for overage. Mix by pipetting 10x and keep on ice:

40 µl	RT/SS buffer
20 µl	dNTP
5 µl	RNase inhibitor
10 µl	RT enzyme
125 µl	Nuclease-free water
200 µl	**Total volume per reaction**

3. Using a pair of clean tweezers, remove the tile from the Hybridization Reaction Mix and dip it in 1X RT wash buffer for 3 sec.
4. Transfer the tile to a new 1.5 ml tube containing 200 µl of RT reaction mix. Make sure the tile is completely submerged.
5. Incubate the tube at room temperature for 10 min.
6. Move the tube to a heat block set at 52°C and incubate for 30 min.

D. Tissue Clearing and Seeker Bead Resuspension

1. If precipitation is observed, heat the TC buffer at 37°C for five 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 following the table below, plus 5% of the total volume for overage. Mix by pipetting 10x and keep at room temperature:

196 µl	TC buffer
4 µl	TC enzyme
200 µl	**Total volume per reaction**

4. Add 200 µl of tissue clearing reaction mix to the tube containing the tile and RT reaction mix. To mix without disrupting the tile, set p200 to 190 µl, pipette from the bottom of the tube and dispense onto the side of the tube. Repeat 10X.
5. Incubate at 37°C for 30 min.

NOTE: 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.

6. Following the incubation, add 200 µl of bead wash buffer, pipette-mix repeatedly to dissociate beads from the glass slide, and mechanically shear the tissue.

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 center of 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. If the tissue persists, you may place it back at 37°C for an additional 10 min.

– 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 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.

7. Guide the remaining piece of glass to the top rim of the 1.5 ml tube with your pipette tip. Remove it from the tube with tweezers or forceps and discard.
8. Transfer the contents to a new 1.5 ml tube to ensure effective pelleting of the beads.

9. Spin the beads down for 2 min at 3,000*g* at room temperature. A white bead pellet should be visible to the eye (Figure 8).

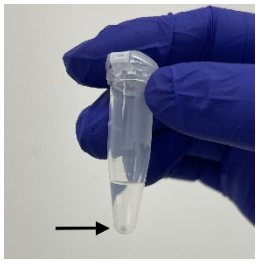

Figure 8. Example of the bead pellet after dissociation and centrifugation.

10. Remove any bubbles from the top of the supernatant.
11. Carefully remove and discard the supernatant without disturbing the bead pellet as shown above.

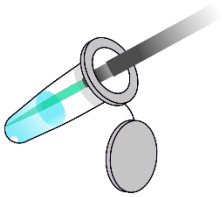

Figure 9. Example of pipette placement for supernatant removal from around the bead pellet.

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 supernatant. If the pellet begins to slide, spin for an additional 30 sec at 3,000*g*. **THIS APPLIES TO ALL BEAD WASH STEPS IN THIS PROTOCOL**.

NOTE: To retain as many beads as possible, it is not necessary to remove all the supernatant from the initial washes. 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.

12. Resuspend the bead pellet in 200 µl of bead wash buffer and pellet the beads by centrifuging for 2 min at 3,000*g* at room temperature.
13. Immediately remove and discard the supernatant (You may leave <10 µl of Bead Wash Buffer to preserve the bead pellet) and resuspend the bead pellet in 200 µl of bead wash buffer.

SAFE STOPPING POINT: Beads can be stored at 4°C for up to three 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, plus 5% of the total volume for overage. Mix by pipetting 10X and keep at room temperature.

Second-Strand Mix:

40 µl	RT/SS buffer
20 µl	dNTP
2 µl	SS primer
5 µl	SS enzyme
133 µl	Nuclease-free water
200 µl	**Total volume per reaction**

NOTE: 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 the beads down for 30 sec at 3,000*g* at room temperature and carefully remove and discard the supernatant.

IMPORTANT: Remove the supernatant immediately after centrifugation. THIS APPLIES TO ALL REMAINING STEPS IN SECTION E.

8. Immediately resuspend the beads in 200 µl of the Second-Strand Mix.
9. Incubate at 37°C for one hour.
10. Add 200 µl of bead wash buffer.
11. Spin the beads down for 2 min at 3,000*g* at room temperature and **immediately** remove and discard the supernatant.
12. Resuspend the beads in 200 µl of bead wash buffer.

F. cDNA Amplification

1. Thaw the following reagents at room temperature, vortex, briefly centrifuge, and then keep on ice until ready for use:
   a. cDNA amp buffer
   b. cDNA amp primer mix
2. Keep the cDNA amp enzyme on ice until ready for use.
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 following the table below, plus 5% of the total volume for overage. Mix by pipetting 15X and keep on ice:

cDNA Amplification Mix:

100 µl	cDNA amp buffer
8 µl	cDNA amp primer mix
4 µl	cDNA amp enzyme
88 µl	Nuclease-free water
200 µl	**Total volume per reaction**

5. Spin the beads down for 2 min at 3,000*g* at room temperature and immediately remove and discard the supernatant.
6. Add 200 µl of the cDNA amplification mix to the beads.
7. Split the cDNA amplification mix and beads into four PCR tubes (50 µl each).
8. Pipette-mix each PCR tube before placing the tubes into the thermal cycler.
9. Immediately run the cDNA amplification program on the preheated thermocycler as follows:

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 may be required during Phase 2. 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 reactions from the four PCR tubes into a single 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 reamplification.

5. Vortex the SPRIselect reagent for 30 sec and carefully add 120 µl to the tube with supernatant (0.6X volume of amplification volume). Pipette mix.
6. Vortex to mix for 10–15 sec.
7. Incubate at room temperature for 5 min.
8. Briefly centrifuge the tubes and place the tubes on the magnetic rack. Once the solution is clear, carefully aspirate and discard the supernatant.
9. Keeping the tube on the magnetic stand, add 500 µl of 80% ethyl alcohol.
10. Wait 30 sec and remove the supernatant.
11. Add 500 µl 80% ethyl alcohol.
12. Wait 30 sec and remove the supernatant.
13. Briefly spin the tube to collect the remaining ethyl alcohol at the bottom of the tube.
14. Place the tube back on the magnetic rack and remove the remaining ethyl alcohol carefully.
15. Let the SPRIselect reagent dry at room temperature until the beads appear matte (1–2 min).
16. 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.
17. Incubate at room temperature for 1 min.
18. Place the tube back on the magnetic rack.
19. 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

20. Vortex the SPRIselect reagent for 30 sec and carefully add 30 µl to the tube (0.6X volume of amplification volume). Pipette mix.
21. Vortex to mix for 10–15 sec.
22. Incubate at room temperature for 5 min.
23. Briefly centrifuge the tubes and place the tubes on the magnetic rack. Once the solution is clear, carefully aspirate and discard the supernatant.

24. Keeping the tube on the magnetic stand, add 200 µl of 80% ethyl alcohol.
25. Wait 30 sec and remove the supernatant.
26. Add 200 µl of 80% ethyl alcohol.
27. Wait 30 sec and remove the supernatant.
28. Briefly spin the tube to collect the remaining ethyl alcohol at the bottom of the tube.
29. Place the tube back on the magnetic rack and remove the remaining ethyl alcohol.
30. Let the SPRIselect reagent dry at room temperature until the beads appear matte (30 sec–2 min).
31. Remove the tube from the magnetic rack and add 20 µl of nuclease-free water to elute. Pipette the beads to mix well and incubate at room temperature for 1 min.

    TIP: If your sample is expected to have low cDNA yield, elute with 10 µl of nuclease-free water.

32. Place the tubes on a magnetic rack.
33. Once the solution is clear, transfer the supernatant to a new tube. Discard the used beads.
34. 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 or TapeStation High Sensitivity D5000 assay or TapeStation D5000 assay).

    Concentrations in the range of **0.2 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 18–32.

TapeStation D5000

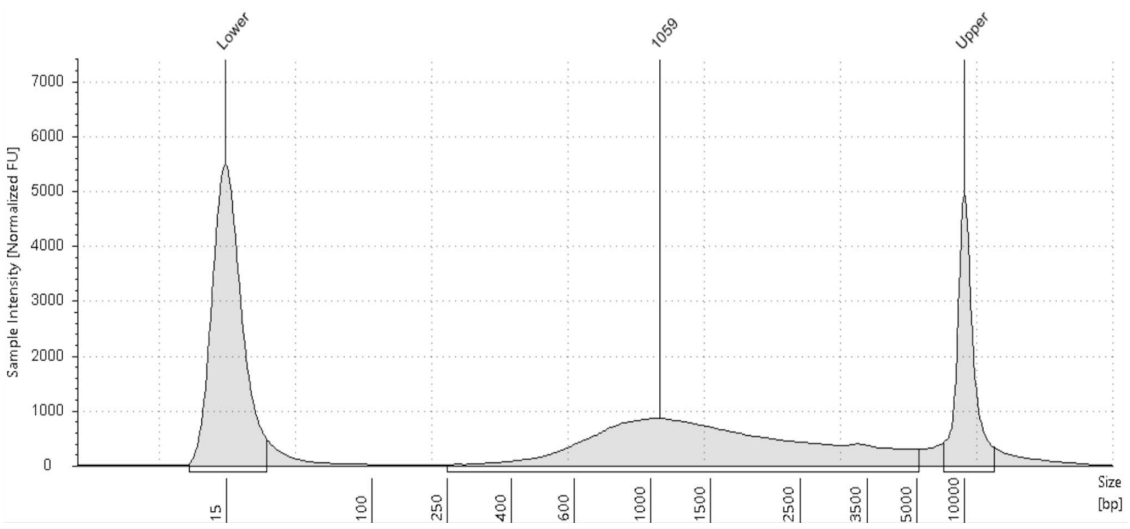

Figure 10. Example TapeStation D5000 electropherogram results after cDNA amplification and purification.

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:
   - Dual Indexing Primers
   - Tagment DNA Buffer (**TD**) buffer
3. Keep Amplicon Tagment Mix (**ATM**) and Nextera PCR Master Mix (**NPM**) on ice.
4. In a 0.2 ml PCR tube, add 600 pg of cDNA. Top off to a total volume of 5 µl with nuclease-free water. For example, if your cDNA amplified product has a concentration of 0.5 ng/µl, use 1.2 µl and top off with 3.8 µl of water. If the calculated volume is <1 µl, dilute the products to avoid pipetting low volumes.
5. Add 10 µl of the **TD** buffer.
6. Add 5 µl of **ATM**. Pipette to mix.
7. Briefly centrifuge the tube.
8. Incubate at 55°C for 5 min.
9. After 5 min, **immediately** add 5 µl of **Neutralize Tagment Buffer (NT)**. Mix by pipetting ~5 times and spin down.
10. Incubate at room temperature for 5 min.
11. Add 15 µl of **NPM** to each tube.

WARNINGS:

– In step 12, please ensure that each sample that will be sequenced together has a unique combination of F and R primers.

– 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

12. Add 5 µl of index primer F and 5 µl of index primer R from the Seeker Dual Indexing Primer Kit v2 (Cat. No. K006) to the tube.
13. Pipette to mix and briefly centrifuge the tube.
14. Run the indexing PCR program according to the table below (~35 min total run time).

72°C	3 min
95°C	30 sec
12 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. Vortex the SPRIselect reagent at high speed for 30 sec. The beads should appear homogeneous and uniform in color.
3. Perform 0.6X SPRI cleanup by carefully adding 30 µl of SPRIselect reagent to 50 µl of the total sample. Pipette mix.
4. Vortex to mix for 10–15 sec.
5. Incubate at room temperature for 5 min.
6. Briefly centrifuge the tubes and place the tubes on the magnetic rack. Once the solution is clear, carefully aspirate and discard the supernatant.
7. Keeping the tube on the magnetic stand, add 200 µl of 80% ethyl alcohol.
8. Wait 30 sec and remove the supernatant.
9. Add 200 µl of 80% ethyl alcohol.
10. Wait 30 sec and remove the supernatant.
11. Briefly spin the tube to collect the remaining ethyl alcohol at the bottom of the tube.
12. Place the tube back on the magnetic rack and remove the remaining ethyl alcohol.
13. Let the SPRIselect reagent dry at room temperature until the beads appear matte (30 sec–2 min).
14. Remove the tube from the magnetic rack and add 50 µl of TE to elute. Pipette beads to mix well and incubate at room temperature for 1 min.
15. Place tubes on a magnetic rack and incubate for 1 min.
16. Transfer the supernatant to a new PCR tube.

2. Second 0.8X Bead Purification

17. Add 40 µl of SPRI beads to the tube (0.8X of the eluted volume).
18. Vortex to mix for 10–15 sec.
19. Incubate at room temperature for 5 min.
20. Briefly centrifuge the tube.
21. Place the tube on the magnetic rack. Once the solution is clear, carefully aspirate, and discard the supernatant.
22. Keeping the tube on the magnetic stand, add 200 µl of 80% ethanol.
23. Wait 30 sec and remove the supernatant.
24. Add 200 µl of 80% ethanol.
25. Wait 30 sec and remove the supernatant.
26. Briefly spin the tube to collect the remaining ethanol at the bottom of the tube.
27. Place the tube back on the magnetic rack and remove the remaining ethanol.

28. Let the SPRI beads dry at room temperature until the beads appear matte (30 sec–2 min).
29. 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 min.
30. Place the tube on a magnetic rack and incubate for 1 min.
31. Transfer the supernatant to a new 0.2 ml PCR tube.
32. 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 above **1 ng/µl** are acceptable. See the example library trace in Figure 10

    TapeStation D5000

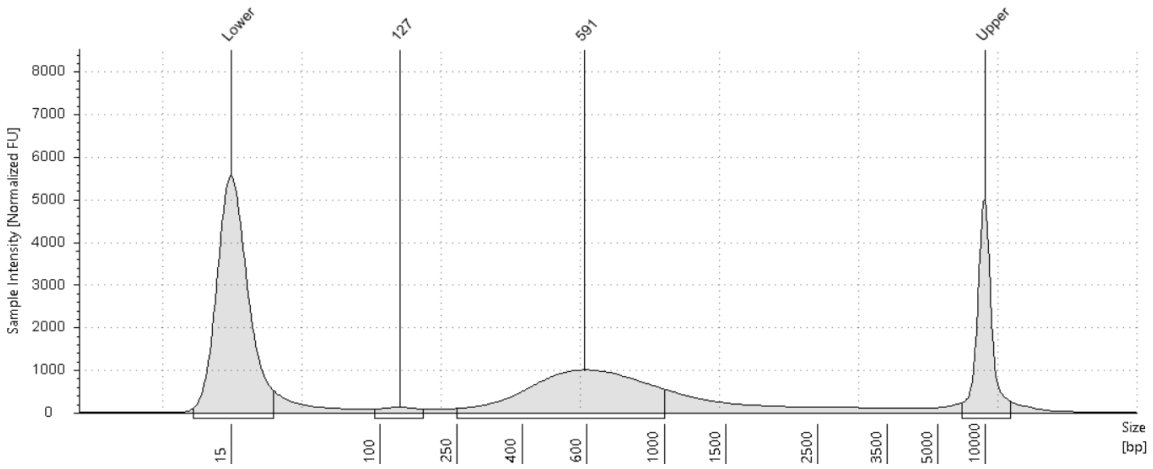

Figure 14. Example TapeStation D5000 electropherogram results after library clean-up.

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, ~3.0 x 10^7 reads per tile is recommended.
- For **deep sequencing**, 2–6 x 10^8 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:
  - 5% PhiX spike-in when pooling with non-Seeker libraries
  - 10% PhiX spike-in when sequencing 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 11. Seeker 3x3 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	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 (<0.2 ng/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.

Problem	Possible Explanation	Solution
cDNA amplification product yield is low (<0.2 ng/µl) *(continued from previous page)*	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. Repeat centrifugation to ensure tight pelleting.
	SPRIselect reagents are not properly mixed before use or after being added to the sample.	The SPRIselect reagent is viscous and requires full resuspension of the magnetic beads by vortexing before pipetting. Carefully pipette the viscous SPRI regent after complete resuspension by vortexing to ensure the correct volume is added to the sample. Pipette-mix the SPRI reagent with the sample until homogenous.
	80% Ethyl alcohol is not fresh or is at the wrong concentration	Always make fresh ethyl alcohol the day of the bead cleanup since ethyl alcohol evaporates quickly. Ethyl alcohol may evaporate from a closed tube which changes the concentration and may affect your cDNA purification yield.
	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
	An incorrect PCR program was used or entered	Ensure the correct PCR program is used
	Buffer precipitation occurred during bead wash steps after tissue clearing	Ensure the bead wash buffer and centrifuge are >20°C. If working in a workspace 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.
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

Problem	Possible Explanation	Solution
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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