SEEKGENE

SeekSpace™ Single Cell Spatial Transcriptome-seq Kit User Manual

V1.0

REF: K02501-02 (2 tests), K02501-08 (8 tests)

K02501 - 0201 & K02501 - 0202 & K02501 - 0203 & K00202 - 0201 & K00801 - 0202 & K00801 - 0203 & K00801 - 0803 & K02101 - 0204 & K00202 - 0205 & K02101 - 0206 & K02101 - 0207 & K02401 - 0808 & K02401 - 0209

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Beijing SeekGene BioSciences Co., Ltd

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Disclaimer

1. The results obtained from this kit are influenced by factors such as the source of the sample, the collection process, sample quality, transportation conditions, and pre-treatment procedures. Additionally, the quality of cell nuclei and the operational environment may impose limitations. Users should be aware of potential errors and the limitations of accuracy inherent in the detection process.

2. After use, dispose of the waste materials as per the relevant medical waste disposal regulations.

1. Product Introduction

1.1 Product Overview

The SeekSpace™ Single-Cell Spatial Transcriptome-seq technology, developed independently by Beijing SeekGene BioSciences Co., Ltd, is an efficient platform for single-cell spatial transcriptomics with tissue location information. Its principle involves labeling each cell nucleus in tissue sections with breakable positional nucleic acid sequences on spatial chips, preparing them into single-nucleus suspensions. This is followed by high-throughput co-detection of single-nucleus transcriptomics and positional sequence using the SeekOne™ Digital Droplet platform. By integrating the shared cell barcodes, this technology precisely matches single-cell expression information with spatial location information, overcoming the technical bottleneck of conventional spatial transcriptomics that cannot achieve real single-cell resolution. The technology is complemented by the SeekSpace™ Tools, an analysis software, providing you a comprehensive single-cell spatial transcriptomics solution.

The SeekSpace™ Single-Cell Spatial Transcriptome-Seq Kit includes: SeekSpace™ Single-Cell Spatial Chip (SeekSpace™ Chip, referred to as the spatial chip), Space Chamber, Tissue Scraper, spatial labeling reagents, water-in-oil chip (SeekOne™ DD Chip S3, referred to as Chip S3), Gasket, Carrier Oil, SeekOne™ DD Barcoded Beads (referred to as Barcoded Beads), reverse transcription reagents, multiplexing reagents, TSO reagents, barcode ligation reagents, decrosslinking reagents, library amplification reagents, and purification reagents.

1.2 Intended Usage

This kit uses spatial chips with positional nucleic acid sequences to spatially label the cell nuclei in tissue sections derived from OCT-embedded samples. After preparing single-nucleus suspensions, Oligo-dT and random primers with group tags are used to label the mRNA and spatial oligos in single nuclei. Subsequently, nucleic acid-modified gel beads are employed to label the mRNA and spatial oligos with different group tags from cell nuclei. The final result will include a high-throughput single-nucleus transcriptome library and spatial barcode library that are compatible with Illumina and GeneMind sequencing platforms. This kit is suitable for research into tumor microenvironment cell interactions, cell development mapping, and the mechanisms of disease occurrence and progression.

1.3 User Requirement

The operators of this kit should primarily be laboratory technicians.

Operators should possess a foundamental knowledge of molecular biology theory and practical skills. Only individuals who have undergone proper training and have been certified as competent should operate this kit.

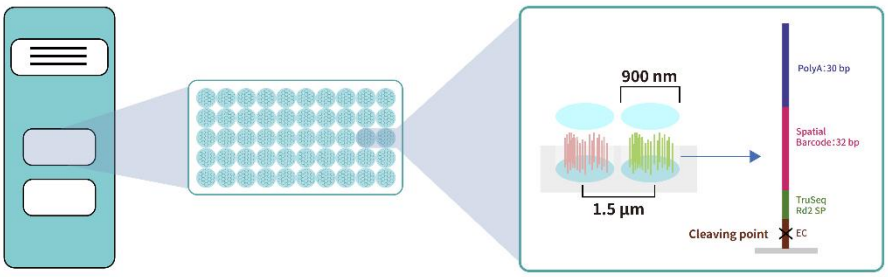
1.4 Limitations of the Test Method

This reagent kit is intended solely for research purposes and the results should NOT be used directly as the results of in vitro diagnostic tests.

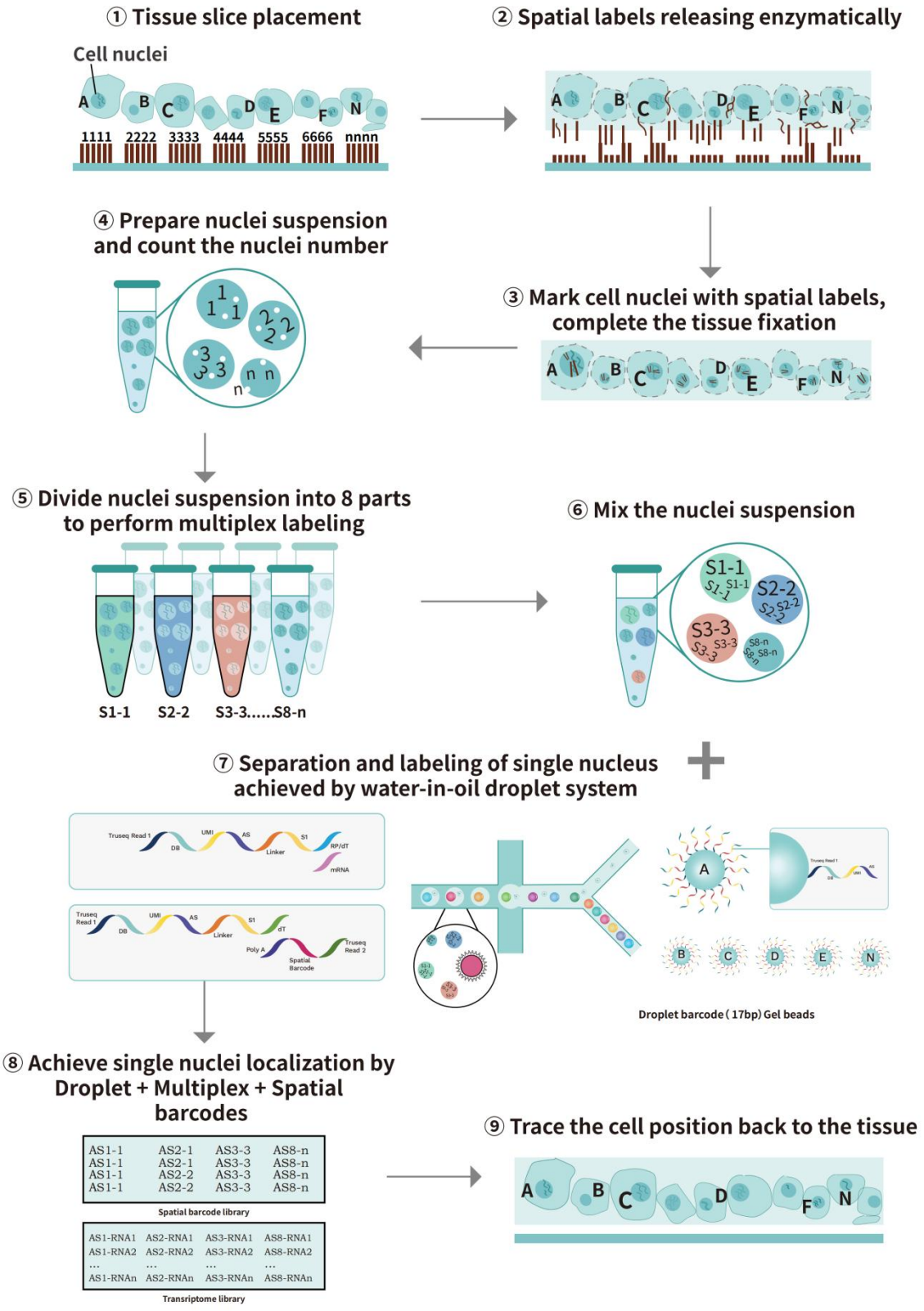
1.5 Spatial Chip and Labels

The SeekSpace™ Spatial Chip has 2 labeling regions, each measuring 5.5 × 15 mm and comprising approximately 30 million types of clusters of spatial labels. Each cluster having a diameter of about 900 nm with the center points of two adjacent clusters spaced approximately 1.5 μm apart.

The spatial label sequence consist of EC, TruSeq Read2 Sequence Primer (TruSeq Rd2 SP), Spatial Barcode, and Poly A components. The EC is a nucleic acid sequence with conditionally breakable sites for spatial label to be released, TruSeq Rd2 SP represents the TruSeq Read2 sequencing primer sequence, Spatial Barcode (32 bp) denotes the spatial barcode sequence, and Poly A (30 bp) serves as the binding site for group multiplex primers.



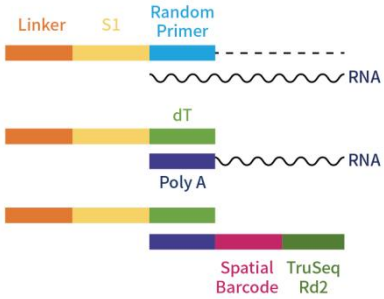
1.6 Experimental Principle and Workflow Diagram



1.7 Library Construction Process

1) Preliminary Reverse Transcription for Group Tagging:

After spatially labeling the cell nuclei, the tissue slices are fixed, and nuclei suspensions are prepared. Group tags are then used to perform preliminary reverse transcription (pre-RT) on the nuclei suspensions, achieving grouped tagging of mRNA and spatial lables.



Group tags consist of three nucleic acid sequences: linker, tag sequence, and primer sequence.

- **Linker**: This connecting sequence is used to facilitate bridge connection with gel bead droplet labeling.
- **Tag Sequence**: There are eight types of tag sequences in total. After spatial labeling, the nuclei suspensions are divided into eight parts for group tagging, with S1 as one of these tag sequences.
- **Primer Sequence**: This includes two types of primers: Random Primer and dT Primer.
    - **dT Primer**: Captures spatial lable sequences and mRNA with Poly A tails.
    - **Random Primer**: Ensures more comprehensive capture of mRNA information.

2) Water-in-oil Droplet-Based Cell Tagging:

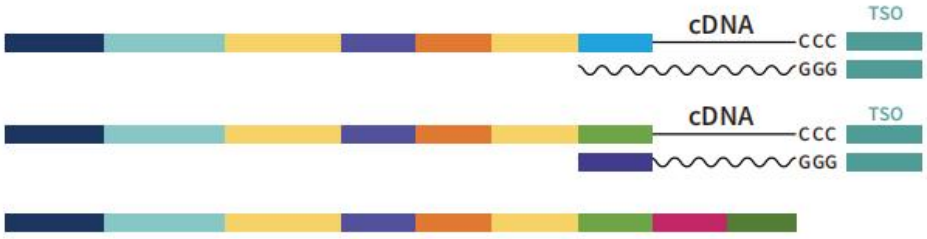
The water-in-oil step bridges droplet bead tags with group tags, ultimately achieving high-throughput single-cell labeling that shares spatial and transcriptome through the combination of droplet tags and group tags.

Bridge joint:

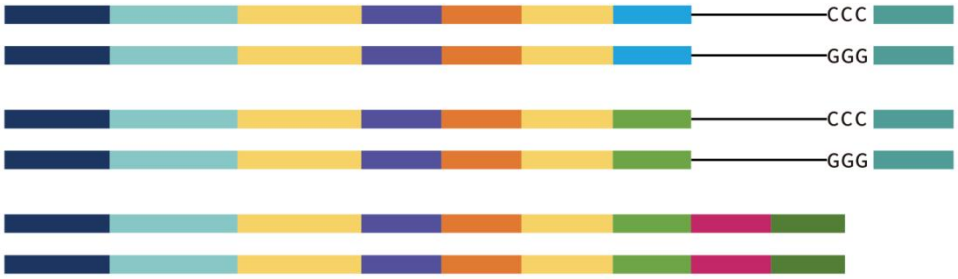
Resulting product:

3) Decrosslinking and Secondary Reverse Transcription:

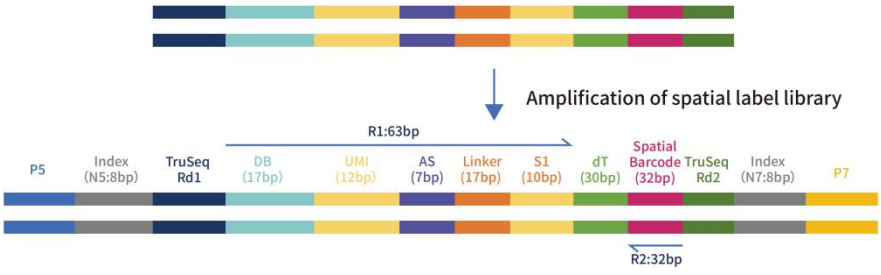
Digest the crosslinked proteins to fully expose previously hidden mRNA, allowing for thorough extension and template switching of cDNA during the secondary reverse transcription.



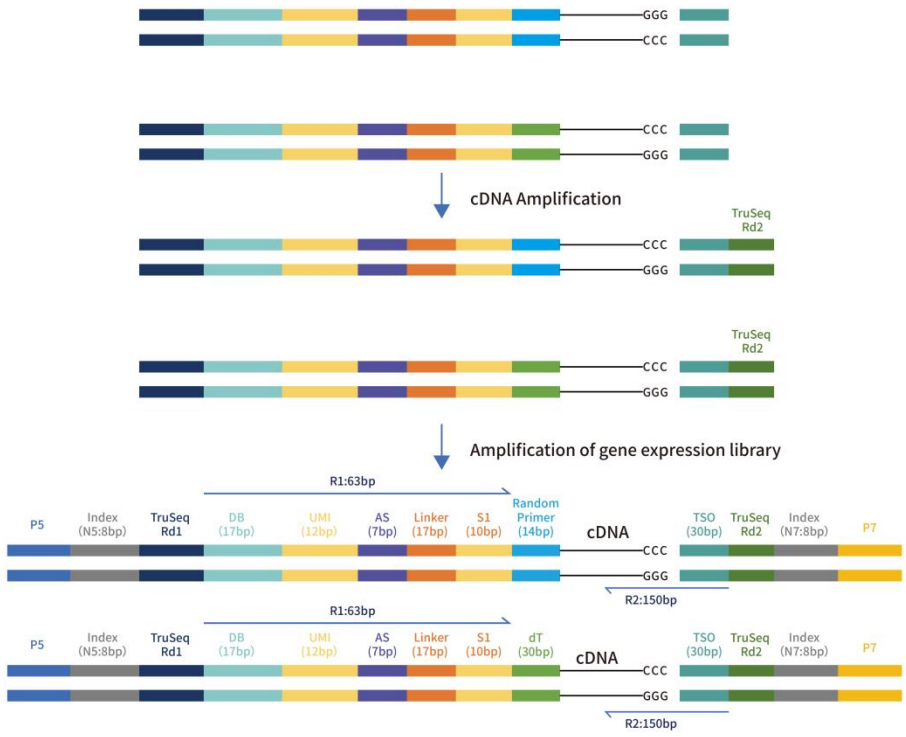
4) Pre-amplification to Enrich Template Strands



5) Construction of Single Cell Spatial labeling Library



6) Construction of Single Cell Transcriptome Library



1.8 Components of the Product Reagents

The SeekSpace™ Single Cell Spatial Transcriptome-seq Kit includes the following components, categorized by their functions and storage conditions (Table 1 and Table2):

1. SeekSpace™ sc-Spatial Chip V1.0, K02501-0201
2. SeekSpace™ sc-Spatial Chip Accessories V1.0, K02501-0202/K02501-0802
3. SeekSpace™ sc-Spatial Labeling Kit V1.0, K02501-0203/K02501-0803
4. SeekOne™ DD Chip S3 Kit V1.0, K00202-0201/K00202-0801
5. SeekOne™ DD Single Cell Cleanup Kit V1.0, K00202-0205/K00202-0805
6. SeekOne™ DD sc-FAST seq Barcoded Beads Kit V1.2, K00801-0202/K00801-0802
7. SeekOne™ DD Single Cell Barcode ligation Kit V1.0, K02101-0204/K02101-0804
8. SeekOne™ DD Single Cell Decrosslinking Kit V1.0, K02101-0206/K02101-0806
9. SeekOne™ DD sc-FAST seq Reverse Transcription Kit V1.2, K00801-0203/K00801-0803
10. SeekOne™ DD Single Cell Sample Multiplexing Kit V1.0, K02401-0808
11. SeekOne™ DD Single Cell TSO Kit V1.0, K02401-0209
12. SeekOne™ DD Single Cell Library Amplification Kit V1.0, K02101-0207

Table 1 SeekSpace™ Single Cell Spatial Transcriptome-seq Kit, 2 tests, REF: K02501-02

Name & PN & Storage	Package	Cap Color	Component	CN	Quantity/ Volume
SeekSpace™ sc-Spatial Chip V1.0 K02501-0201 2-8℃	1	-	SeekSpace™ Chip	-	1
SeekSpace™ sc-Spatial Chip Accessories V1.0 K02501-0202 Room temperature	1	-	Space Chamber	SP00055	2
		-	Tissue Scraper	SP00056	1 bag
SeekSpace™ sc-Spatial Labeling Kit V1.0 K02501-0203 -20±5℃	1		10× Labeling Buffer	R0015201	50 μL
			Labeling Enzyme	R0015301	25 μL
			Labeling Enhancer	R0015401	50 μL
			RNase Inhibitor-B	R0015501	12 μL
			NLB	R0012902	1.5 mL×2
			Fix Buffer B	R0015601	1.6 mL
			Buffer T	R0012601	0.5 mL
SeekOne™ DD Chip S3 Kit V1.0 K00202-0201 Room temperature	1	-	SeekOne™ DD Chip S3	R0003001	2
		-	Gasket	R0003101	2
			Carrier Oil	R0003201	0.6 mL
			Demulsion Agent	R0003301	0.5 mL
SeekOne™ DD scFAST-seq Barcoded Beads V1.2 K00801-0202 -80℃	1		Single Cell Whole Transcriptome Barcoded Beads	R0008601	45 μL ×2
			TSO	R0003601	10 μL
SeekOne™ DD scFAST-seq Reverse Transcription kit V1.2 K00801-0203 -20±5℃	1		3× RT Buffer	R0008401	80 μL
			RT Enzyme	R0003801	15 μL
			Reducing Buffer	R0003901	100 μL
SeekOne™ DD scFAST-seq Reverse Transcription kit V1.2 K00801-0803 -20±5℃	1		3× RT Buffer	R0008402	280 μL
			RT Enzyme	R0003802	50 μL
			Reducing Buffer	R0003901	100 μL
SeekOne™ DD Single Cell Barcode Ligation V1.0 K02101-0204 -20±5℃	1		DNA Ligase Mix	R0011801	15 μL
			RNase Inhibitor	R0011901	25 μL
			Primer R	R0012001	10 μL
			FFPE RT primers	R0012101	10 μL
			10× Ligation Buffer	R0012201	100 μL

Name & PN & Storage	Package	Cap Color	Component	CN	Quantity/ Volume
SeekOne™ DD Single Cell Decrosslinking Kit V1.0 K02101-0206 Room temperature	1	Purple	DCL Buffer	R0012301	0.5 mL
		Red	Enzyme K1	R0012401	90 μL
		Green	Buffer S	R0012501	0.5 mL
		Orange	Buffer T	R0012601	0.5 mL
SeekOne™ DD Single Cell Library Amplification Kit V1.0 K02101-0207 -20±5℃	1	Red	2× PCR Master Mix	R0002102	240 μL
		Purple	Pre-Primers	R0012701	10 μL
		Green	Post-Primers	R0012801	10 μL
		Orange	N501	R0004601	25 μL
		Orange	N502	R0004701	25 μL
		Orange	N701	R0005001	25 μL
		Orange	N702	R0005101	25 μL
SeekOne™ DD Single Cell Cleanup Kit V1.0 K00202-0205 2-8℃	2	White	Cleanup Beads	R0003401	0.5 mL
SeekOne™ DD Single Cell Sample Multiplexing Kit V1.0 K02401-0808 -20±5℃	1	Red	S1	R0013501	35 μL
		Purple	S2	R0013601	35 μL
		Green	S3	R0013701	35 μL
		Orange	S4	R0013801	35 μL
		White	S5	R0013901	35 μL
		White	S6	R0014001	35 μL
		Blue	S7	R0014101	35 μL
		Black	S8	R0014201	35 μL
		Red	S9	R0014301	35 μL
		Purple	S10	R0014401	35 μL
		Green	S11	R0014501	35 μL
		Orange	S12	R0014601	35 μL
		White	S13	R0014701	35 μL
		White	S14	R0014801	35 μL
		Blue	S15	R0014901	35 μL
		Black	S16	R0015001	35 μL
SeekOne™ DD Single Cell TSO Kit V1.0 K02401-0209 -80℃	1	White	TSO	R0003602	20 μL

Table 2 SeekSpace™ Single Cell Spatial Transcriptome-seq Kit, 8 tests, REF: K02501-08

Name & PN & Storage	Package	Cap Color	component	CN	Quantity /Volume
SeekSpace™ sc-Spatial Chip V1.0 K02501-0201 2-8℃	4	-	SeekSpace™ Chip	-	4
SeekSpace™ sc-Spatial Chip Accessories V1.0 K02501-0802 Room temperature	1	-	Space Chamber	SP00055	8
		-	Tissue Scraper	SP00056	1 bag
SeekSpace™ sc-Spatial Labeling kit V1.0 K02501-0803 -20±5℃	1		10× Labeling Buffer	R0015202	200 μL
			Labeling Enzyme	R0015302	100 μL
			Labeling Enhancer	R0015401	50 μL
			RNase Inhibitor-B	R0015502	50 μL
			NLB	R0012902	1.5 mL×8
			Fix Buffer B	R0015601	1.6 mL
			Buffer T	R0012601	0.5 mL
SeekOne™ DD Chip S3 Kit V1.0 K00202-0801 Room temperature	1	-	SeekOne™ DD Chip S3	R0003001	8
		-	Gasket	R0003101	8
			Carrier Oil	R0003202	1.2 mL×2
			Demulsion Agent	R0003302	1.8 mL
SeekOne™ DD scFAST-seq Barcoded Beads V1.2 K00801-0802 -80℃	1		Single Cell Whole Transcriptome Barcoded Beads	R0008601	45 μL ×8
			TSO	R0003602	20 μL
SeekOne™ DD scFAST-seq Reverse Transcription kit V1.2 K00801-0803 -20±5℃	5		3× RT Buffer	R0008402	280 μL
			RT Enzyme	R0003802	50 μL
			Reducing Buffer	R0003901	100 μL
SeekOne™ DD Single Cell Barcode Ligation kit V1.0 K02101-0804 -20±5℃	1		DNA Ligase Mix	R0011802	50 μL
			RNase Inhibitor	R0011902	100 μL
			Primer R	R0012002	30 μL
			FFPE RT primers	R0012102	30 μL
			10× Ligation Buffer	R0012201	100 μL
SeekOne™ DD Single Cell Decrosslinking Kit V1.0 K02101-0806 Room temperature	1		DCL Buffer	R0012301	0.5 mL×2
			Enzyme K1	R0012401	90 μL×2
			Buffer S	R0012501	0.5 mL
			Buffer T	R0012601	0.5 mL

Name & PN & Storage	Package	Cap Color	component	CN	Quantity/ Volume
SeekOne™ DD Single Cell Library Amplification Kit V1.0 K02101-0807 -20±5℃	1	🔴	2× PCR Master Mix	R0002102	240 μL×4
		🟣	Pre-Primers	R0012702	30 μL
		🟢	Post-Primers	R0012802	30 μL
		🟠	N501	R0004601	25 μL
		🟠	N502	R0004701	25 μL
		🟠	N503	R0004801	25 μL
		🟠	N504	R0004901	25 μL
		🟠	N701	R0005001	25 μL
		🟠	N702	R0005101	25 μL
		🟠	N703	R0005201	25 μL
		🟠	N704	R0005301	25 μL
SeekOne™ DD Single Cell Cleanup Kit V1.0 K00202-0805 2-8℃	2	⚪	Cleanup Beads	R0003402	1.75 mL
SeekOne™ DD Single Cell Sample Multiplexing Kit V1.0 K02401-0808 -20±5℃	1	🔴	S1	R0013501	35 μL
		🟣	S2	R0013601	35 μL
		🟢	S3	R0013701	35 μL
		🟠	S4	R0013801	35 μL
		⚪	S5	R0013901	35 μL
		⚪	S6	R0014001	35 μL
		🔵	S7	R0014101	35 μL
		⚫	S8	R0014201	35 μL
		🔴	S9	R0014301	35 μL
		🟣	S10	R0014401	35 μL
		🟢	S11	R0014501	35 μL
		🟠	S12	R0014601	35 μL
		⚪	S13	R0014701	35 μL
		⚪	S14	R0014801	35 μL
		🔵	S15	R0014901	35 μL
		⚫	S16	R0015001	35 μL
SeekOne™ DD Single Cell TSO Kit K02401-0209 -80℃	4	⚪	TSO	R0003602	20 μL

1.9 Storage and Transportation Conditions

Name	Storage temperature	Transportation temperature
SeekSpace™ sc-Spatial Chip	2-8℃	Ambient
SeekOne™ DD Single Cell Cleanup Kit	2-8℃	Ambient
SeekSpace™ sc-Spatial Chip Accessories	Room temperature	Ambient
SeekOne™ DD Chip S3 Kit	Room temperature	Ambient
SeekOne™ DD Single Cell Decrosslinking Kit	Room temperature	Ambient
SeekSpace™ sc-Spatial Labeling Kit	-20±5℃	Dry ice
SeekOne™ DD Single Cell Sample Multiplexing Kit	-20±5℃	Dry ice
SeekOne™ DD ScFAST-seq Reverse Transcription Kit	-20±5℃	Dry ice
SeekOne™ DD Single Cell Barcode Ligation Kit	-20±5℃	Dry ice
SeekOne™ DD Single Cell Library Amplification Kit	-20±5℃	Dry ice
SeekOne™ DD scFAST-seq Barcoded Beads Kit	-80℃	Dry ice
SeekOne™ DD Single Cell TSO Kit	-80℃	Dry ice

NOTE: Upon receiving the product shipped with dry ice, please check if there is any remaining dry ice.

1.10 Parameter Specifications

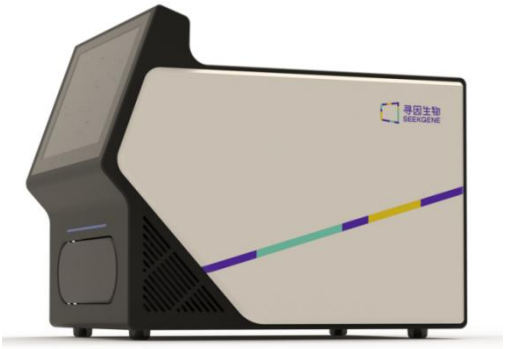
1. Sample Throughput: There are 2 labeling regions per spatial chip.
2. Individual labeling region size: 5.5 × 15 mm.
3. Chip S3 is a single-channel chip that can flexibly run 1-8 samples in parallel as needed.
4. Water-in-oil generation rate: 150,000 water-in-oil droplets generated within 3 minutes.

Note: Spatial chips are packaged in vacuum-sealed aluminum bags and transported at room temperature. Upon receiving the product, unopened spatial chips should be immediately stored at 2-8°C. If the bag is opened and chips are not used, place the chips back in the chip box, seal them in a dry condition, and store them at 2-8°C. The resealed spatial chips should not be stored for more than one month.

1.11 Compatible Instruments and Consumables

1. **SeekSpace™ sc-Spatial Chip Holder,** also called **Space Holder**: Used in conjunction with spatial chips.

2. **SeekOne™ Digital Droplet System** (SeekOne™ DD, REF: M001A)



3. **SeekOne™ DD Accessories**: Each SeekOne™ DD instrument is equipped with one set of this accessory, which includes the following two parts:

1) **SeekOne™ DD Chip Holder,** abbreviated as **Chip Holder**: Used in conjunction with SeekOne™ Digital Droplet system and SeekOne™ DD Chip S3 Kit.

2) **Placed Chip, abbreviated as Chip P:** Placed in the chip holder (each SeekOne™ DD instrument comes with 8 Chip Ps); when the sample quantity is less than 8, Chip P is placed in the sample-free slots. It serves as a replacement for Chip S3.

4. **SeekSpace™ High-Frequency Ultrasonic Device** (REF: M005A)

By adjusting the operating frequency, power, and duration, ultrasound oscillation effectively reduces aggregation in cell suspensions, thereby increasing cell nucleus yield.

Note: Specific operational requirements for the SeekSpace™ High-Frequency Ultrasound Device are detailed in the "SeekSpace™ High-Frequency Ultrasound Device User Manual."

1.12 Self-provided Instruments and Reagents

1.12.1 Self-provided Instruments and Consumables

Name of self-provided instruments and consumables	Recommended brands and product numbers
Cell counting device	SeekMate Tinitan, M002C
	Countstar, IN030101
Cryostat	Leica, CM1950
	Dakewe Biotech, CT520
Fluorescence microscope	Leica, DMi8
High-speed cryogenic centrifuge	Thermo Fisher Scientific, 75009880
	Xiangyi Instrument, H1850R
Nucleic acid fragment analyzer	Agilent, G2991AA (Agilent 4200 TapeStation system)
	Agilent, G2939BA (Agilent 2100 Bioanalyser)
	Bioptic, Qsep400
Thermal cycler capable of heating 100 µl volumes	Thermo Fisher Scientific, 4375786
	LongGene, A300
	BioRad, 1851196 (C1000)
Qubit 4.0 Fluorometer	Thermo Fisher Scientific, Q33238
Microcentrifuge	Tiangen Biochemical, OSE-MP25
Vortex Mixer	IKA, MS3 basic
Thermo Shaker	Ruiwell, TCS10
Pipette	Eppendorf, 2.5 µL/10 µL/20 µL/200 µL/1,000 µL
	RAININ, 2.5 µL/10 µL/20 µL/200 µL/1,000 µL
0.2 mL Magnetic rack	Mich Scientific, Magpow-24
1.5 mL Magnetic rack	Thermo Scientific DynaMag, 12321D
Grinding pestle	KIMBLE, D8938

Name of self-provided instruments and consumables	Recommended brands and product numbers
40 μm Cell strainer	NEST, 258369
	Corning, 352340
30 μm Cell strainer	MACS® Smart Strainers 30 μm, 130-110-915
20 μm Cell strainer	PluriStrainer 20μm, 43-50020-03
0.2 mL PCR tubes	Axygen, PCR-02-L-C
0.2 mL 8-channel PCR tubes	Yeasen Biotechnology, 83602ES03
50 mL Centrifuge tube	Corning, 430829
5 mL Centrifuge tube	Axygen, MCT-500-C
1.5 mL Centrifuge tube	Axygen, MCT-150-L-C
0.5 mL PCR tubes with flat cap	Axygen, PCR-05-C
DNase/RNase-free Low-bind Eppendorfs	Axygen, MCT-150-L-C
Low retention tip	Axygen, T-300-L-R-S/T-200-C-L-R-S/ T-1000-C-L-R-S
Compressed air duster	MATIN, M-6318

1.12.2 Self-provided Reagents/Kits

Name of self-provided reagents/kits	Recommended brands and product numbers
1× PBS buffer	Sangon, B540626
RNAse Inhibitor-A (40 U/μL)	SeekGene, R0015801
	Enzymatics, Y9240-40000
	Vazyme Biotech, R301-03
OCT Compound	Sakura, 4583
	Scigen, 4586
AO/PI staining kit	SeekGene, K01701 (for use with SeekMate Tinitan, M002C)
Qubit ssDNA Assay Kit	Invitrogen, Q10212
	ABP, FP006
Anhydrous ethanol (analytical grade)	Millipore Sigma, E7023-500ML
Nuclease-Free Water	Thermo Fisher Scientific, AM9937
Lint-free paper	KIMTECH, 34155
DNA selection beads	Beckman, B23318/A63882
	Vazyme Biotech, N411-03
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, Q32854
Fragment analyzer kit High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents S1-High Resolution Quantitative Cartridge Kit	Agilent, 5067-5592/5067-5593 Agilent, 5067-5584/5067-5585 Bioptic, C105202/C105802/C405102

1.13 Sample Requirement

1.13.1 Applicable Sample Types

Suitable for OCT-embedded fresh frozen tissues (solid tissues); not suitable for samples that are difficult to section such as bone tissues, samples with high impurities after nuclei extraction like heart tissues, or highly degradable RNA samples like pancreatic tissues.

1.13.2 Considerations for Sample Collection

- Avoid sampling from areas with necrosis, calcification, hardening, or fibrosis, especially in diseased tissues.
- If the tissue is cut using an electrosurgical knife during surgery, remove the tissue that is necrotic due to contact with the knife.
- Ensure that fresh samples are processed for embedding within 30 minutes post-surgery in laboratory conditions to minimize RNA degradation within tissues.
- Ensure that tissues are fully embedded without exposure to prevent sample degradation.
- Support experiments on cryopreserved tissues after embedding.
- Tissue slice area should be no less than 3 × 3 mm, and tissue thickness should be no less than 3 mm.
- Consider experimental models on cells beforehand; for instance, silica particles injected into mouse lungs have similar density to cells and are challenging to be removed completely later.
- Consider potential effects of drug treatments on cells; for example, fragments formed after apoptosis of most tumor cells post-treatment are difficult to distinguish from cells, affecting the quality control of the nucleated cell rate.

1.13.3 Sample Storage

Place OCT-embedded tissues in a sealed bag and store them in a -80°C freezer. It is recommended not to store for more than 3 months.

1.13.4 Sample Transportation

Transport the sealed OCT-embedded tissue in an insulated container with dry ice, ensuring it is placed stably.

1.13.5 Sample Quality

To ensure sample quality and reduce experimental risk, it is recommended to cut 10-20 slices of tissue at 10 μm thickness, for Total RNA extraction and quality testing. It is strongly advised to proceed with subsequent experimental operations only on tissue samples with an RQN/RIN ≥ 7.

1.13.6 Recommended Sample Loading Amount

- For each sample labeling region, the total number of cell nuclei undergoing pre-RT should not exceed 240,000. The cell nuclei loading amount for each individual tube of pre-RT reaction should be no less than 600 and no more than 30,000.
- For each water-in-oil ligation reaction, the cell nuclei loading amount should not exceed 120,000.

2. Operation Steps and Recommendations

Total Duration	Operation Step	Step Duration
Step 1 Preparation of Nuclei Suspensions with Spatial Labels		
~ 3 h	Step 1-1 Pre-experiment Preparation	~30 min
	Step 1-2 Spatial Chip Preparation	~20 min
	Step 1-3 Tissure Sections Preparation	~5 min
	Step 1-4 Tissue Section Placement	~30 min
	Step 1-5 Tissue Labeling	15 min
	Step 1-6 Fluorescence Imaging of Tissues	~12 min
	Step 1-7 Nuclei Extraction From Tissue Sections	~60 min
	Step 1-8 Nuclei Suspension Quality Control	~10 min
Step 2 Preliminary Reverse Transcription Reaction		
~ 2 h	Step 2-1 Pre-experiment Preparation	~15 min
	Step 2-2 Pre-RT Reaction	100 min
Step 3 Water-in-oil Droplet Formation and Barcode Labeling		
~ 2.5 h	Step 3-1 Pre-experiment Preparation	~50 min
	Step 3-2 Single-nucleus Mixture Preparation	~10 min
	Step 3-3 Add Reagents to Chip S3	~5 min
	Step 3-4 Run SeekOne™ DD System	4.5 min
	Step 3-5 Transfer the Formed Water-in-oil Droplet	~5 min
	Step 3-6 Water-in-oil Droplet Ligation Reaction	70 min
	STOP ***Stopping point:4℃ overnight, or store at -80℃ ≤4 days***	
Step 4 Decrosslinking and Purification		
~1.5 h	Step 4 -1 Pre-experiment Preparation	~15 min
	Step 4-2 Water-in-oil Droplet Demulsification	~2 min
	Step 4-3 Decrosslinking	30 min
	STOP ***Stopping point: After breaking the emulsion, the liquid can be mixed with reagents, shaken thoroughly, and stored at -80°C overnight.*** *After decrosslinking, the sample can be stored overnight at -80°C.*	
	Step 4-4 Purification	~40 min
Step 5 Secondary RT and cDNA Purification		
~2.5 h	Step 5-1 Pre-experiment Preparation	~20 min
	Step 5-2 Secondary RT Reaction	90 min
	STOP ***Stopping point:Store at 4℃ ≤ 72 h, or store at -20℃ ≤ 1week.***	
	Step 5-3 Purification of the Secondary RT Product	~30 min

Total Duration	Operation Step	Step Duration
	STOP ***Stopping point:Store at 4℃ ≤ 72 h, or store at -20℃ ≤ 1week.***	
Step 6 Pre-amplification and Purification of cDNA and Spatial Label		
~1 h	Step 6-1 Pre-experiment Preparation	~10 min
	Step 6-2 Pre-amplification	25 min
	Step 6-3 Purification of Pre-amplification Products	~30 min
	STOP ***Stopping point:Store at 4℃ ≤ 72 h, or store at -20℃ ≤ 1 month***	
	Step 6-4 Quantification of Pre-amplification Products	-
Step 7 Transcriptome cDNA Amplification and Expression Library Construction		
~2.5 h	Step 7-1 Pre-experiment Preparation	~10 min
	Step 7-2 cDNA Amplification	30 min
	Step 7-3 cDNA Purification	~30 min
	STOP ***Stopping point: store at -20℃ ≤ 1 month***	
	Step 7-4 cDNA Product Quality Control	~15 min
	Step 7-5 Expression Library Amplification	30 min
	Step 7-6 Expression Library Purification	~30 min
	STOP ***Stopping point: store at -20℃ ≤ 6 months***	
	Step 7-7 Expression Library Quality Control	~15 min
Step 8 Spatial Label Library Amplification and Purification		
~2 h	Step 8-1 Pre-experiment Preparation	~10 min
	Step 8-2 Library Amplification	50 min
	Step 8-3 Library Purification	~30 min
	STOP ***Stopping point: store at -20℃ ≤ 1 month***	
	Step 8-4 Spatial Label Library Quality Control	~15 min

Step 1 Preparation of Nuclei Suspensions with Spatial Labels

Step 1-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekSpace™ sc-Spatial Labeling Kit	10× Labeling Buffer	●	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	NLB	●			
	Labeling Enzyme	●			Centrifuge briefly and store on ice for later use.
	RNase Inhibitor-B	●			

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
	Buffer T			Ambient	Vortex thoroughly and centrifuge briefly, then store at room temperature for later use.
	Fix Buffer B			Ice	Thaw on ice, vortex thoroughly, centrifuge briefly, and store on ice for later use.
	Labeling Enhancer				
SeekSpace™ sc-Spatial Chip	SeekSpace™ Chip	-	2-8℃	Ambient	After removing the record number, rinse with Nuclease-Free Water, air dry, avoid touching the labeled area, and place in the chip box at room temperature for later use.
SeekSpace™ sc-Spatial Chip Accessories	Tissue Scraper	-	Room temp	Ambient	Tissure scraper
	Space Chamber	-	Room temp	Ambient	Space Chamber
Self-prepared reagents	1× PBS	-	Room temp	Ambient	-
	ssDNA Reagent	-	2-8℃	Ice	-
	Nuclease-Free Water	-	Room temp	Ambient	-
	RNase Inhibitor-A	-	-20℃	Ice	Brief centrifugation, then store on ice for later use.
	OCT Compound	-	Room temp	Ice	Pre-chill on ice
	Dry ice	-	-	-	-
Self-prepared consumables	Brush	-	Ambient	-20℃	Pre-cool in the cryostat beforehand.
	Blade	-	Ambient	-20℃	Pre-cool in the cryostat beforehand.
	Dust-free paper	-	Ambient	Ambient	-
	Air canister	-	Ambient	Ambient	-
	Grinding rod	-	Ambient	Ambient	Clean with 1× PBS-R1 before use.
	40 μm Cell strainer	-	Ambient	Ambient	Rinse with Wash Buffer before use.
	30 μm Cell strainer	-	Ambient	Ambient	Rinse with Wash Buffer before use.
	50 mL Centrifuge tube	-	Ambient	Ambient	-
	5 mL Centrifuge tube	-	Ambient	Ambient	-
	1.5 mL Centrifuge tube	-	Ambient	Ambient	-
	Pipettes	-	Ambient	Ambient	-

2. Equipment Preparation

a. **Thermal cycler 1**: Set temperature to 4°C Hold, 4°C for 5 min, 37°C for 10 min, with a lid temperature of 42°C. **Thermal Cycler 2**: Set temperature to 37°C Hold, without lid heating.

b. **Cryostat**: Pre-cool the chamber to -20°C, and pre-cool the sample head to -15°C to -10°C.

c. **Ultrasonic device**: Set frequency to 20 kHz, power to 50 W, with water filled to 1/4 of the ultrasonic bath capacity; keep the heating off.

d. **Fluorescence microscope**: Turn on and prepare for use before the experiment.

e. **High-speed refrigerated centrifuge**: Cool to 4°C and prepare for use.

f. **Cell counter**: Turn on and prepare for use.

g. **Microcentrifuge**: Turn on and prepare for use.

h. **Vortex mixer**: Turn on and prepare for use.

i. **Thermo Shaker**: Turn on and prepare for use.

3. Reagent Preparation

a.Wash Buffer and 1× PBS-RI:

Component	Wash Buffer	1× PBS-RI
1× PBS (Self-prepared)	18 mL	11 mL
RNAse Inhibitor-A (Self-prepared)	450 µL	275 µL
Buffer T	180 µL	/
Total	**18.63 mL**	**11.275 mL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 2 labeling regions.

b.Fixation solution preparation:

Component	Fixation solution
1× PBS (Self-prepared)	120 µL
Fix Buffer B	360 µL
Total	**480 µL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 2 labeling regions.

c.N-1 reaction mixture preparation:

Component	N-1
10× Labeling Buffer	33 μL
Labeling Enzyme	22 μL
Labeling Enhancer	3.3 μL
RNase Inhibitor-B	8.25 μL
ssDNA Reagent (Self-prepared)	1 μL
Nuclease-Free Water (Self-prepared)	262.45 μL
Total	**330 μL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 2 labeling regions.

d.Nuclei extraction buffer preparation:

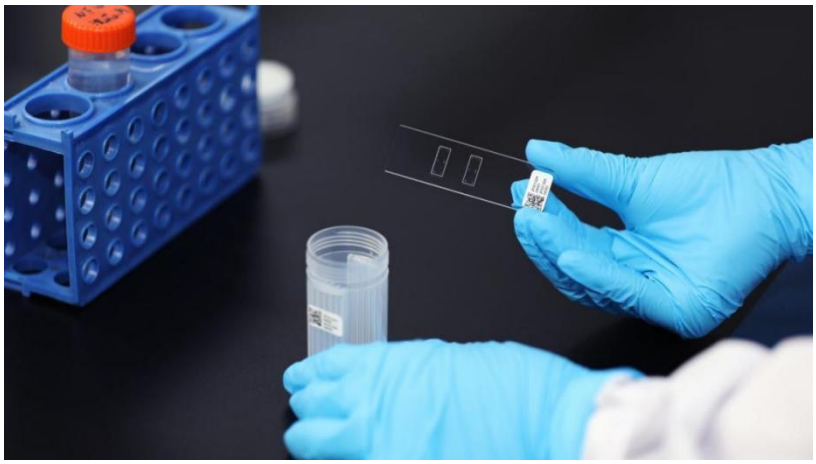
Component	Nuclei Extraction Buffer
NLB	3 mL
RNAse Inhibitor-A (Self-prepared)	75 μL
Total	**3.075 mL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 2 labeling regions.

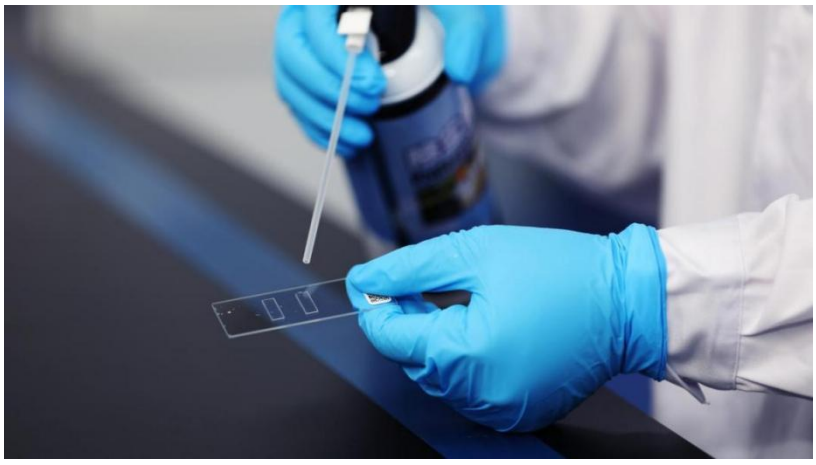
Step 1-2 Spatial Chip Prepration

1. Remove the spatial chip: Take the spatial chip out from the vacuum-dried aluminum foil bag, record the number on the front side of the spatial chip. The front side of the marked area contains spatial barcodes used for marking cell nuclei positions. Do not touch the front side of the marked area.



2. Hold the spatial chip by the numbered area and perform 5-10 cycles of gentle up-and-down rinsing in a centrifuge tube containing 40-50 mL of Nuclease-Free Water.

3. After rinsing, use lint-free paper to absorb excess liquid from the back of the spatial chip and around the marked area. Then, use an air canister to blow dry the front of the marked area. or allow it to air dry directly.



4. Once the front of the marked area is free of impurities, visible marks or any liquid residue, and wavy textures, you can prepare section placement.

Step 1-3 Tissue Sections Preparation

1. Remove the tissue block embedded in optimal cutting temperature compound (OCT) from the -80°C freezer and equilibrate it in the cryostat for 30 minutes.

2. Use OCT to secure the tissue block with the expected cutting surface facing upwards onto the specimen holder that fits the cryostat. Then, secure the specimen holder onto the cryostat.

3. Use the cryostat to trim the OCT from the tissue block, exposing the tissue and determining the cutting position.

4. Use a blade to trim the tissue to the final desired dimensions (section area should be less than 5.5 × 15 mm), ensuring the tissue section fits well with the marked area, then proceed with cryosectioning.

Note 1: Typical section thickness is 10-20 μm.

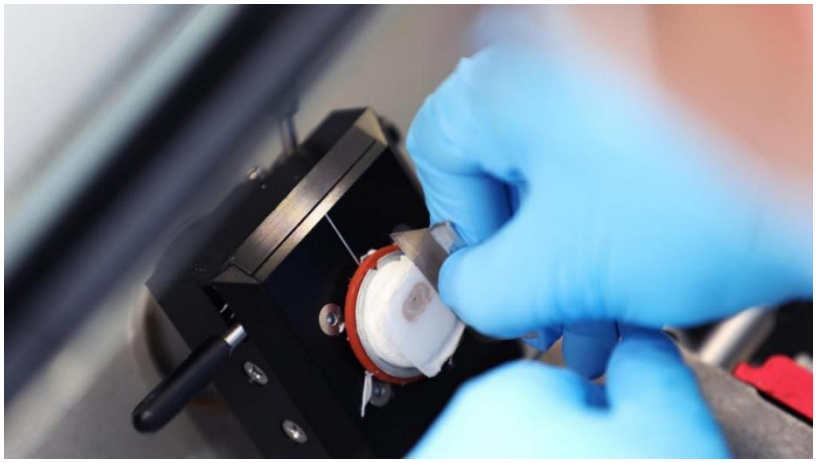
- *For tissues with cell nuclei diameter of 3-5 μm, it is recommended to section at a thickness of 10 μm.*
- *For tissues with cell nuclei diameter of 6-10 μm, it is recommended to section at a thickness of 14 μm.*
- *For tissues with cell nuclei diameter greater than 10 μm, it is recommended to section at a thickness of 20 μm.*
- *For tissues with a small number of cell nuclei, it is recommended to section at a thickness of 14-20 μm.*

Note 2: Before sectioning, ensure all samples are trimmed close to the target area to minimize the trimming time between samples.

Note 3: If a labeling region needs to be shared by multiple sections, no more than 3 sections should be attached to an individual marked area. Arrange the samples to be tiled in sequence. It is recommended to leave at least a 2 mm gap between two tissue sections, and for each additional slice, leave at least a 2 mm gap to prevent overlapping or extending beyond the marked area during placement.

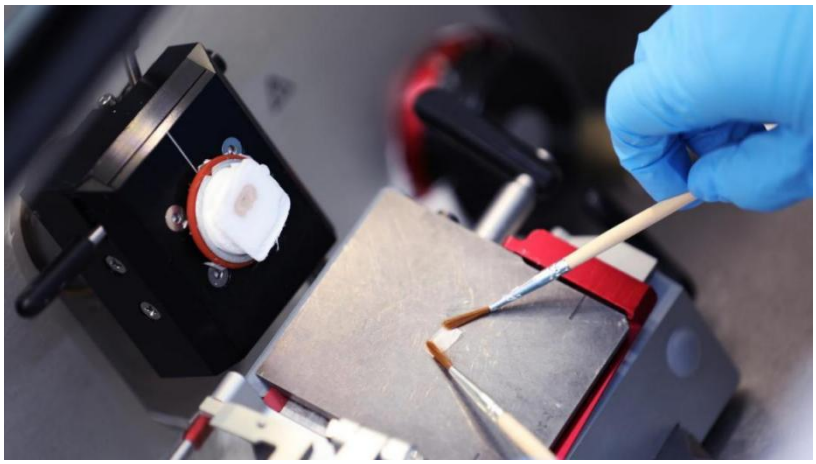
Note 4: It is recommended to select samples with the same tissue type and similar histopathological structures for tiling.

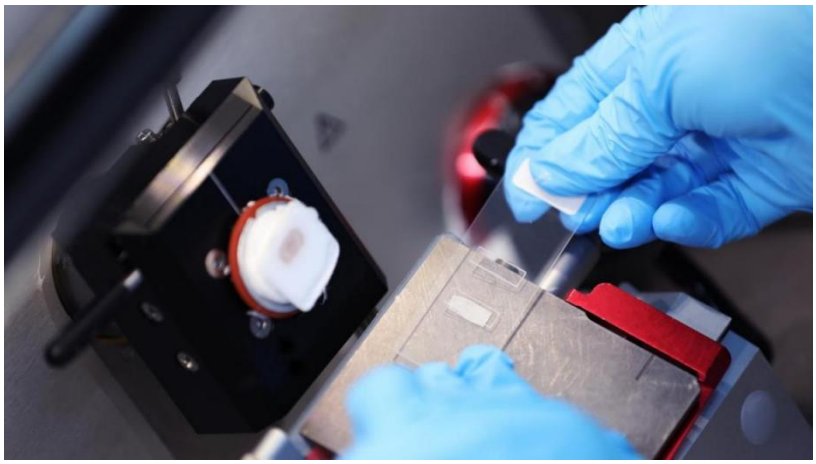
Note 5: Adjacent sections can be selected for HE staining as required.



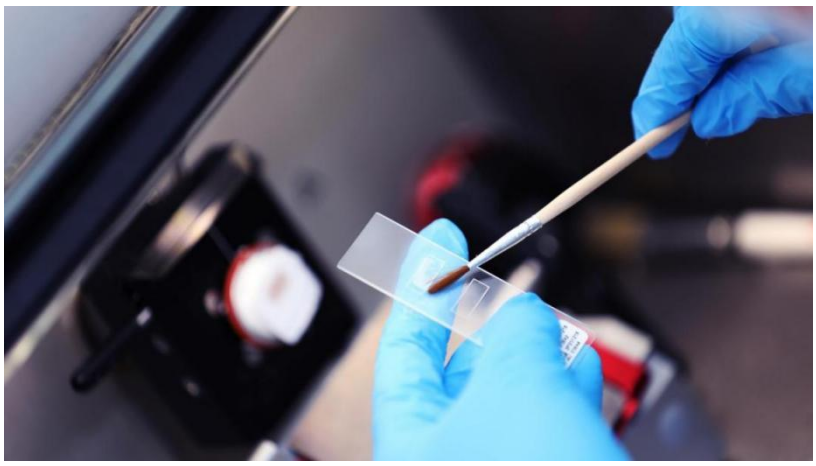
Step 1-4 Tissue Section Placement

1. Place the spatial chip with the front side up into the cryostat, and pre-cool for 2 minutes.
2. Cut the tissue section, then gently flatten the surrounding OCT with a pre-cooled brush. Move the tissue section from the target area to the right edge of the platform. Pick up the spatial chip by the numbered area, align it face down with the slice, and gently press down.

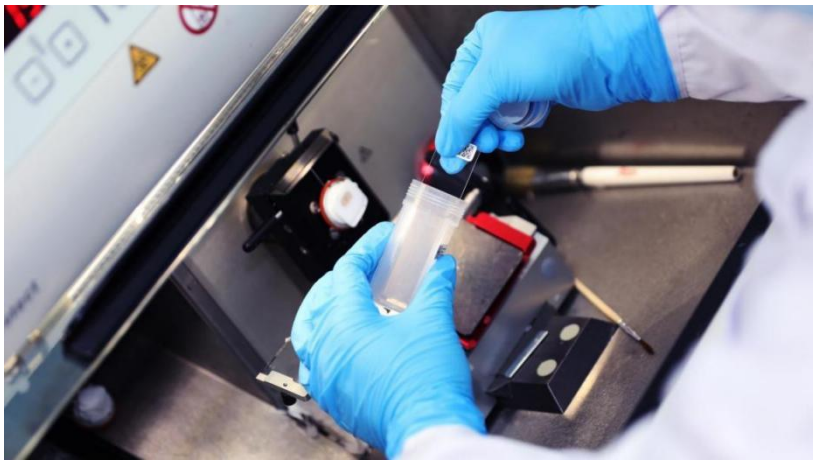




3. Immediately take up the spatial chip and gently warm the back of it with your fingertips for a few seconds until the section adheres.



4. Place the spatial chip with the tissue section facing up back into the chip box and store the box in dry ice.



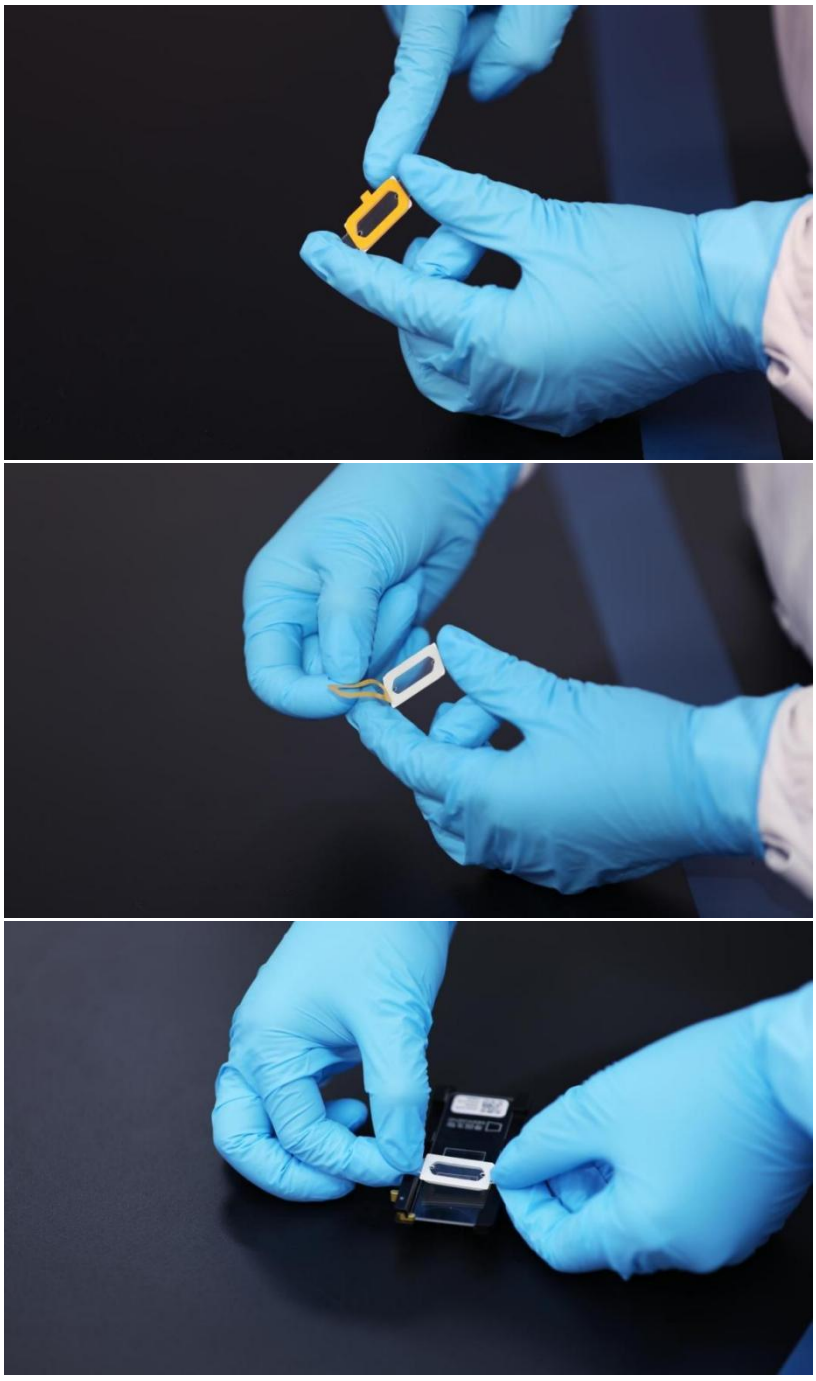
5. Repeat steps 2-4 until all sections are placed on the chip. Aim to complete the placement within 30 minutes from the first section to the last.

Note: It is crucial to manage the placement time for each section. If the time interval is too long, tissue sections may dry and shrink, leading to RNA degradation.

Step 1-5 Tissue Labeling

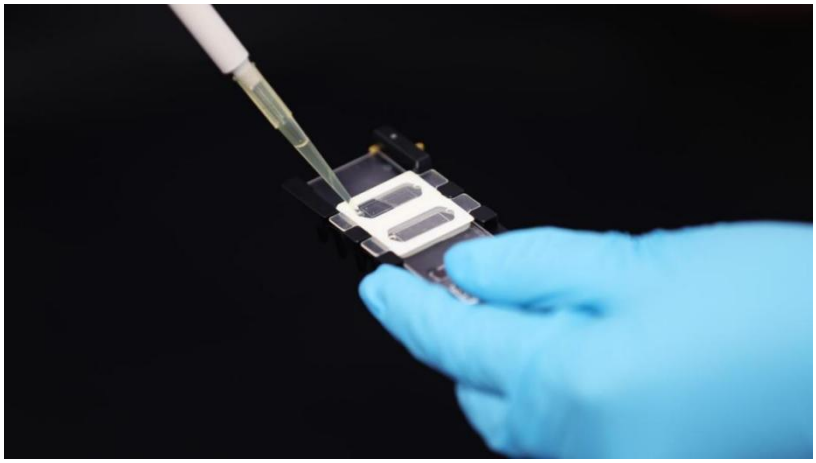
1. Warm the slide: Quickly place the spatial chip onto the SeekSpace™ chip holder, secure it with the safety latch, and incubate it in thermal cycler 2 at 37°C for 90 seconds without covering the lid.

2. During warming, remove the green film and yellow sticker from the Space Chamber. After tissue incubation is done, attach the Space Chamber onto the marked area of the chip, ensuring that the wings of the Space Chamber are positioned between the SeekSpace™ chip holder's alignment pillars. Gently press around the edges of the Space Chamber to ensure it adheres tightly to the chip.



3. Add 150 µL of N-1 reaction mixture into the reaction chamber. Gently pipette once to ensure that the N-1 solution covers all marked areas on the spatial chip. Then, place the SeekSpace™ chip holder with the spatial chip into thermal cycler 1 which should be pre-set to the following program(lid temperature 42°C):

Temperature	Time
4℃	5 min
37℃	10 min



4. After the reaction is complete, remove the N-1 solution from the reaction chamber. Wash the marked areas twice with 150 μL of 1×PBS-RI solution each time.

5. Remove the PBS-RI solution and add 150 μL of fixation solution into the reaction chamber, ensuring it immerses all marked areas on the spatial chip. Incubate at room temperature for 12 minutes.

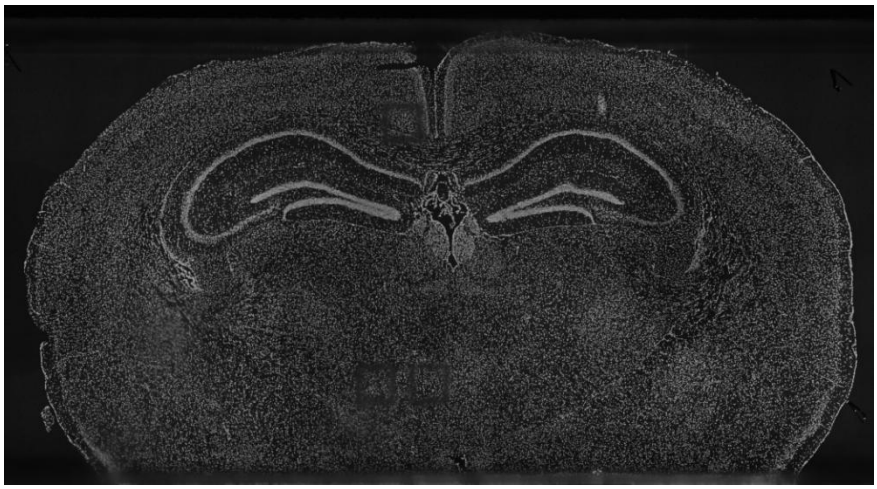
Step 1-6 Fluorescence Imaging of Tissues

1. During the fixation process, perform fluorescence imaging of the tissue. Press and hold the latch on the SeekSpace™ chip holder to remove the spatial chip. Place the chip upright on the stage of the fluorescence microscope (LEICA DMi8).

Note: Avoid prolonged fluorescence exposure of the chip with the attached tissue section. Turn off the laser when not taking photos to prevent extended exposure.

2. Set the microscope to a 10× objective lens. Choose “Black and White Mode” and select “Image Format”, with “Live Format” set to “bin 2 × 2.” It is recommended to use manual focusing. Focus on the four corners of the labeled area of the space chip and within the tissue to obtain clear images of both the chip and the stained tissue. The photographed area should include the entire labeled region of the space chip and the tissue. The ssDNA image should have a clear presentation, with distinct tissue boundaries, as shown in the reference image below.

Note 1: The fluorescence imaging should be completed within 12 minutes during the fixation process. If the imaging is not completed, you can replace the fixation solution with 150 μL of nuclei extraction buffer and continue photographing, but this must be finished within 10 minutes.



3. During manual focusing on the tissue, reduce the gain to 1, set FIM to 100%, and adjust the light intensity to prevent overexposure of the tissue area.

Note 1: The fluorescence microscope should have image stitching functions if using a different model.

Note 2: During the imaging process, ensure that the edges of the chip and the tissue area are simultaneously clear and that the tissue area is not overexposed.

Step 1-7 Nuclei Extraction from Tissue Sections

1. After fixation, remove all liquid from the chamber and add 150 μL of nuclei extraction buffer.

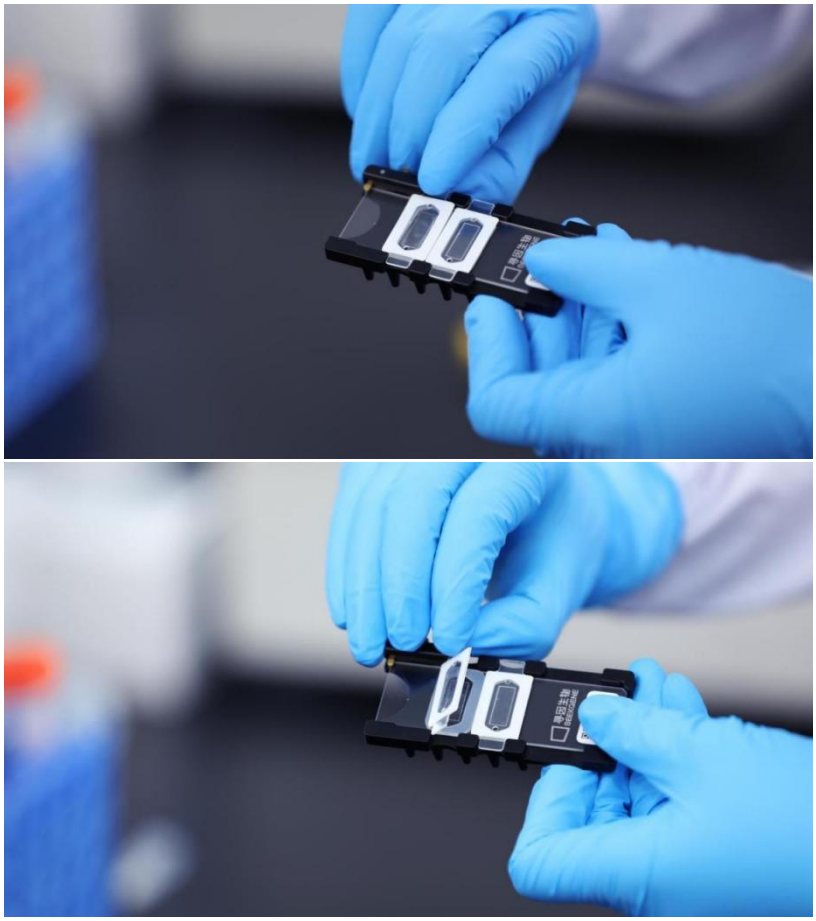
Note: If 150 μL of nuclei extraction buffer was already added during the imaging step, skip this step.

2. Transfer the nuclei extraction buffer from the chamber into a new 1.5 mL centrifuge tube. Remove the top cover of the Space Chamber to expose the tissue.

Note 1: After removing the Space Chamber cover, there will be a circle of silicone adhering around the marked area.

Note 2: Complete step 1-7-3 for the first marked area before removing the cover for the second marked area and repeating step 1-7-3.

3. Immediately pipette 150 µL of fresh nuclei extraction buffer onto the tissue on the marked area, taking care to prevent the tissue form drying out during this process.



4. Open the sealed tissue scraper and adjust its angle to approximately 20° angle relative to the marked area of the spatial chip. **Gently** (**with very light pressure, just enough for the scraper to touch the surface of the tissue**) scrape off the white tissue in a single direction, ensuring that the tissue is fully removed. Use a 200 µL pipette to collect the scraped tissue into a centrifuge tube containing the nuclei extraction buffer.

Note: Avoid scraping back and forth or scraping areas without tissue to prevent contamination of the cell nuclei with spatial labels from other marked areas on the chip, which could affect the accuracy of spatial labeling.

5. Then use 150 µL of nuclei extraction buffer to wash the chip and collect the buffer.

6. If any tissue remains on the chip, repeat step 1-7-3 and 1-7-4 once more.

7. Transfer all the collected nuclei extraction buffer containing tissue to a grinding tube. Add an additional 300 µL of nuclei extraction buffer to rinse the original centrifuge tube, then transfer this buffer to the grinding tube. The total volume of nuclei extraction buffer in the grinding tube should not exceed 1 mL.

8. Place the grinding tube on ice or a cooling block. Use grinding rod A to gently grind up-and-down 20-25 times, then use grinding rod B to grind up-and-down 20-25 times. Transfer the resulting suspension to a 5 mL centrifuge tube.

9. Wash the grinding tube and rod with 1 mL of 1× PBS-RI solution. Recover the 1× PBS-RI solution into the 5 mL centrifuge tube containing the nuclei suspension.

10. Repeat step 1-7-9 twice.

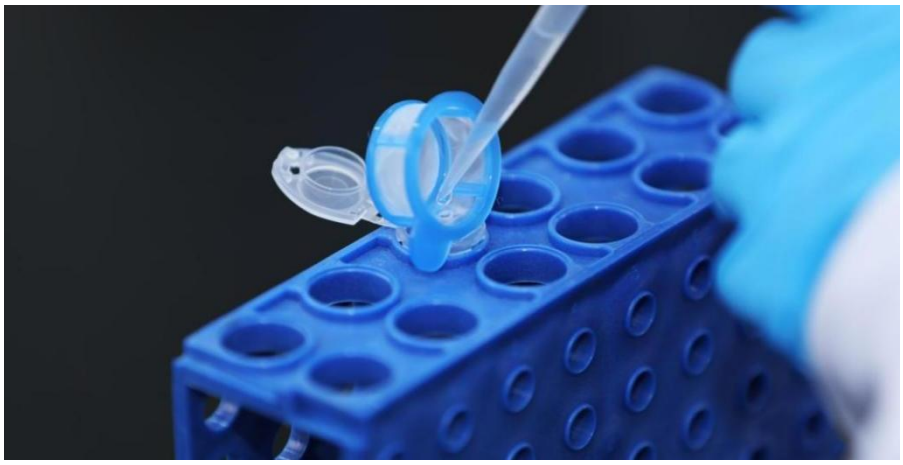
11. After grinding, add 7.5 μL of Buffer T to the 5 mL centrifuge tube containing the nuclei suspension. Gently mix the nuclei suspension by pipetting up and down. Centrifuge at 1000 g, 4°C for 5 minutes. Remove the supernatant, leave approximately 50 μL to avoid disturbing the cell nuclei pellet.

12. Gently mix the nuclei suspension by pipetting up and down. Transfer it to a 1.5 mL centrifuge tube. Wash the 5 mL centrifuge tube with 100 μL of 1× PBS-RI solution, then transfer the wash solution to the 1.5 mL centrifuge tube. Mix the nuclei suspension well by gentle pipetting.

13. Place the 1.5 mL centrifuge tube containing the nuclei suspension on top of the ultrasonic probe of the SeekSpace™ High Frequency Ultrasound Device (REF: M005A) set at 20 kHz frequency and 50 W power. Add water to the ultrasonic device's water tank to fill 1/4 of its capacity without turning on the heat. Perform ultrasonication for 10 seconds.

14. Resuspend the sonicated nuclei suspension in 1 mL of Wash Buffer. Gently mix the nuclei suspension by pipetting up and down with a pipette.

15. Pre-wet a 40 μm cell strainer with 300 μL of Wash Buffer. Filter the 1.1 mL of nuclei suspension through the 40 μm cell strainer into a new 5 mL centrifuge tube.



16. Wash the cell strainer with 1 mL of Wash Buffer, filtering it into the 5 mL centrifuge tube containing the nuclei suspension.

17. Repeat step 1-7-16.

18. Centrifuge at 1000 g, 4°C for 5 minutes. Remove the supernatant and resuspend the nuclei pellet in 1× PBS-RI solution to a final volume of 190 μL(ensure the remaining suspension is not less than 175 μL after quality control).

Step 1-8 Nuclei Suspension Quality Control

1. The total number of nuclei for pre-RT in a labeling region should not exceed 240,000. The number of nuclei for pre-RT for nulcei input in a single tube should be no less than 600 and not exceed 30,000.

2. Nucleation rate should be greater than 5%. A low nucleation rate can affect the number of captured cells and genes.

3. Aggregation rate should be less than 30%.

Note: If the aggregation rate exceeds 30%, large tissue clumps should be removed, re-ground with grinding rod B for 10-20 times, or filtered through a 20 μm or 30 μm cell strainer, then combine with the single-nucleus suspension.

Step 2 Preliminary Reverse Transcription Reaction

Step 2-1 Pre-experiment Preparation

1. Prepare Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	ExpTemp	Pre-use Handling
SeekOne™ DD scFAST-seq Reverse Transcription Kit	3× RT Buffer	🔵	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	Reducing Buffer	⚪	-20℃		
	RT Enzyme	🔵	-20℃		Centrifuge briefly and store on ice for later use.
SeekOne™ DD Single Cell Sample Multiplexing Kit	S1	🔴	-20℃		Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	S2	🟣	-20℃		
	S3	🟢	-20℃		
	S4	🟠	-20℃		
	S5	⚪	-20℃		
	S6	○	-20℃		
	S7	🔵	-20℃		
	S8	⚫	-20℃		
SeekOne™ DD Single Cell TSO Kit	TSO	○	-80℃		Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
SeekOne™ DD Single Cell Decrosslinking Kit	Buffer T	🟠	Room temp	Ambient	Vigorously vortex, centrifuge briefly.
	Buffer S	🟢			
Self-prepared reagents	1× PBS	-	Room temp	Ambient	-
	RNase Inhibitor-A	-	-20℃	Ice	Centrifuge briefly and store on ice for later use.
Self-prepared consumables	Pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	50 mL Centrifuge tube	-	Ambient	Ambient	-
	1.5 mL Centrifuge tube	-	Ambient	Ambient	-
	0.2 mL 8-channel PCR tubes	-	Ambient	Ambient	-

2. Equipment Preparation

a. **Thermal cycler**: Plug in and prepare for use.

b. **Ultrasonic device**: Set frequency to 20 kHz, power to 50 W. Fill the water tank to 1/4 capacity, but do not turn on the heating setting.

c. **Cell counter**: Plug in and prepare for use.

d. **Microcentrifuge**: Plug in and prepare for use.

e. **Vortex mixer**: Plug in and prepare for use.

f. **Thermo Shaker**: Plug in and prepare for use.

3. Reagent Preparation

a. Preliminary reverse transcription (pre-RT) reaction mix:

Prepare the Mix on ice, mix thoroughly by pipetting up and down 15 times, and perform a short spin (ensure following the table below to prepare the reaction Mix before use).

Component	Single Sample
3× RT Buffer	106.4 μL
RT Enzyme	20.8 μL
TSO	8 μL
Reducing Buffer	6.4 μL
Total	**17.7 μL* 8 wells**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

Step 2-2 Pre-RT Reaction

1. Divide an individual sample into 8 parts for pre-RT reactions. Add 17.7 μL of the pre-RT Mix into each tube of a 0.2 mL 8-strip tubes. Then, add 1.5 μL of a different reverse transcription sample multiplex primer (S1, S2, ... S8) to each tube, and label accordingly.

2. Determine the number of nuclei to be used. The total number of nuclei for pre-RT in an individual sample should not exceed 240,000, with each tube containing between 600 and 30,000 nuclei. Calculate the volume of nuclei suspension to be added for each tube. For each tube, first add [20.8 μL - volume of nuclei suspension (μL)] μL of 1× PBS-RI to the pre-RT Mix and mix thoroughly by pipetting up and down. Repeat this for all 8 tubes. Then add the corresponding volume of single-nucleus suspension (mix thoroughly by pipetting up and down before adding). The final total volume of reaction mixture in each tube should be 40 μL. Proceed with the reverse transcription reaction using the following program (lid temperature at 85°C):

Cycle number	Temperature	Time	Temperature change rate
15	8℃	12 s	
	15℃	45 s	1.5℃/s
	20℃	45 s	1.5℃/s
	30℃	30 s	1.5℃/s
	42℃	3 min	1.5℃/s
	42℃	5 min	
	4℃	Hold	

3. After the pre-RT reaction, add 1.5 μL Buffer S to each tube of the 8-strip, mix thoroughly by pipetting up and down, then transfer the all 8 reverse transcription reaction mixtures of the same sample to a new 1.5 mL centrifuge tube. Add 100 μL Wash Buffer to the first tube of the 8-strip for washing, then transfer the washing solution to the second tube for washing. Repeat this process for all 8 tubes and transfer all the washing solution to the 1.5 mL centrifuge tube containing the reverse transcription reaction mixture.

4. Add 1 mL of Wash Buffer, mix thoroughly by pipetting up and down, centrifuge at 1000 g for 5 minutes at 4°C, and remove the supernatant.

Note 1: The volume of Wash Buffer used in this step should be at least twice the total volume of the reverse transcription reaction.

Note 2: When discarding the supernatant, leave about 50 μL of liquid to avoid pipetting the cell pellet, which could lead to cell loss.

Repeat the above wash step (Step 2-2-4) twice, then resuspend the nuclei solution in 90 μL 1× PBS-RI (ensure the remaining suspension is not less than 70 μL after quality control).

5. Set the ultrasonic device to a frequency of 20 kHz and a power of 50 W. Fill the water tank to 1/4 capacity, but do not turn on the heating setting. Place the 1.5 mL centrifuge tube above the ultrasonic probe at a distance of 1 cm and sonicate for 5 seconds.

6. Count the nuclei.

Step 3 Water-in-oil Droplet Formation and Barcode Labeling

Step 3-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD	10×Ligation Buffer	○	-20℃	Ice	Thaw on ice, vigorously

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
Single Cell Barcode Ligation Kit	Primer R	🟢	-20℃		vortex, centrifuge briefly, and store on ice for later use.
	DNA Ligase Mix	🔴	-20℃		
SeekOne™ DD scFAST-seq Reverse Transcription Kit	Reducing Buffer	⚪	-20℃		
SeekOne™ DD scFAST-seq Barcoded Beads Kit	Single Cell Whole Transcriptome Barcoded Beads	○	-80℃	Ambient	Equilibrate 30 minfor later use.
SeekOne™ DD Chip S3 Kit	SeekOne™ DD Chip S3	-	Room temp	Ambient	Replace the Chip P with Chip S3 in the chip holder.
	Gasket	-			Cover on the upper layer of the chip holder.
	Carrier Oil	●			Inject into the well corresponding to label 3.
Self-prepared reagents	Pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	0.2 mL PCR tubes	-	Ambient	Ambient	-
	1.5 mL Centrifuge tubes	-	Ambient	Ambient	-
	20 μm Cell strainer	-	Ambient	Ambient	-

2. Equipment Preparation

a.**SeekOne™ DD**: Ensure the instrument is properly positioned, operating at room temperature, and free from vibration or collisions. Turn on the SeekOne™ DD, place the Chip Holder with all Chip Ps, run the self-check program, and wait for successful self-check completion before proceeding with further experiments.

Note: If experiment is suspended before running the digital droplet system, ensure that the pre-prepared gel beads are allowed to equilibrate at room temperature for 30 minutes until completely thawed before storing them back in the -80°C freezer. Rapid freezing and thawing can easily alter the density and viscosity of the gel beads.

b.**Thermal cycler**: Plug in and prepare for use.

c.**Microcentrifuge**: Plug in and prepare for use.

d.**Vortex Mixer**: Plug in and prepare for use.

e.**Thermo Shaker**: Plug in and prepare for use.

3. Reagent Preparation

Mix preparation: Prepare the Mix on ice, mix thoroughly by pipetting up and down 15 times, and perform a short spin (ensure following the table below to prepare the reaction Mix before use).

Component	Volumn/Sample
10× Ligation Buffer	8 μL
DNA Ligase Mix	5.2 μL
Primer R	2.4 μL
Reducing Buffer	1.6 μL
Total	**17.2 μL**

Note 1: DNA Ligase Mix has high viscosity. When pipetting, avoid inserting the pipette tip too deeply into the liquid surface. Slowly pipette to prevent wall hanging that may result in insufficient reagent adding.

Note 2: Prepared solutions should be placed on ice for storage.

Note 3: The amounts specified above are for one sample.

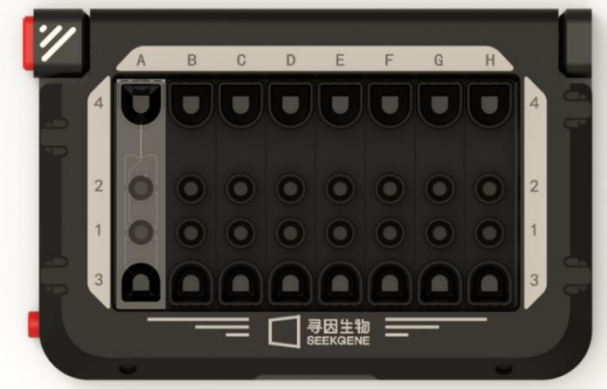
Step 3-2 Single-nucleus Mixture Preparation

1. For each individual water-in-oil nuclei sample, ensure the nuclei input dose not exceeding 120,000. Calculate the input volume and add the corresponding volume [62.8 μL - volume of single-nucleus suspension added in μL] μL of 1× PBS-RI to the Mix and gently mix by pipetting. Then add the corresponding single-nucleus suspension (ensure mixing by pipetting before adding the single-nucleus suspension). The final volume of the single-nucleus mixture should be 80 μL.

Note: Aggregation rate should be ≤ 20%; if aggregation rate > 20%, filter through a 20 μm cell strainer, as high aggregation rates may cause blockage in the water-in-oil system.

Step 3-3 Add reagent to Chip S3

1. Take out the Chip S3 based on your sample number (one sample to one chip). Replace the Chip P with Chip S3 in the chip holder. Then close the chip holder (as shown in the diagram).



Note 1: If no sample is added, Chip P must be placed in the slot.

Note 2: Chip S3 should be used within 24 hours after opening from the packaging.

2. Mix the single-nucleus suspension thoroughly with a pipette and draw up 78 μL. Insert the pipette tip vertically into the bottom center of the well corresponding to Label 1, slightly above the plane of the bottom. Slowly dispense the suspension without generating bubbles and let it stand for 30 seconds.

3. Vortex the equilibrated gel barcoded beads thoroughly for 30 seconds and briefly centrifuge for 2 seconds to ensure no air bubbles are present in the liquid. Draw up 38 μL using a pipette, insert the pipette tip vertically into the bottom center of the well corresponding to Label 2 with the tip just slightly above the bottom surface, and inject slowly without creating bubbles.

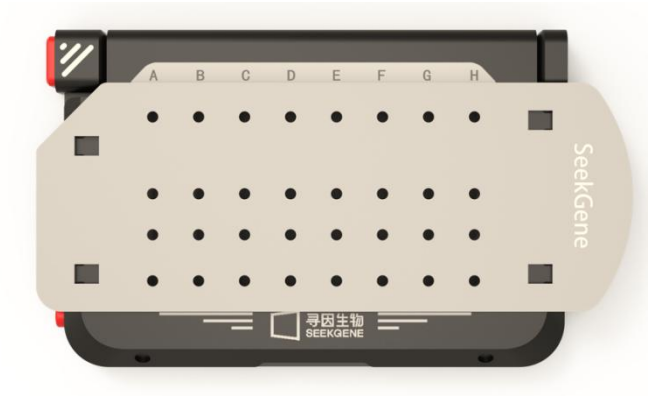
Note 1: When adding reagents, keep the pipette tip within 3 mm below the liquid surface as it moves, to prevent bubble formation.

Note 2: Due to the viscous nature of the gel bead solution, after drawing up to the specified volume range, wait for 5 seconds with the pipette tip in the reagent tube before removing it for sample addition.

4. Using a 200 μL pipette, draw up 120 μL of Carrier Oil. Insert the pipette tip into the well corresponding to Label 3 at an angle against the inner wall. Slowly dispense the oil without creating bubbles. Repeat this step once to add a total of 240 μL of Carrier Oil.

Note: Failure to add Carrier Oil properly may result in the failure of water-in-oil formation or damage to the equipment.

5. As shown in the illustration below, attach the Gasket over the Chip Holder, ensuring alignment between the Gasket wells and the chip wells. The cut-off corner should be on the left upper side.



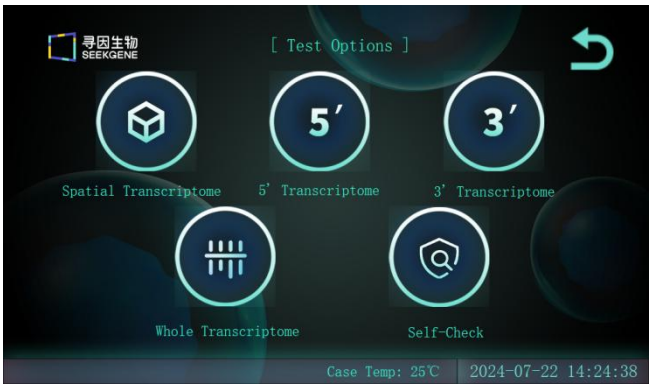
Note: Avoid touching the smooth surface of the Gasket with your hands.

Step 3-4 Running SeekOne™ DD system

1. Click the "Open" button on SeekOne™ DD to eject the tray.

2. According to the illustration below, place the chip holder with the covering gasket into the tray of SeekOne™ DD, ensuring the chip holder is placed horizontally. Click the "Close" button to retract the tray.

3. Click on the "Spatial Transcriptome" program on the instrument screen, then click "Confirm" to initiate the program.



4. After the program finishes running, click on the "Open" button to remove the chip holder. Proceed immediately to the next step of the experiment.

Note: For detailed operating instructions and troubleshooting guidelines for SeekOne™ DD system, please refer to the User Manual of "SeekOne™ Digital Droplet System".

Step 3-5 Transfer the Formed Water-in-oil Droplet

1. Place a new 0.2 mL PCR tube on ice.

2. Discard the Gasket, press and hold the squared PUSH button as shown in the illustration below, open the chip holder all the way, and position the chip at a 45° angle to the horizontal.

3. Observe the volume of single-nucleus mixture and Barcoded Beads solution in Well 1 and Well 2. Any abnormal remaining volume in either well indicates a blockage in the chip:

Note: Remaining volume in well 1 should be <10 μL, and in well 2 should be <15 μL. Total volume of the emulsion product should be approximately 120 μL.

4. Slowly pipette all water-in-oil droplet from the Well 4.

Note 1: When pipetting out water-in-oil emulsion, the tip of the pipette tip should be kept suspended in the liquid without touching the bottom of the well. If there is any excess carrier oil (clear) at the bottom, it can be removed using a 0.5-10 μL pipette tip, taking care not to aspirate the water-in-oil liquid.

Note 2: When running multiple chips simultaneously, there is a low probability of observing bubbles in the Wells 4 in individual chips. This is a normal occurrence and does not affect the library preparation and subsequent results.

5. Observe the liquid inside the pipette tip; normal liquid phase (upper phase) should appear uniformly opaque and turbid.

6. Slowly (~20 sec) inject the water-in-oil droplet from the pipette tip along the walls of the new 0.2 mL PCR tube placed on ice. Do not centrifuge or vortex. Proceed the ligation reaction immediately with the water-in-oil droplet.

Step 3-6 Water-in-oil Droplet Ligation Reaction

1. Place the PCR tubes containing water-in-oil droplet from Step 3-5 into the thermal cycler and run the following program: PCR thermal lid at 85°C, volume 100 μL.

Temperature	Time
20℃	60 min
65℃	10 min
4℃	Hold

Note: If the liquid level of the tube is higher than the height of the heating block, split the water-in-oil product uniformly into two tubes for ligation reaction. After the reaction, centrifuge and combine them into one tube again.

STOPPING POINT: The products generated in step 3-6 can be stored at 4℃ for 16-18 hours or at -80℃ for 4 days.

Step 4 Decrosslinking and Purification

Step 4-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD scFAST-seq Reverse Transcription Kit	Reducing Buffer	○	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
SeekOne™ DD Single Cell Cleanup Kit	Cleanup Beads	○	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use.
SeekOne™ DD Chip S3 Kit	Demulsion Agent	○	Room temp	Ambient	vigorously vortex, centrifuge briefly.
SeekOne™ DD Single Cell Decrosslinking Kit	DCL Buffer	●			
	Enzyme K1	●			
Self-prepared reagents	80% ethanol	-	Ambient	Ambient	Prepare and use on the same day.
	DNA selection beads	-	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use.
	Nuclease-Free Water	-	Ambient	Ambient	-
Self-prepared consumables	pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	1.5 mL Centrifuge tubes	-	Ambient	Ambient	-

2. Equipment preparation

a. **Thermo Shaker**: Plug in and prepare for use.

b. **1.5 mL Magnetic rack**: Prepare for use.

c. **Microcentrifuge**: Plug in and prepare for use.

d. **Vortex Mixer:** Plug in and prepare for use.

Step 4-2 Water-in-oil Droplet Demulsification

1. At room temperature, add 100 µL of Demulsion Agent to each tube containing the water-in-oil droplet. Allow to stand at room temperature for 2 minutes.

2. Remove and discard 130 µL Demulsion Agent/Carrier Oil mixture by slowly pipetting from the bottom of the PCR tube, leaving 2-5 µL transparent mixture at the bottom of the tube to avoid taking any of the aqueous phase.

Note 1: If the upper aqueous phase remains turbid after the de-emulsification step, repeat steps 1 and 2 for a second oil breaking.

Note 2: If the upper aqueous phase remains turbid after the second de-emulsification, after removing the bottom transparent mixture, retain the turbid liquid together with the aqueous phase and directly proceed to the next decrosslinking step.

Step 4-3 Decrosslinking

1. Transfer all the aqueous phase reaction liquid from Step 4-2 to a new 1.5 mL centrifuge tube. Add 90 µL of DCL Buffer and 18 µL of Enzyme K1. Vortex to mix thoroughly.

Note: If the DCL Buffer solution forms white solid or crystals during use, heat it to 55°C until clarified.

STOPPING POINT: After this step, samples can be stored overnight at -80°C.

2. Place the samples in a thermo shaker and run the following program: 55°C, 500 rpm for 15 minutes, followed by 80°C, 500 rpm for 15 minutes.

STOPPING POINT: After this step, samples can be stored overnight at -80°C.

Step 4-4 Purification

1. Prepare the Cleanup Mix according to the table below:

Component	Volume/Sample
○ Cleanup Beads	390 µL
Reducing Buffer	10 µL
Total	**400 µL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

2. Add 396 μL of the vortexed Cleanup Mix to each sample tube. Gently and slowly pipette up and down at least 15 times, then incubate at room temperature for 10 minutes.

 ***Note: If the reaction mixture appears white solid or crystalline after adding the Cleanup Mix, heat at 55°C until clear, then continue incubating at room temperature.***

3. After incubation, place the centrifuge tube on a 1.5 mL magnetic rack until the solution becomes clear and discard the supernatant.

4. Close the lid and briefly centrifuge (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 μL pipette to remove any remaining supernatant

5. Add 50 μL of Nuclease-Free Water to the centrifuge tube, then add 80 μL (1.6×) of DNA selection beads and pipette to mix thoroughly.

Note: 1.6× refers to the ratio of the added DNA selection beads to the product volume, i.e., 80 μL/50 μL = 1.6×.

6. After incubating the mixed product at room temperature for 5 minutes, place it on a 1.5 mL magnetic rack until the solution becomes clear. Discard the supernatant.

Note: When adsorbing, gently mix by pipetting on the opposite side of the magnetic bead surface five times with the pipette tip to accelerate magnetic adsorption.

7. While still on the magnetic rack, add 800 μL of 80% ethanol. After approximately 30 seconds, carefully remove the supernatant. Repeat this step once.

8. Close the lid and briefly centrifuge (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 μL pipette to remove any remaining supernatant.

9. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 46 μL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

10. Place on the magnetic rack until the solution becomes clear, then transfer 44.6 μL of the supernatant to a new 0.2 mL PCR tube.

Note: When adsorbing, gently mix by pipetting on the opposite side of the magnetic bead surface five times with the pipette tip to accelerate magnetic adsorption.

Step 5 Secondary RT and cDNA Purification

Step 5-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD scFAST-seq Reverse Transcription Kit	3× RT buffer	●	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	Reducing Buffer	●	-20℃		
	RT Enzyme	●	-20℃		Centrifuge briefly, and store on ice for later use.
SeekOne™ DD scFAST-seq Barcoded Beads Kit	TSO	○	-80℃		Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
Self-prepared reagents	80% Ethanol	-	Ambient	Ambient	Prepare and use on the same day.
	DNA selection beads	-	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use.
	Nuclease-Free Water	-	Ambient	Ambient	-
Self-prepared consumables	Pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	0.2 mL PCR tubes	-	Ambient	Ambient	-
	1.5 mL Centrifuge tubes	-	Ambient	Ambient	-

2 . Equipment Preparation

a. **Thermal cycler**: Plug in and prepare for use.

b. **0.2 mL Magnetic rack:** Prepare for use.

c. **Microcentrifuge**: Plug in and prepare for use.

d. **Vortex Mixer**: Plug in and prepare for use.

3. Reagent Preparation

Secondary reverse transcription (RT) reaction Mix preparation:

Prepare the Mix on ice, mix thoroughly by pipetting up and down 15 times, and perform a short spin (ensure following the table below to prepare the reaction Mix before use).

Component	Volume/Sample
3× RT Buffer	26.6 μL
RT Enzyme	5.2 μL
TSO	2 μL
Reducing Buffer	1.6 μL
Total	**35.4 μL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

Step 5-2 Secondary RT Reaction

1. Add the above reagents to the 44.6 μL cleaned-up cDNA product in step 4-4, mix by pipetting 10 times, centrifuge briefly, and then perform the reverse transcription reaction. PCR lid temperature 85℃, volume 80 μL, reverse transcription reaction procedure is as follows:

Temperature	Time
42℃	90 min
4℃	Hold

STOPPING POINT: The products generated in step 5-2 can be stored at 4℃ for up to 72 hours or -20℃ for up to 1 week.

Step 5-3 Purification of Secondary RT product

1. Add 144 μL (1.8×) of DNA selection beads, mix by pipetting 10 times or vortexing, then briefly centrifuge.

Note: 1.8× refers to the volume ratio of added DNA selection beads to the reaction product, i.e., 144 μL / 80 μL = 1.8×.

2. Allow the mixed product to stand at room temperature for 5 minutes, then place on a 0.2 mL magnetic rack until the solution becomes clear and remove the supernatant.

Note: During adsorption, gently pipette 5 times on the opposite side of the bead-facing surface to accelerate magnetic adsorption.

3. While keeping the tubes on the magnetic rack, add 200 μL of 80% ethanol, wait approximately 30 seconds, then carefully remove the supernatant. Repeat this step once.

4. Briefly centrifuge with the lid closed (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation) and use a 10 μL pipette to remove all remaining supernatant.

5. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 24 μL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

6. Place on a magnetic rack until the solution becomes clear, then transfer 23 μL of supernatant to a new 0.2 mL PCR tube.

Note: During adsorption, gently pipette 5 times on the opposite side of the bead-facing surface to accelerate magnetic adsorption.

STOPPING POINT: The products generated in step 5-3 can be stored at -20℃ for up to 1 week or 4℃ for up to 72 hours.

Step 6 Pre-amplification and Purification of cDNA and Spatial Label

Step 6-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD Single Cell Library Amplification Kit	2× PCR Master Mix	●	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	Pre-Primers	●	-20℃		
Self-prepared reagents	80% Ethanol	-	Ambient	Ambient	Prepare and use on the same day.
	DNA selection beads	-	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use.
	Nuclease-Free Water	-	Ambient	Ambient	-
Self-prepared consumables	Pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
	0.2 mL PCR tubes	-	Ambient	Ambient	-
	1.5 mL Centrifuge tubes	-	Ambient	Ambient	-

2. Equipment preparation

a.**Thermal cycler**: Plug in and prepare for use.

b.**0.2 mL Magnetic rack**: Prepare for use.

c.**Qubit 4.0 Fluorometer**: Plug in and prepare for use.

d.**Microcentrifuge**: Plug in and prepare for use.

e. **Vortex Mixer**: Plug in and prepare for use.

3. Reagent preparation

Pre-amplification Mixpreparation:

Component	Volume
2× PCR Master Mix	25 μL
Pre-Primers	2 μL
Total	**27 μL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

Step 6-2 Pre-amplification

1. Add 27 μL of the prepared pre-amplification mix to the 23 μL cDNA product purified in Step 5. Mix thoroughly by pipetting up and down 10 times, then briefly centrifuge. Set and proceed with PCR program as follows: lid temperature at 105°C, reaction volume of 50 μL.

Amplification cycle number	Temperature	Time
	98℃	3 min
7	98℃	10 sec
	63℃	15 sec
	72℃	1 min
	72℃	5 min
	4℃	Hold

Step 6-3 Purification of the Pre-amplification Product

1. Add 80 μL (1.6×) of DNA selection beads to the PCR product from Step 6-2. Mix thoroughly by pipetting up and down 10 times or by vortexing.

Note: 1.6× indicates the volume ratio of the added DNA selection beads to the product, i.e., 80 μL/50 μL = 1.6×.

2. Allow the mixture to stand at room temperature for 5 minutes, then briefly centrifuge. Place the tube on a 0.2 mL magnetic rack until the solution becomes clear and remove the supernatant.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

3. While keeping the tube on the magnetic rack, add 200 μL of 80% ethanol. After approximately 30 seconds, carefully remove the supernatant. Repeat this step once.

4. Briefly centrifuge with the cap closed (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 μL pipette to remove any remaining supernatant.

5. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 101 μL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

6. Place the tube on the magnetic rack until the solution becomes clear, and transfer 100 μL of the supernatant to a new 0.2 mL PCR tube.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

STOP ***STOPPING POINT: The products generated in step 6-3 can be stored at -20℃ for up to 1 month or 4℃ for up to 72 hours.***

Step 6-4 Quantification of the Pre-amplification Product

Measure the product concentration using Qubit to determine the amount of pre-amplified product to add for transcriptome cDNA amplification and spatial library construction.

Step 7 Transcriptome cDNA Amplification and Expression Library Construction

Step 7-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD Single Cell Library Amplification Kit	2× PCR Master Mix	🔴	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	Post-Primers	🟢	-20℃		
	N5 & N7	🟠	-20℃		
Self-prepared reagents	80% Ethanol	-	Ambient	Ambient	Prepare and use on the same day.
	DNA selection beads	-	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use
	Nuclease-Free Water	-	Ambient	Ambient	-
	Reagents for Nucleic Acid Fragment Analyzer	-	4℃	Ambient	-
Self-prepared consumables	Pipette	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	0.2 mL PCR tubes	-	Ambient	Ambient	-
	1.5 mL centrifuge tubes	-	Ambient	Ambient	-

2. Equipment preparation

a. **Thermal cycler**: Plug in and prepare to use.

b. **0.2 mL Magnetic rack**: Prepare for use.

c. **Qubit 4.0 Fluorometer**: Plug in and prepare to use.

d. **Nucleic Acid Fragment Analyzer**: Plug in and prepare to use.

e. **Microcentrifuge**: Plug in and prepare for use.

f. **Vortex Mixer**: Plug in and prepare for use.

Step 7-2 cDNA Amplification

1. Transcriptome cDNA amplification Mix preparation:

Component	Volume/Sample
2× PCR Master Mix	25 μL
Post-Primers	1 μL
Step 6-3 cleanup product≤ 20 ng	24 μL
Total	**50 μL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

2. Using product purified in Step 6-3 for transcriptome cDNA amplification, take 24 μL (maximum input is 20 ng. If the concentration of pre-amplified product exceeds 0.83 ng/μL, take only 20 ng and dilute to 24 μL with Nuclease-Free Water. If the concentration is less than 0.83 ng/μL, directly take 24 μL.) Add this to the transcriptome cDNA amplification mix, vortex to mix thoroughly, briefly centrifuge, and proceed with PCR using the following program: lid temperature at 105°C, reaction volume of 50 μL.

Amplification cycle	Temperature	Time
	98℃	3 min
8-10 (refer to the table below)	98℃	10 sec
	63℃	15 sec
	72℃	1 min
	72℃	5 min
	4℃	Hold

cDNA input	Recommended cycle number
≤ 7 ng	10
8-15 ng	9
16-20 ng	8

Step 7-3 cDNA Purification

1. Take 40 μL (0.8×) of DNA selection beads and add them to the cDNA amplification product. Mix thoroughly by pipetting up and down 10 times or vortexing, then briefly centrifuge.

Note: 0.8× refers to the volume ratio of the added DNA selection beads to the product, i.e., 40 μL/50 μL = 0.8×.

2. Allow the mixture to stand at room temperature for 5 minutes, then place it on a 0.2 mL magnetic rack until the solution becomes clear. Remove the supernatant.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

3. While keeping the tube on the magnetic rack, add 200 µL of 80% ethanol. After approximately 30 seconds, carefully remove the supernatant. Repeat this step once.

4. Briefly centrifuge with the cap closed (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 µL pipette to remove any remaining supernatant.

5. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 41 µL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high ***or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.***

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

6. Place the tube on the magnetic rack until the solution becomes clear and transfer 40 µL of the supernatant to a new 0.2 mL PCR tube.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

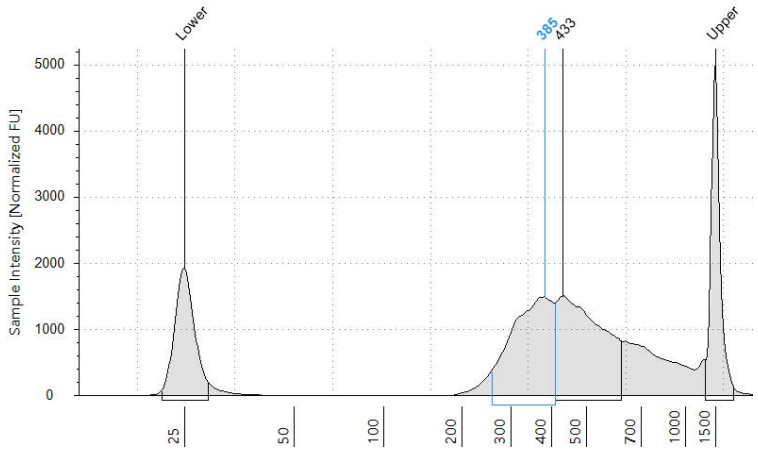
STOPPING POINT: The products generated in step 7-3 can be stored at -20°C for up to 1 month.

Step 7-4 cDNA Product Quality Control

1. If the concentration of enriched cDNA product (measured by Qubit) is ≥ 1 ng/µL, with the peak size ranging from 200-2500 bp and the main peak within 250-1000 bp (Agilent 4200 TapeStation), it is considered qualified.

2. If the concentration of enriched cDNA product ranges from 0.5 ng/µL to 1 ng/µL, with the peak size ranging from 200-2500 bp and the main peak within 250-1000 bp (Agilent 4200 TapeStation), it suggests at risk.

3. If the concentration of enriched cDNA product is ≤ 0.5 ng/µL, it is considered disqualified.



Step 7-5 Expression Library Amplification

1. Expression library amplification Mix preparation:

Component	Volume/Sample
2× PCR Master Mix	25 μL
N5	1 μL
N7	1 μL
Step 7-3 cDNA product after purification≤ 20 ng	10 μL
Nuclease-Free Water	13 μL
Total	**50 μL**

Note 1: Place prepared solutions on ice for later use.

Note 2: The quantities specified above are for the amount needed for 1 sample.

2. Take 10 μL (maximum input is 20 ng) of purified cDNA. If the concentration of the purified cDNA product exceeds 2 ng/μL, take only 20 ng and dilute to 23 μL with Nuclease-Free Water. If the concentration is less than 2 ng/μL, directly take 10 μL. Add this to the expression library amplification mix, vortex to mix thoroughly, briefly centrifuge, and proceed with PCR using the following program: lid temperature at 105°C, reaction volume of 50 μL.

Amplification cycle	Temperature	Time
	98℃	3 min
7-9 (refer to the table below)	98℃	20 sec
	54℃	30 sec
	72℃	1 min
	72℃	5 min
	4℃	Hold

cDNA input	Recommended cycle number
5-10 ng	9
11-30 ng	8
31-50 ng	7

Step 7-6 Expression Library Purification

1. Take 40 μL (0.8×) of DNA selection beads and add them to the cDNA amplification product. Mix thoroughly by pipetting up and down 10 times or vortexing, then briefly centrifuge.

Note: 0.8× refers to the volume ratio of the added DNA selection beads to the product, i.e., 40 μL/50 μL = 0.8×.

2. Allow the mixture to stand at room temperature for 5 minutes, then place it on a 0.2 mL magnetic rack until the solution becomes clear. Remove the supernatant.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

3. While keeping the tube on the magnetic rack, add 200 µL of 80% ethanol. After approximately 30 seconds, carefully remove the supernatant. Repeat this step once.

4. Briefly centrifuge with the cap closed (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 µL pipette to remove any remaining supernatant.

5. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 31 µL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

6. Place the tube on the magnetic rack until the solution becomes clear and transfer 30 µL of the supernatant to a new 0.2 mL PCR tube.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

STOPPING POINT: The products generated in step 7-6 can be stored at -20°C for up to 6 months.

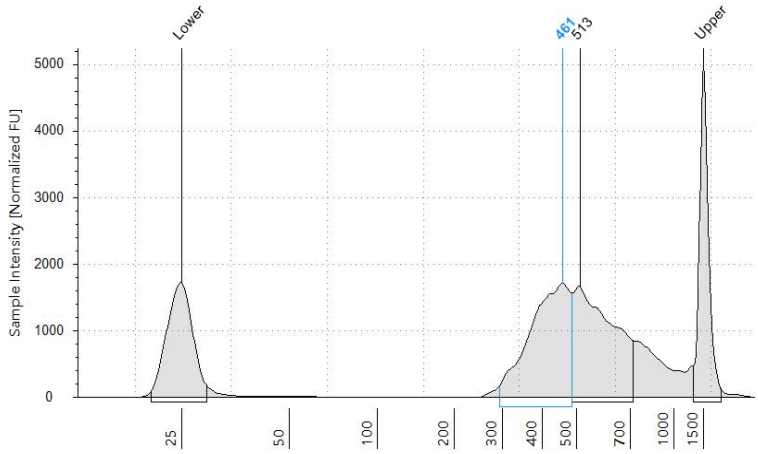
Step 7-7 Expression Library Quality Control

1. If the library concentration (measured by Qubit) is ≥ 5 ng/µL, with no contamination from large fragments and the main peak within the range of 300-1000 bp (Agilent 4200 TapeStation), it is considered qualified.

2. If the library concentration ranges from 1 ng/µL to < 5 ng/µL, with the main peak within the range of 300-1000 bp and no contamination from large fragments, it suggests a risk for sequencing.

3. If the library concentration is ≥ 5 ng/µL, with the main peak size ranging from 300-1000 bp, but there is contamination from fragments larger than 2000 bp, and their height is lower than the height of the target fragments, additional purification is recommended until no large fragments are detected, but risky for sequencing

4. If the library concentration is < 1 ng/μL, or if the peak analysis shows no target fragments within the range of 300-1000 bp, no distinct main peak is observed, or if large fragment contamination exceeds the target fragments, it is considered disqualified.



Step 8 Spatial Label Library Amplification and Purification

Step 8-1 Pre-experiment Preparation

1. Preparation of Reagents and Consumables

Kit	Reagent/ Consumable	Cap Color	Take from:	Exp Temp	Pre-use Handling
SeekOne™ DD Single Cell Library Amplification Kit	2×PCR Master Mix	●	-20℃	Ice	Thaw on ice, vigorously vortex, centrifuge briefly, and store on ice for later use.
	N5 & N7	●	-20℃		
Self-prepared reagents	80% Ethanol	-	Ambient	Ambient	Prepare and use on the same day.
	DNA selection beads	-	2-8℃	Ambient	Equilibrate and mix thoroughly at room temperature for further use.
	Nuclease-Free Water	-	Ambient	Ambient	-
	Reagents for Nucleic Acid Fragment Analyzer	-	4℃	Ambient	-
Self-prepared consumables	Pipettes	-	Ambient	Ambient	-
	DNase/RNase-free Low-bind Eppendorfs	-	Ambient	Ambient	-
	Low retention tip	-	Ambient	Ambient	-
	0.2 mL PCR tubes	-	Ambient	Ambient	-
	1.5 mL centrifuge tubes	-	Ambient	Ambient	-

2. Equipment preparation

a. **Thermal cycler**: Plug in and prepare to use.

b. **0.2 mL Magnetic rack**: Prepare for use.

c. **Qubit 4.0 Fluorometer**: Plug in and prepare to use.

d. **Nucleic Acid Fragment Analyzer**:Plug in and prepare to use.

e. **Microcentrifuge**: Plug in and prepare for use.

g. **Vortex Mixer**: Plug in and prepare for use.

3. Reagent preparation

Mix preparation:

Component	Volume/Sample
2× PCR Master Mix	25 μL
N5	1 μL
N7	1 μL
Step 6-3 cleanup product≤ 20 ng	23 μL
Total	**50 μL**

Note: Place prepared solutions on ice for later use.

Step 8-2 Library Amplification

1. Using product purified in Step 6-3 for spatial label library construction, take 23 μL (maximum input is 20 ng). If the concentration of the pre-amplification product exceeds 0.87 ng/μL, take only 20 ng and dilute to 23 μL with Nuclease-free Water. If the concentration is less than 0.87 ng/μL, directly take 23 μL. Add this to the pre-configured Mix, vortex to mix thoroughly, briefly centrifuge, and proceed with PCR using the following program: lid temperature at 105°C, reaction volume of 50 μL.

Amplification cycle	Temperature	Time
	98℃	3 min
13-16 (refer to the table below)	98℃	20 sec
	54℃	30 sec
	72℃	20 sec
	72℃	5 min
	4℃	Hold

pre-amplification product input	Recommended cycle number
≤5 ng	16
6-10 ng	15
11-15 ng	14
16-20 ng	13

Step 8-3 Library Purification

1. After completing the reaction, briefly centrifuge the tube. Add 60 μL of DNA selection beads (1.2×) and mix thoroughly by pipetting up and down 10 times or vortexing.

Note: 1.2× refers to the volume ratio of added DNA selection beads to the product, i.e., 60 μL/50 μL = 1.2×.

2. Allow the mixture to stand at room temperature for 5 minutes, then place it on a 0.2 mL magnetic rack until the solution becomes clear. Remove the supernatant.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface ***to accelerate magnetic adsorption.***

3. While keeping the tube on the magnetic rack, add 200 μL of 80% ethanol. After approximately 30 seconds, carefully remove the supernatant. Repeat this step once.

4. Briefly centrifuge with the cap closed (ensure beads face outward to prevent them from sticking to the tube walls during centrifugation). Use a 10 μL pipette to remove any remaining supernatant .

5. Allow the ethanol to evaporate completely at room temperature (magnetic beads appear dull, not cracked, approximately 3-5 minutes). Add 31 μL of Nuclease-free Water to fully suspend the magnetic beads and incubate at room temperature for 5 minutes.

Note 1: Adjust drying time based on the dryness state of the magnetic beads if the room temperature is too high or too low. Over-drying of the beads may cause clumping and difficulty to be resuspended.

Note 2: For thorough bead resuspension, recommend vortexing for 10-15 seconds and then briefly centrifuging before pipetting up and down 15 times.

6. Place the tube on the magnetic rack until the solution becomes clear and transfer 30 μL of the supernatant to a new 0.2 mL PCR tube.

Note: During adsorption, use the pipette tip to gently mix 5 times on the side opposite the magnetic bead surface to accelerate magnetic adsorption.

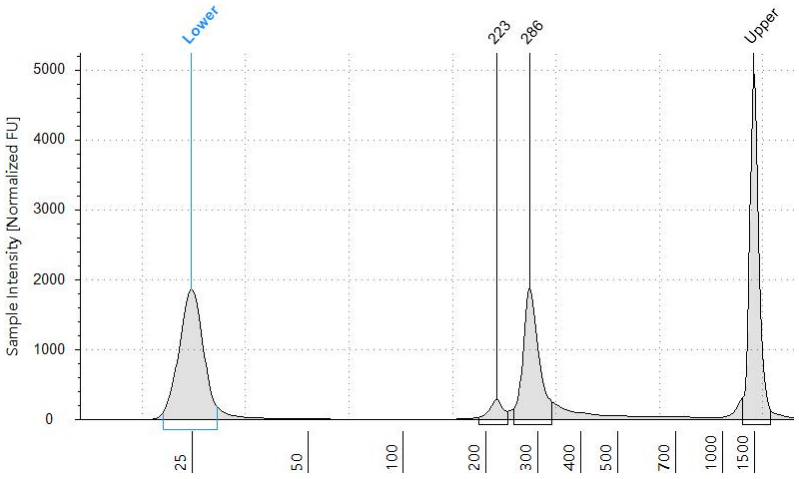
STOPPING POINT: The products generated in step 8-3 can be stored at -20°C for up to 6 months.

Step 8-4 Spatial Label Library Quality Control

1. If the library concentration (measured by Qubit) is ≥ 1 ng/μL, with the main peak size ranging from 220-330 bp (Agilent 4200 TapeStation), and there is no contamination from large fragments, it is considered qualified.

2. If the library concentration (measured by Qubit) is ≥ 1 ng/μL, with the main peak size ranging from 220-330 bp, but there is contamination from large fragments where the proportion of large fragment molar mass is less than 20%, it suggests risk for sequencing.

3. If the library concentration (measured by Qubit) is < 1 ng/μL, or if the peak analysis shows no desired fragments within the 220-330 bp range, or if the proportion of large fragment molar mass exceeds 20%, it is considered disqualified.



Appendix 1: Multiplex Label Sequence and Index Sequence

Multiplex label sequences

Sample Label	Forward sequences
S1	GTGATAAGCA
S2	TCCTATTAGG
S3	CATGGCGTAC
S4	AGACCGCCTT
S5	CGTGCCGATT
S6	TTCAGGTGGC
S7	AAGTTACTCG
S8	GCACATACAA
S9	GGACTGTGGA
S10	CCTAATAACG
S11	AAGGCACTAT
S12	TTCTGCGCTC
S13	GCTCTGCTTC
S14	TAGTCATACG
S15	CTAAGTACGA
S16	AGCGACGGAT

Index sequences

Index number	Forward sequences
N501	ACTAGAGC
N502	TGCCTATA
N503	GCAGCTGT
N504	ACGTTAAG
N701	TCAAGTAT
N702	CACTTCGA
N703	GCCAAGAC
N704	AAACATCG

Note 1: Forward Index sequence refers to the sequence direction consistent with Illumina's official provided sequences. If sequencing on the HiSeq X Ten platform, the reverse complementary sequence for Index N5 should ***be provided.***

Note 2: Index sequences provided from this kit are capable of simultaneously labeling up to 16 library samples.

Note 3: The adapter sequences for the library are as follows:

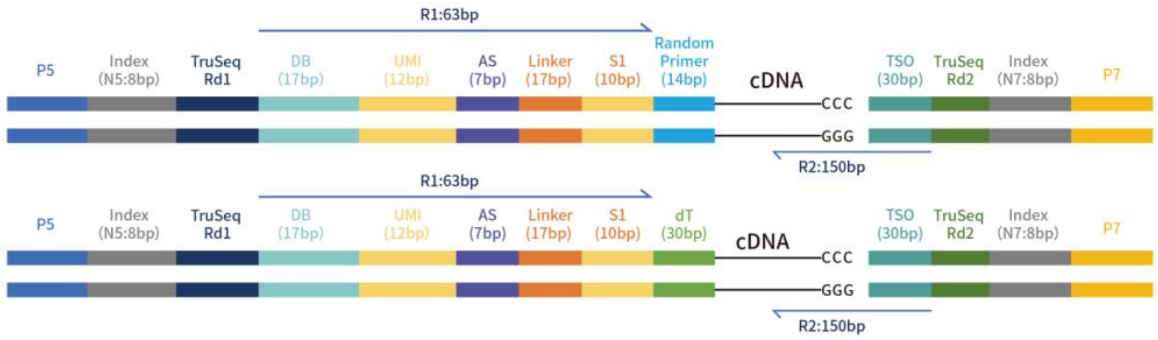
N5	5'AATGATACGGCGACCACCGAGATCTACAC[N5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
N7	5'GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[N7]ATCTCGTATGCCGTCTTCTGCTTG 3'

Appendix 2: High-throughput Sequencing

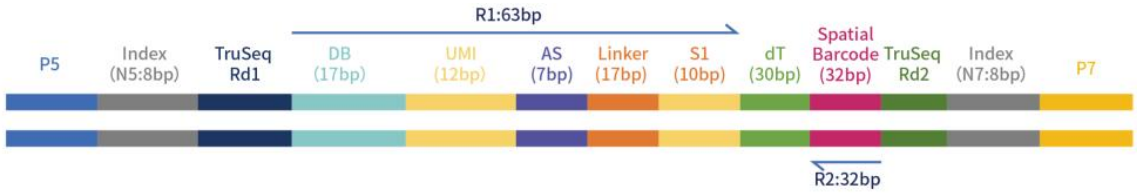
1. Sequencing Library:

The sequencing library constructed using the SeekSpace™ Single-Cell Spatial Transcriptome-seq Kit includes both a transcriptome library and a spatial label library. Both libraries start with P5 and end with P7 sequences. The Cell Barcode consists of a 17 bp droplet barcode followed by a 10 bp multiplex label. The UMI is 12 bp long, the linker sequence is 24 bp, and the template switching sequence is 30 bp. Each sample has dual-end Index sequences, N5 and N7, each consisting of 8 bp. Through the sequencing of the library, basic data FASTQ for standard single-cell analysis can be obtained.

Transcriptome Library:



Spatial Label Library:



2. Sequencing platform

The single-cell libraries constructed using this kit can be adapted for sequencing on the following platforms:

GeneMind platform:SURFSeq 5000.

Illumina platform: MiSeq, NextSeq 500/550, NextSeq 1000/2000, HiSeq 2500(Rapid Run), HiSeq 3000/4000, NovaSeq.

3. Sequencing Library Data and Run Parameters

Sequencing Strategy	Single-Cell Transcriptome Library	Spatial label Library
Sequencing Data Volume	≥ 120 G	≥ 30 G
Sequencing Type	Paired-End Sequencing	Paired-End Sequencing

Sequencing Strategy	Single-Cell Transcriptome Library	Spatial label Library
Read Lengths	Read 1: 63 bp N7 Index: 8 bp N5 Index: 8 bp Read 2: 150 bp	Read 1: 63 bp N7 Index: 8 bp N5 Index: 8 bp Read 2: 32 bp

4. Library Loading Amounts

Platform	Equipment	Loading Concentration (pM)	PhiX (%)
Illumina	MiSeq	11	1
	NextSeq 500/550	1.8	1
	NextSeq 1000/2000	650	1
	HiSeq 2500(RR)	11	1
	HiSeq 4000	240	1
	NovaSeq	150*/300	1
GeneMind	SURFSeq 5000	160	1

Note: Use 150 pM loading concentration for Illumina XP workflow。

For further information on other sequencing platforms, please contact us at info@seekgene.com for additional assistance.

5. Library Pooling

Considering that gene expression libraries and spatial label libraries from different samples may be pooled into a single lane for sequencing, it is crucial that the same index sequences are not used within the same lane. Samples with identical index sequences cannot be separated in subsequent data analysis.

When sequencing pooled gene expression and spatial label libraries on the same lane, the libraries may have varying sequencing depth requirements. Therefore, mixing libraries based on their molar ratios is advisable. The gene expression libraries are recommended to be initially sequenced to a depth of at least 120 G and then determine whether additional data is needed based on sequencing saturation.

Library Type	Sequencing Data Volume	Library Molar Ratio
Gene Expression Library	≥ 120 G	5
Spatial Tag Library	≥ 30 G	1

Appendix 3: Bioinformatics Analysis

Analysis Software:

SeekSpace™ Tools is a software developed by SeekGene for processing single-cell spatial transcriptomics data. This software builds upon the SeekSoul™ Tools module used for single-cell transcriptomics analysis, including Cell Barcode quantification, cell identification, and generation of the cell expression matrix for downstream analysis.

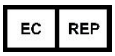
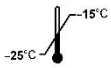
For spatial libraries, SeekSpace™ Tools can precisely locate cells on the chip based on the correlation between spatial barcodes and cell barcodes. It also segments tissues from background in tissue images, performs downstream clustering and differential analysis on successfully localized cells, and enables spatial visualization of clustering results.

Input Files: Expression FASTQ(clean data), Spatial FASTQ(clean data), HDMI FASTQ, Slide Image(tiff format: HE/DAPI), gtf, genomeDir

Output Files: bam, html, csv, rds, zarr, matrix(filtered_feature_bc_matrix), image(_DAPI.png/_HE.png/_HE_TIMG.png)

Operating System: Linux

Appendix 4: Packaging Symbols Explanation

	Manufacturer		EU Representative
	In Vitro Diagnostic Medical Device		Use-by date
	Batch code		Catalogue number
PN	Kit Part Number	CN	Bottle Part Number
	Unique Device Identifier		Consult Instructions for Use
	Keep dry		Keep away from sunlight
	Do Not Use If Packaging is Damaged		Cautions
	Biological Risks		Fragile, handle with care
	Store at -25 to -15℃		CE Mark
	Store at 2 to 8℃		Store at -80℃
	Store at 0 to 25℃		

Appendix 5: Revision History

Serial Number	Change Type	Modified Clauses and Main Content	Modifier	Reviewer	Effective Date
1	New	Create new document			May 21th, 2024