USER GUIDE

Curio Seeker 3x3 Spatial Mapping Kit

For fresh frozen tissues

Document # CB-000470-UG v2.2b
May 2024
CURIO BIOSCIENCE PROPRIETARY

Table of Contents

Version summary

Revision date	Version	Major changes
Aug. 2023	2.1	• Addition of Qubit in PCR Product QC Steps in sections G and I • Included SparQ Beads as an alternative to SPRISelect Reagent for bead cleanup steps in sections G and I • Added new workflow steps to include a CryoCube section in section B • Added notes about Precipitation in RT/SS Buffer and TC Buffer • Updated Bead Dissociation Tips in section D • Provided additional cDNA Amp Cycle Number Recommendations in section F • Updated indexing PCR Reaction Volumes to reflect changes in the Dual Indexing Primer Kit v2 in section H • Updated Sequencing Read Recommendation in section J • Updated Phix Spike-In Percentage for Sequencing in section J • Added an Appendix with Oligo Sequences
Feb. 2024	2.2	• Edited Appendix with minor changes • Add PCR tube magnetic rack • Added static electricity warning • Added storage temperature recommendations for master mixes • Corrected Curio Seeker Dual Indexing Kit v1 instructions • RIN score addendum • Added CryoCube guidelines in section B • Increased hybridization time from 15 minutes to 30 minutes • Added buffer temperature guidelines for the bead dissociation step in section D
May 2024	2.2b	• Minor edition on schematics

Overview

This user manual describes the 3x3 Curio Seeker Spatial Mapping Kit protocol, hereby referred to as the Curio 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 Curio 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 Curio 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.

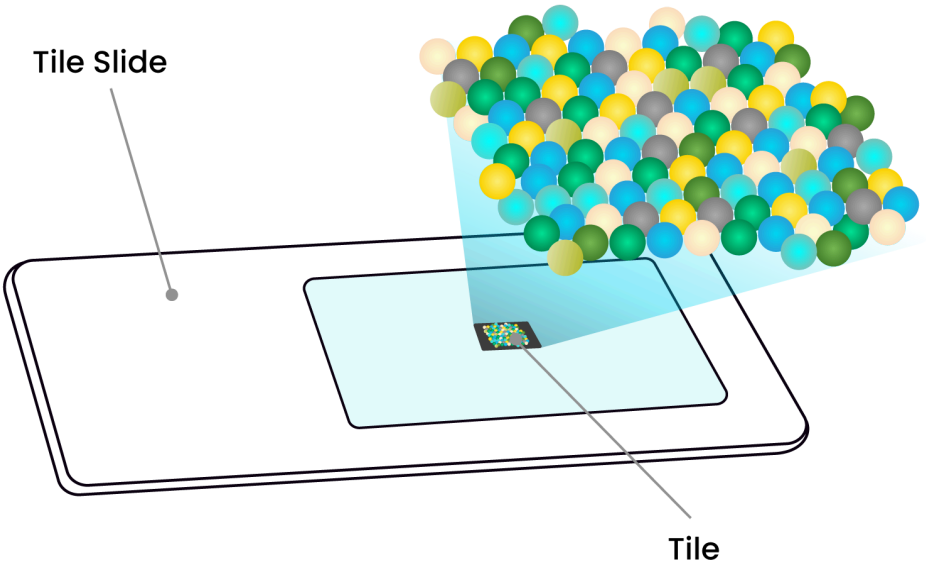


Figure 1. Curio Seeker tile

Curio Seeker workflow

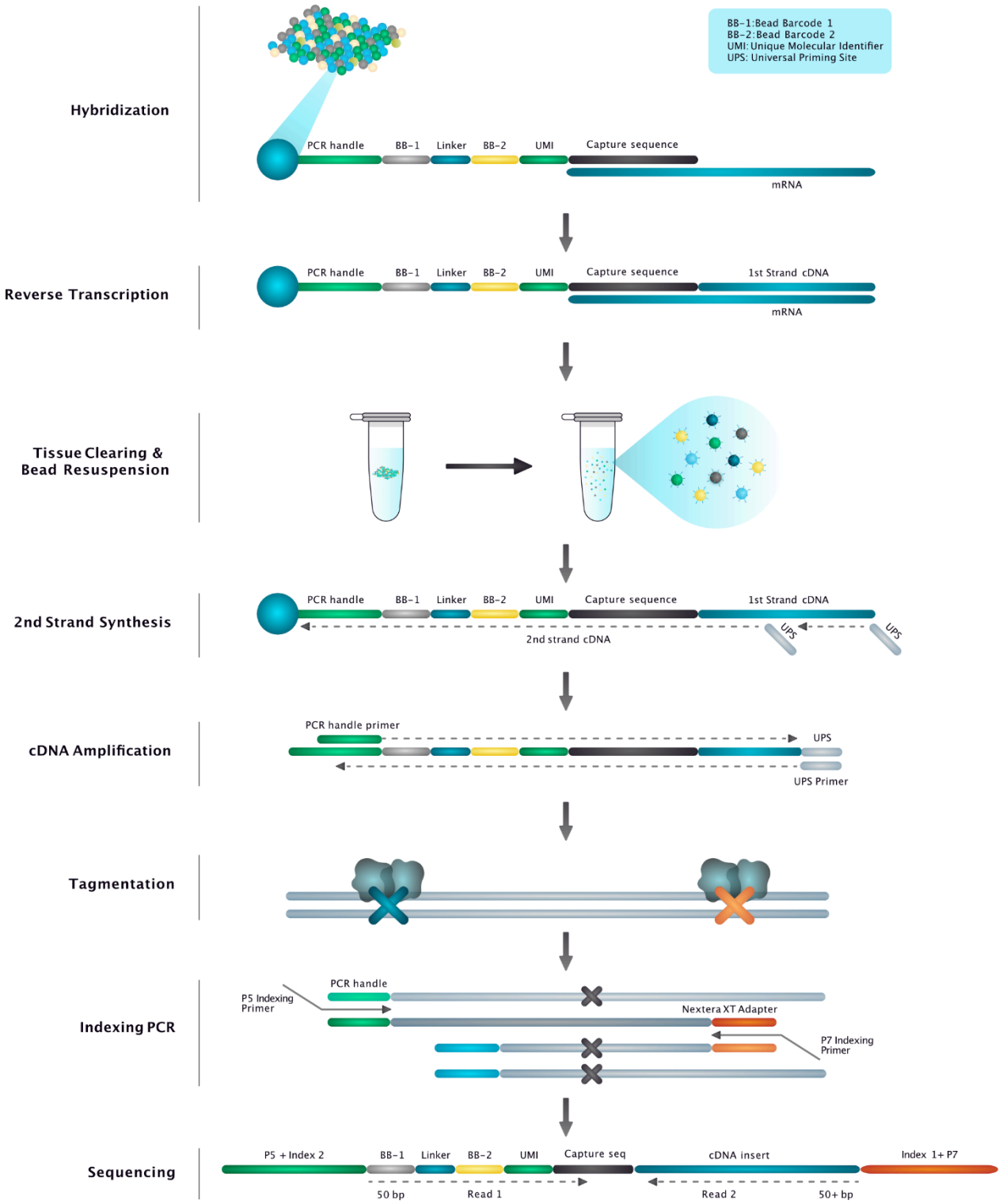


Figure 2. Curio Seeker workflow. Please refer to supplementary sections at the end of this protocol for more detailed sequence information.

Estimated workflow timing

Figure 2. Estimated workflow timing

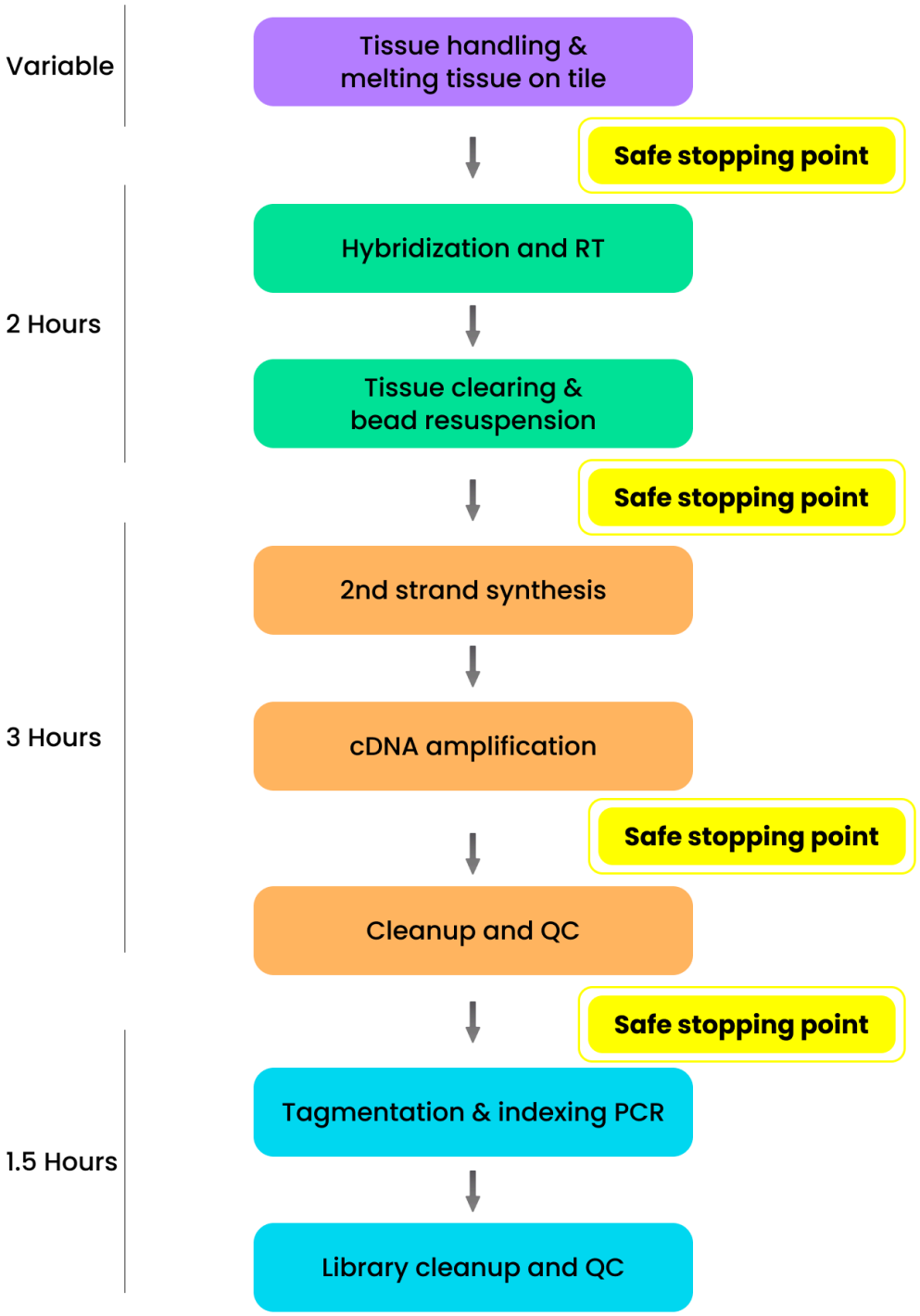


Figure 3. Estimated workflow timing

Materials

Curio Seeker 3x3 Kit components (8 reactions)

Component	Part number
Pouch 1 (4°C)	**SQB002**
3x3 Seeker Tile v1.1	SQ003
Box 1 (RT)	**K001**
Hyb Buffer	B005
TC Buffer	B004
Nuclease-free Water	B001
Bead Wash Buffer	B003
Box 2 (-20°C)	**K002**
RNase Inhibitor	E001
RT/SS Buffer	B006
dNTP	N001
RT Enzyme	E003
TC Enzyme	E002
SS Primer	P002
SS Enzyme	E007
cDNA Amp Buffer	B007
cDNA Amp Primer Mix	P003
cDNA Amp Enzyme	E006
TE	B009
Pouch 2 (-80°C)	
CryoCube	JW001

Variations of the Curio Seeker Dual Indexing Primer Kit: v1 and v2

To improve the workflow and ensure success for our users, we have updated the Curio Seeker Dual Indexing Primer Kit with a new version. Both v1 and v2 dual indexing primer kits support up to 40 3x3 Curio Seeker tiles. Please follow the appropriate portion in this user guide for setting up the indexing PCR in section H based on the Dual Indexing Primer Kit version you are using.

Curio Seeker Dual Indexing Primer Kit v1 components

Component	Part number
Seeker Dual Indexing Primer Kit v1 (-20°C)	**K003**
Index Primer F1	P005
Index Primer F2	P006
Index Primer F3	P007
Index Primer F4	P008
Index Primer F5	P009
Index Primer R1	P010
Index Primer R2	P011
Index Primer R3	P012
Index Primer R4	P013
Index Primer R5	P014

Note: Do not substitute with any other primers.

Curio Seeker Dual Indexing Primer Kit v2 components

Component	Part number
Seeker Dual Indexing Primer Kit v2 (-20°C)	**K006**
Index Primer F1	P016
Index Primer F2	P017

Index Primer F3	P018
Index Primer F4	P019
Index Primer F5	P020
Index Primer R1	P021
Index Primer R2	P022
Index Primer R3	P023
Index Primer R4	P024
Index Primer R5	P025

Note: Do not substitute with any other primers.

Required reagents not included

Component	Vendor	Part number
Ethyl Alcohol	Sigma Aldrich	459844-1L
SPRIselect Reagent or sparQ PureMag beads	Beckman Coulter Quanta Bio	B23318 95196-005
Nextera XT Library Prep Kit	Illumina	FC-131-1024
Bioanalyzer High Sensitivity DNA kit or	Agilent	5067-4626
TapeStation High Sensitivity DNA D5000 ScreenTape	Agilent	5067-5592
TapeStation High Sensitivity DNA D5000 Reagents	Agilent	5067-5593
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher	Q33230

Note: Do not substitute with any other library prep kits.

Equipment

Component	Vendor	Part number
Cryostat	Leica	CM3050S
Single-channel pipette: 10 µL, 20 µL, 200 µL, and 1,000 µL	Rainin	17014388 17014392 17014391 17014382
Eight-channel or 12-channel pipette: 20 µL and 200 µL	Rainin	17013803 17013805
Mini centrifuge for 1.5 mL tubes	Major suppliers	-
Mini centrifuge for 0.2 mL tubes	Major suppliers	-
Eppendorf Centrifuge 5415 D or equivalent	Eppendorf	5425-55001
2 heat blocks for 1.5 mL tubes	Major suppliers	-
96-well PCR chiller rack or 96-well aluminum block	MIDSCI Light Labs	5640-T4 A-7079
C1000 Touch Thermal Cycler	Bio-Rad	1851148
Vortexer	Major suppliers	-
Tweezers	Ted Pella	58083-NM
2100 Bioanalyzer or 4200 TapeStation	Agilent Agilent	G2939BA/G2953CA G2991AA
DynaMag™-2 Magnet (1.5 mL)	Invitrogen	12321D
12-Tube Magnetic Separation Rack (PCR tube)	NEB	S1515S
Qubit 4 Fluorometer	Thermo Fisher	Q33238

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.

Consumables

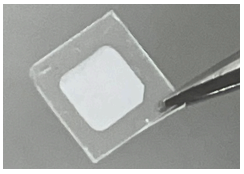
Component	Vendor	Part number
Filter pipette tips: 20 µL, 200 µL, and 1,000 µL	Rainin	30389226 30389240 30389213
DNA LoBind 1.5 mL tubes	Eppendorf	022431021
0.2 mL PCR strip tubes	USA Scientific	1402-4700
Optimal Cutting Temperature compound (OCT compound)		
Qubit assay tubes	Thermo Fisher	Q32856

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 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 Curio Seeker workflow to provide information on tissue structural context and sectioning quality.
- Run a pilot Curio Seeker tile experiment on new tissue samples and perform shallow sequencing of 30 million reads to assess library quality.

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 the example below:



- 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.
- 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 seconds 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.*

Protocol

A. Preparation before starting

1. Thaw the following reagents at room temperature 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 minutes and briefly vortex before use.
    b. dNTP
2. Set one heat block to 52°C and another to 37°C.

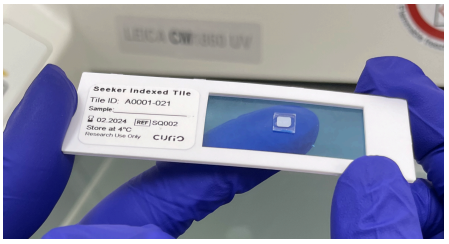
B. Tissue sectioning and hybridization to the Curio Seeker tile

1. Equilibrate the fresh frozen tissue and CryoCube to -18 °C in a cryostat (such as Leica CM3050S) for at least 20 minutes 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 Curio Seeker tile slide in the cryostat to chill for at least one minute.
4. Record the tile ID (ex: A0001_001) of the Curio Seeker tile as shown in the example on the right.

IMPORTANT: Each Curio 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 Curio Seeker tile slide in the cryostat to chill for one minute
        ii. Place the chilled Curio 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.



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. Remove the tissue block and replace it with the CryoCube.
9. Section a 30 µm section of the CryoCube. Alternatively, 60 µm can be used for tissues with higher expression levels.
10. Move the CryoCube section onto the tile so that it is covering the entire tile.
11. 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.
12. 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.
13. Remove the remaining block of tissue and CryoCube from the cryostat and store them at -80°C.

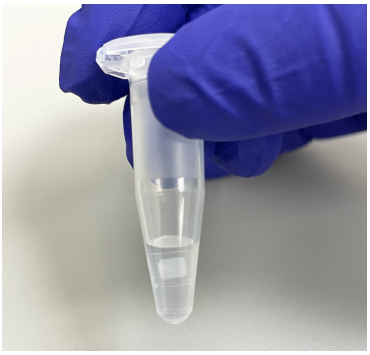
TIP: It is recommended to cover the exposed tissue with a drop of OCT and freeze prior to storage to prevent desiccation of the tissue.

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

14. Prepare the **hybridization reaction mix** following the table below and keep at room temperature:

Component	1 Reaction (µL)	Plus 5% overage (µL)
○ Hyb buffer	190	199.5
○ RNAse inhibitor	10	10.5
Total	200	210

15. 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.
16. 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 the photo below.**



17. Incubate for 30 minutes at room temperature.

C. Reverse transcription

1. Prepare the **1X RT wash buffer** following the table below for washing the tile prior to RT and keep at room temperature:

Component	1 reaction (µL)	Plus 5% overage (µL)
○ RT/SS buffer	40	42
○ Nuclease-free water	160	168
Total	200	210

2. Prepare the **RT reaction mix** following the table below and keep on ice:

Component	1 Reaction (µL)	Plus 5% overage (µL)
○ RT/SS buffer	40	42
○ dNTP	20	21
○ RNase inhibitor	5	5.25
○ RT enzyme	10	10.5
○ Nuclease-free water	125	131.25
Total	200	210

3. Using a pair of clean tweezers, remove the tile from the hybridization reaction mix and dip it in 1X RT wash buffer for three seconds.
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 minutes.
6. Move the tube to a heat block set at 52°C and incubate for 30 minutes.

D. Tissue clearing and Curio Seeker bead resuspension

1. If precipitate is observed, heat the TC buffer at 37°C for five minutes 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 and keep at room temperature:

Component	1 reaction (µL)	Plus 5% overage (µL)
TC buffer	196	205.8
TC enzyme	4	4.2
Total	200	210

4. Add 200 µL of tissue clearing reaction mix to the tube containing the tile and RT reaction mix. Carefully pipette-mix 10 times without disrupting the tile.
5. Incubate at 37°C for 30 minutes.

TIP: If your tissue is difficult to digest or contains plant cell walls, you may wish to increase tissue clearing incubation from 30 minutes to a total duration of one hour.

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.

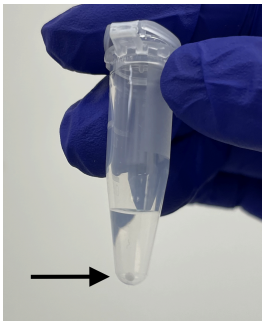
TIP: Aim the pipette tip at the center of the bead patch when dissociating the beads from the glass slide. You should see patches of beads detach from the glass slide as you pipette. Occasionally, the residual tissue and beads may still be loosely associated after detachment from the glass slide. Pipet 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 minutes.

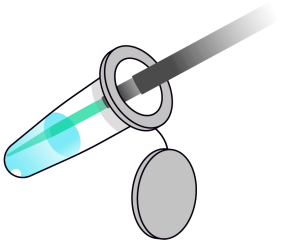
IMPORTANT: There should be no visible patches of beads remaining on the glass. It is important to completely dissociate the beads from the glass slide.

TIP: 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 pipet 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 two mins at 3000 x *g*. A white bead pellet should be visible to the eye.





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. .

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 seconds at 3000 x *g*. **THIS APPLIES TO ALL BEAD WASH STEPS IN THIS PROTOCOL**.

TIP: In order to retain as many beads as possible, it is not necessary to remove all the supernatant from 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 minutes at 3000 x *g*.
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 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 minutes.
6. Prepare the **second strand mix** and keep at room temperature.

Component	1 reaction (µL)	Plus 5% overage (µL)
RT/SS buffer	40	42
dNTP	20	21
SS primer	2	2.1
SS enzyme	5	5.25
Nuclease-free water	133	139.7
Total	200	210

7. After 5 minutes of incubation at 95°C, immediately spin the beads down for 30 seconds at 3000 x *g* and carefully remove and discard the supernatant.

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

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.

8. Immediately resuspend the beads in 200 µL of 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 minutes at 3000 x *g* 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 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.
4. Prepare the **cDNA amplification mix** following the table below and keep on ice:

Component	1 reaction (µL)	Plus 5% overage (µL)
cDNA amp buffer	100	105
cDNA amp primer mix	8	8.4
cDNA amp enzyme	4	4.2
Nuclease-free water	88	92.4
Total	200	210

5. Spin the beads down for 2 minutes at 3000 x *g* 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 pre-heated thermocycler as follows:

Temperature ramp rate: 3°C/s	Time	Cycle
98°C	2 min	
98°C	20 sec	4 cycles (phase 1)
65°C	45 sec	
72°C	3 min	
98°C	20 sec	9* cycles (phase 2)
67°C	20 sec	
72°C	3 min	
72°C	5 min	
4°C	Hold	

IMPORTANT: * 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.

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 minutes prior to use.

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. Vortex the SPRIselect reagent for 30 seconds and carefully add 120 µL to the tube (0.6X volume of amplification volume). Pipette mix.
4. Vortex to mix for 10-15 seconds.
5. Incubate at room temperature for 5 minutes.
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 500 µL of 80% ethyl alcohol.
8. Wait 30 seconds and remove the supernatant.
9. Add 500 µL 80% ethyl alcohol.
10. Wait 30 seconds 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 carefully.
13. Let the SPRIselect reagent dry at room temperature until the beads appear matte (1 -2 minutes).
14. 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.
15. Incubate at room temperature for one minute.
16. Place the tube back on the magnetic rack.
17. Once the solution is clear, transfer the supernatant to a new **0.2 mL PCR tube**. Discard the used beads.

Second 0.6X bead purification

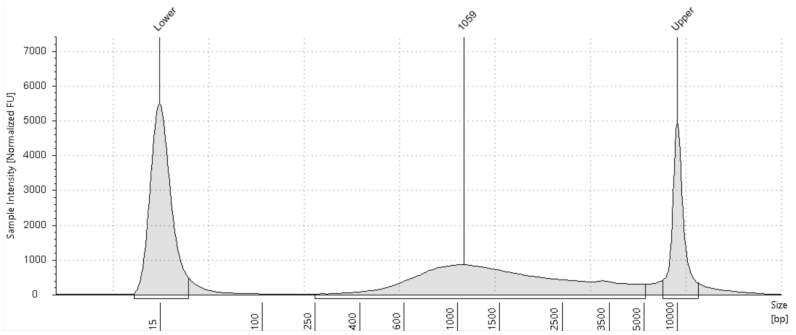
18. Vortex the SPRIselect reagent for 30 seconds and carefully add 30 µL to the tube (0.6X volume of amplification volume). Pipette mix.
19. Vortex to mix for 10-15 seconds.
20. Incubate at room temperature for 5 minutes.
21. Briefly centrifuge the tubes and place the tubes on the magnetic rack. Once the solution is clear, carefully aspirate and discard the supernatant.
22. Keeping the tube on the magnetic stand, add 200 µL of 80% ethyl alcohol.
23. Wait 30 seconds and remove the supernatant.
24. Add 200 µL of 80% ethyl alcohol.
25. Wait 30 seconds and remove the supernatant.
26. Briefly spin the tube to collect the remaining ethyl alcohol at the bottom of the tube.
27. Place the tube back on the magnetic rack and remove the remaining ethyl alcohol.

28. Let the SPRIselect reagent dry at room temperature until the beads appear matte (30 seconds -2 minutes).
29. 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 one minute.

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

30. Place the tubes on a magnetic rack.
31. Once the solution is clear, transfer the supernatant to a new tube. Discard the used beads.
32. Quantify the cDNA products using Qubit (1X dsDNA HS assay kit) and a Bioanalyzer or TapeStation following 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



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

H. Tagmentation (with Nextera XT Library Prep Kit)

1. Preheat a thermocycler to 55°C and heat the lid to 105°C.
2. Thaw the following reagents at room temperature and then keep on ice:
    a. Dual Indexing Primers
    b. 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.
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 minutes.
9. After 5 minutes, **immediately** add 5 µL of **Neutralize Tagment Buffer (NT)**. Mix by pipetting ~5 times and spin down.
10. Incubate at room temperature for 5 minutes.

IMPORTANT: Identify the part number of your Curio Seeker dual indexing primer kit. If you are using the Curio Seeker dual indexing primer kit (K003), proceed to Appendix A. If you are using the Curio Seeker dual indexing primer kit v2 (K006, released August 2023), proceed to step 11 below.

11. Add 15 µL of **NPM** to each tube.
12. Add 5 µL of index primer F and 5 µL of index primer R from the **Curio Seeker dual indexing primer v2 kit (K006)** to the tube.

WARNING: Please ensure that each sample that will be sequenced together have a unique combination of F and R primers.

13. Pipette to mix and briefly centrifuge the tube.
14. Run the indexing PCR program according to the table below:

Temperature	Time	Cycle
72°C	3 mins	
95°C	30s	
95°C	10s	12 cycles
55°C	30s	
72°C	30s	
72°C	5 mins	
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 minutes prior to use.

First 0.6X bead purification

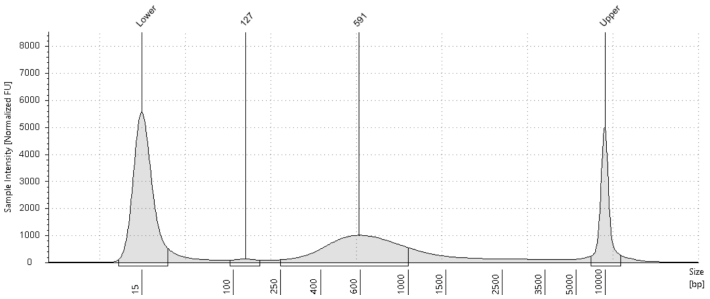
1. Prepare fresh 80% ethyl alcohol.
2. Vortex the SPRIselect reagent at high speed for 30 seconds. The beads should appear homogeneous and uniform in color.
3. Perform 0.6X SPRI cleanup by carefully adding 30 µL of SPRIselect reagent to 50 µL of the total sample. Pipette mix.
4. Vortex to mix for 10-15 seconds.
5. Incubate at room temperature for 5 minutes.
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 seconds and remove the supernatant.
9. Add 200 µL of 80% ethyl alcohol.
10. Wait 30 seconds 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 seconds - 2 minutes).

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 one minute.
15. Place tubes on a magnetic rack and incubate for one minute.
16. Transfer supernatant to a new PCR tube.

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 seconds.
19. Incubate at room temperature for 5 minutes.
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 seconds and remove the supernatant.
24. Add 200 µL of 80% ethanol.
25. Wait 30 seconds 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 seconds - 2 minutes).
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 one minute.
30. Place the tube on a magnetic rack and incubate for one minute.
31. Transfer the supernatant to a new 0.2 mL PCR tube.
32. 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 above **1 ng/µL** are acceptable. See example library trace below:

TapeStation D5000



SAFE STOPPING POINT. Libraries can be stored -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, ~30M reads per tile is recommended.
- For **deep sequencing**, 200M-600M reads per tile is recommended depending on the specific tissue type and tile coverage.
- **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.

- **Loading concentration recommendations:**
  - Nextseq 1000/2000:
    - Start at 750pM final loading concentration and adjust based on sequencing quality
  - Nextseq 500/550:
    - Start at 1.8pM final loading concentration and adjust based on sequencing quality
  - Novaseq 6000:
    - Start at 250–500pM final loading concentration. Adjust based on sequencing quality

- **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-Curio Seeker libraries
    - 10% PhiX spike-in when sequencing only Curio Seeker libraries

- **Index 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
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

- **Library structure**

- **Oligonucleotide sequences**

Primers	Sequence
SS Primer	AAGCAGTGGTATCAACGCAGAGTGANNNGGNNNB
cDNA Amp Primer Mix	CTACACGACGCTCTTCCGATCT AAGCAGTGGTATCAACGCAGAGT
Index primer F	CAAGCAGAAGACGGCATACGAGAT-N8-GTCTCGTGGGCTCGG
Index primer R	AATGATACGGCGACCACCGAGATCTACAC-N8-ACACTCTTTCCC TACACGACGCTCTTCCGATCT

K. Informatics

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

1) To analyze your data on our cloud based analysis platform, please request access at: https://knowledgebase.curiobioscience.com/bioinformatics/bioinfo-portal/

2) To install the pipeline locally at your institution, please request access at: https://curiobioscience.com/bioinformatics-pipeline/.

You can download your barcode file(s) by Tile ID at: https://curiobioscience.com/support/barcode/.

Troubleshooting

Issue	Likely reasons	Solution
Tissue section curling during melting step	Tissue section 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 tissue block was processed following best practices for your specific tissue type. Assess RNA quality of the tissue block by extracting RNA from 5 - 10 µm thick sections using the Qiagen RNeasy mini kit.
	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 supernatant after centrifugation steps. Repeat centrifugation to ensure tight pelleting.

	SPRIselect reagents are not properly mixed before use or after 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 a 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.
	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.
	Tissue section too thick	Ensure 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 labspace 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 uL of bead wash buffer was left behind prior to resuspending beads in the cDNA amp reaction mix.
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

Difficulty dissociating beads	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.

Appendix A: Indexing PCR with Curio Seeker dual indexing primer kit (K003)

IMPORTANT: Please confirm that you are using the Curio Seeker Dual Indexing Primer Kit (K003). The Curio Seeker Dual Indexing Primer Kit v1 (K003) has been discontinued and replaced with the The Curio Seeker Dual Indexing Primer Kit v2 (K006).

1. Prepare the **Nextera PCR mix** following the table below:

Component	1 reaction (µL)
Nextera PCR mix (NPM, Illumina)	15
Nuclease-free water	8
Total	23

2. Add 23 µL of the Nextera PCR mix to the tube.
3. Add 1 µL of index primer F and 1 µL of Index primer R from the **Curio Seeker dual indexing primer kit (K003)** to the tube.
4. Pipette to mix and briefly centrifuge.
5. Run the indexing PCR program according to the table below:

Temperature	Time	Cycle
72°C	3 mins	
95°C	30s	
95°C	10s	12 cycles
55°C	30s	
72°C	30s	
72°C	5 mins	
4°C	Hold	

6. Proceed to section I. Library cleanup and quantification.

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