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SEEKGENE

User Manual

SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit

REF: K02101-02 (2 tests), K02101-08 (8 tests)

V1.0

K00202-0201& K00801-0202& K00801-0203& K02101-0204& K00202-0205& K02101-0206& K02101-02
K00202-0801& K00801-0802& K00801-0802& K02101-0804& K00202-0805& K02101-0806& K02101-0807

Envision the Future

Beijing SeekGene BioSciences Co.,Ltd

Table of Contents

1. Introduction

1.1 Product Overview

The SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit is a high-throughput single-cell transcriptome solution specifically designed for FFPE (Formalin-Fixed Paraffin-Embedded) samples by Beijing SeekGene BioSciences Co., Ltd (SeekGene). This reagent kit must be used in conjunction with our independently developed SeekOne™ Digital Droplet System (abbreviated as SeekOne™ DD, REF: M001A) to complete the entire process from single cell nucleic acid labeling to transcriptome library construction. Additionally, it is complemented by the SeekSoul Tools (http://seeksoul.seekgene.com/en/index.html) single-cell data analysis software, providing an integrated solution for FFPE single-cell transcriptomics.

The SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit includes: chip (SeekOne™ DD Chip S3, referred to as Chip S3), gasket, carrier oil, gel beads (SeekOne™ DD Barcoded Beads, abbreviated as Barcoded Beads), reverse transcription (RT) reagents, barcode ligation reagents, decrosslinking reagents, library amplification reagents, and single cell data analysis software (SeekSoul Tools).

1.2 Intended Use

The SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit utilizes microfluidic technology to capture both coding and noncoding RNA from FFPE samples using oligo-dT and random primers. This kit separates and captures single cells in water-in-oil droplets, employing nucleic acid-modified barcoded beads to label RNA from different cells. This process results in a high-throughput single-cell transcriptome library compatible with Illumina sequencers, enabling comprehensive single-cell gene expression analysis. It is applicable to various scientific research areas, including tumor research, immunology research, viral infection research, and targeted biomarker screening.

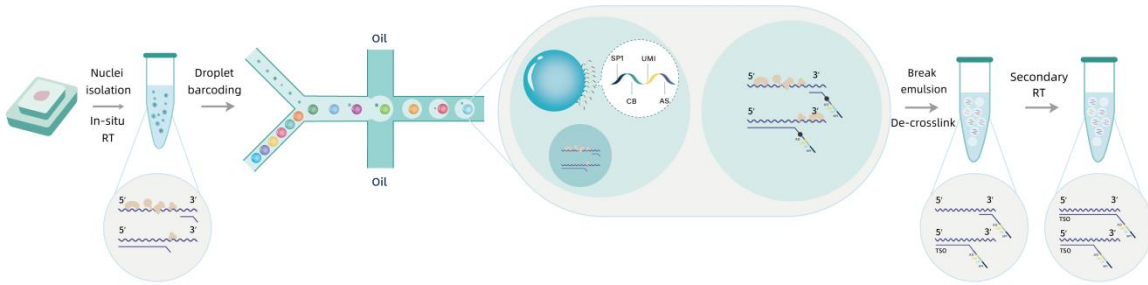
1.3 Intended User

This kit is intended for use by laboratory technicians or individuals with equivalent qualifications. Users must possess a certain level of theoretical knowledge and operational skills in molecular biology. After receiving training and certification from SeekGene, operators will be proficient in performing the procedures associated with this reagent kit.

1.4 Limitations of Test Methods

This reagent kit is intended for use in the pre-processing of samples only and the results should NOT be used directly as the results of in vitro diagnostic tests.

1.5 Experimental Principles



1.6 Parameter Description

1) Sample throughput: Chip S3 is a single-channel chip that can flexibly run 1-8 samples in parallel as needed.

2) Cell capture range: A single channel can capture 500-12,000 cells.

3) Water-in-oil generation rate: 150,000 water-in-oil droplets generated within 3 minutes.

4) Doublet rate: Approximately 0.3% for 1,000 cells.

1.7 Sample Requirements

In the sample preparation step, it is essential to use our proprietary sample pre-processing kit, the SeekMate FFPE Dissociation Kit (REF: K02301-08), to perform nuclei isolation and pre-decrosslinking of the FFPE samples. The resulting single-nucleus suspension will serve as the starting sample for this kit.

1.7.1 Sample Type

☐ **Single Cell Nucleus Suspension**: No large particle precipitation should be present. If there is, filter using a 40 μm cell strainer. No calcium or magnesium ions should be present. FBS (Fetal Bovine Serum) concentration should be ≤2%. Higher FBS concentrations can cause the SeekOne™ DD to clog. BSA (Bovine Serum Albumin) concentration should be ≤0.1%. Higher BSA concentration can lead to incomplete demulsification, affecting cell capture efficiency and gene count.

☐ **Diameter**: 5-40 μm.

1.7.2 Sample Quality

- **RNA Integrity**: DV_{200} > 40%, Main peak > 200 bp (Agilent 4150/4200 TapeStation analysis).

  Note: The "DV_{200}" represents the percentage of RNA fragments > 200 nucleotides.

- **RNA Quantity**: Total RNA from 20,000 nuclei > 25 ng (Qubit 4.0 analysis).

- **Nuclei Count**: Total nuclei > 50,000, minimum 5,000.

  Note: Nuclei count refers to the number of nuclei counted after using our SeekMate FFPE Dissociation Kit and completing Section 3: Sample Pre-decrosslinking.

- **Further requirements**: Aggregation rate ＜25%, Nucleated cell rate ＞10%.

1.7.3 Sample Storage

Store on ice. Complete the in-situ RT experiment within 30 minutes for optimal results. If exceeding 30 minutes, wash once with 1 mL Wash Buffer and re-suspend in 1×PBS-RI (see Step 1).

***Note**: Perform cell counting using a cell counter before starting the experiment with the single-nucleus suspension.*

1.7.4 Recommended Sample Input

- **For In-situ RT**: Recommended number of nuclei for each sample is between 50,000 and 100,000, with a minimum of 5,000 and a maximum of 100,000 nuclei.

- **For Barcode Ligation**: The number of nuclei should not exceed 24,000.

1.8 Product Components and Storage Conditions

SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit (K02101-02, 2 tests; K02101-08, 8 tests) is divided based on their reagent functions and storage conditions, including:

a. SeekOne™ DD Chip S3 Kit, K00202-0201 (2 tests) / K00202-0801 (8 tests)

b. SeekOne™ DD scFAST-seq Barcoded Beads Kit V1.2, K00801-0202 (2 tests) / K00801-0802 (8 tests)

c. SeekOne™ DD scFAST-seq Reverse Transcription Kit V1.2, K00801-0203 (2 tests) / K00801-0803 (8 tests)

d. SeekOne™ DD Single Cell Barcode Ligation Kit V1.0, K02101-0204 (2 tests) / K02101-0804 (8 tests)

e. SeekOne™ DD Single Cell Decrosslinking Kit V1.0, K02101-0206 (2 tests) / K02101-0806 (8 tests)

f. SeekOne™ DD Single Cell Library Amplification Kit V1.0, K02101-0207 (2 tests) / K02101-0807 (8 tests)

g. SeekOne™ DD Single Cell Cleanup Kit, K00202-0205 (2 tests) / K00202-0805 (8 tests)

1.8.1 SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit, 2 tests, REF: K02101-02

Name & PN & Storage	Quantity	Tube lid color	Component	CN	2 tests
SeekOne™ DD Chip S3 Kit, K00202-0201, store at **Room temperature**	1	-	SeekOne™ DD Chip S3	R0003001	2 pieces
		-	Gasket	R0003101	2 pieces
		●	Carrier Oil	R0003201	0.6 mL
		○	Demulsion Agent	R0003301	0.5 mL
SeekOne™ DD scFAST-seq Barcoded Beads Kit V1.2 K00801-0202, store at **-80℃**	1	○	Single Cell Whole Transcriptome Barcoded Beads	R0008601	45 μL ×2 tubes
		○	TSO	R0003601	10 μL
SeekOne™ DD scFAST-seq Reverse Transcription Kit V1.2 K00801-0203, store at **-20 ± 5℃**	2	●	3× RT Buffer	R0008401	80 μL
		●	RT Enzyme	R0003801	15 μL
		○	Reducing Buffer	R0003901	100 μL
SeekOne™ DD Single Cell Barcode Ligation Kit V1.0, K02101-0204, store at **-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
SeekOne™ DD Single Cell Decrosslinking Kit V1.0, K02101-0206, store at **Room temperature**	1	●	DCL Buffer	R0012301	0.5 mL
		●	Enzyme K1	R0012401	90 μL
		●	Buffer S	R0012501	0.5 mL
		●	Buffer T	R0012601	0.5 mL
SeekOne™ DD Single Cell Library Amplification Kit, K02101-0207, store at **-20 ± 5℃**	1	●	2× PCR Master Mix	R0002102	240 μL
		●	Pre-Primers	R0012701	10 μL
		●	Post-Primers	R0012801	10 μL
		●	N501	R0004601	25 μL
		●	N502	R0004701	25 μL
		●	N701	R0005001	25 μL
		●	N702	R0005101	25 μL
SeekOne™ DD Single Cell Cleanup Kit, K00202-0205, store at **2-8℃**	2	○	Cleanup Beads	R0003401	0.5 mL

1.8.2 SeekOne™ DD FFPE Single Cell Transcriptome-seq Kit, 8 tests, REF: K02101-08

Name & PN & Storage	Quantity	Tube lid color	Component	CN	8 tests
SeekOne™ DD Chip S3 Kit, K00202-0801, store at **Room temperature**	1	-	SeekOne™ DD Chip S3	R0003001	8 pieces
		-	Gasket	R0003101	8 pieces
		●	Carrier Oil	R0003202	1.2 mL×2 tubes
		○	Demulsion Agent	R0003302	1.8 mL
SeekOne™ DD scFAST-seq Barcoded Beads Kit V1.2 K00801-0802, store at **-80℃**	1	○	Single Cell Whole Transcriptome Barcoded Beads	R0008601	45 μL ×8 tubes
		○	TSO	R0003602	20 μL
SeekOne™ DD scFAST-seq Reverse Transcription Kit V1.2 K00801-0803, store at **-20±5℃**	2	●	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, store at **-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, store at **Room temperature**	1	●	DCL Buffer	R0012301	0.5 mL×2 tubes
		●	Enzyme K1	R0012401	90 μL×2 tubes
		●	Buffer S	R0012501	0.5 mL
		●	Buffer T	R0012601	0.5 mL
SeekOne™ DD Single Cell Library Amplification Kit, K02101-0807, store at **-20±5℃**	1	●	2× PCR Master Mix	R0002102	240 μL×4 tubes
		●	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, K00202-0805, store at **2-8℃**	2	○	Cleanup Beads	R0003402	1.75 mL

1.8.3 Storage Condition

Name	PN	Transportation	Storage
SeekOne™ DD Chip S3 Kit	K00202-0201/ K00202-0801	Ambient	Room temperature
SeekOne™ DD Single Cell Cleanup Kit	K00202-0205/ K00202-0805	Ambient	2-8℃
SeekOne™ DD scFAST-seq Barcoded Beads Kit V1.2	K00801-0202/ K00801-0802	Dry ice	-80℃
SeekOne™ DD scFAST-seq Reverse Transcription Kit V1.2	K00801-0203/ K00801-0803	Dry ice	-20 ± 5℃
SeekOne™ DD Single Cell Barcode Ligation Kit V1.0	K02101-0204/ K02101-0804	Dry ice	-20 ± 5℃
SeekOne™ DD Single Cell Decrosslinking Kit V1.0	K02101-0206/ K02101-0806	Ambient	Room temperature
SeekOne™ DD Single Cell Library Amplification Kit V1.0	K02101-0207/ K02101-0807	Dry ice	-20 ± 5℃

1.8.4 Index sequence

Index No.	Forward sequence
N501	ACTAGAGC
N502	TGCCTATA
N503	GCAGCTGT
N504	ACGTTAAG
N701	TCAAGTAT
N702	CACTTCGA
N703	GCCAAGAC
N704	AAACATCG

Note 1: *Forward sequence for the index refers to the direction consistent with the sequence provided by Illumina. If sequencing is performed on the HiSeq XTen platform, the reverse complementary sequence for N5 index should be provided.*

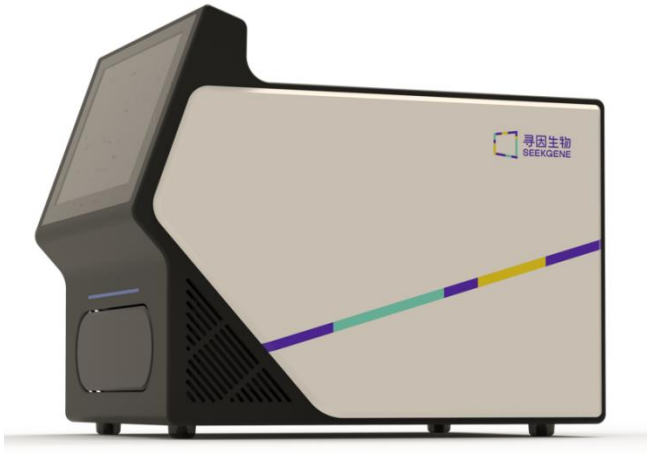
Note 2: *The Index sequence provided by this kit can label up to 16 samples simultaneously.*

Note 3: *The adapter sequence of the library is as follows:*

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

1.8.5 Compatible Instruments and Consumables

1) SeekOne™ Digital Droplet System(SeekOne™ DD, REF: M001A)



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

a. SeekOne™ DD Chip Holder, Abbreviated as Chip Holder: used in conjunction with SeekOne™ Digital Droplet System and Chip S3.

b. Placed Chip, Abbreviated as Chip P: Placed in the chip fixture (8 Chip Ps are included with each instrument). When the sample size is less than 8, the Chip P is used and placed at the position where no sample is added. It serves as a replacement for Chip S3.

1.9 Additional Equipment & Kits, Reagents

1.9.1 Additional Equipment and Consumables

Name	Models	Manufacturer and Item No.
Cell counter equipment	CountStar Rigel S2, SeekMate Tinitan™ FL Cell Counter	Countstar, IN030101, SeekGene, M002C
24-well magnetic separator	24 × 200 μL	Mich Scientific, Magpow-24
12-well magnetic separator	12 × 1.5 mL	
Pipettes	0.1-2.5 μL, 0.5-10 μL, 2-20 μL, 20-200 μL, 100-1,000 μL	Eppendorf, RAININ
Thermal cyclers capable of uniformly heating 100 μl emulsion volumes	C1000 Touch™ Thermal Cycler with 96-Deep Well Fast Reaction Module	BioRad, 1851196
	MasterCycler® Pro	Eppendorf, North America 950030010 International 6321 000.019
	Veriti 96-Well Thermal Cycler	Thermo Fisher, 4375786
	LongGene, A300	LongGene, A300
DNA/RNA quality control equipment	Agilent 4200 TapeStation	Agilent, G2991AA
	Agilent 2100 Bioanalyzer	Agilent, G2939BA
	Bioptic, Qsep400	Bioptic, Qsep400
0.2 mL PCR tubes with flat caps	0.2 mL	Axygen, PCR-02-L-C
8-strip PCR tubes	0.2 mL	Axygen, PCR-0208-FCP-C
50 mL centrifuge tubes	50 mL	Corning, 430829
Qubit 4.0	Qubit 4.0 Fluorometer	Thermo Fisher Scientific, Q33238
Mini centrifuge	-	TIANGEN, OSE-MP25
Vortex mixer	IKA Shakers MS3 (MS3.4/MS3.5)	IKA, -
DNase/RNase-free Low-retention microcentrifuge tubes	1.5 mL	Axygen, MCT-150-L-C
Low retention pipette tips	0.5 - 10 μL/ 1 - 200 μL/ 100 - 1000 μL	Axygen, T-300-L-R-S, Axygen, T-200-C-L-R-S, Axygen, T-1000-C-L-R-S

1.9.2 Additional Kits, Reagents

Name	Manufacturer and Item No.
HyClone phosphate buffered saline (PBS), 1×, 0.0067M PO_4, without calcium, magnesium	HyClone，SH30256.LS
Ethyl alcohol, Pure	Millipore Sigma, E7023-500ML
Nuclease-free Water	Thermo Fisher Scientific, AM9937
SPRIselect / AMPure XP(for Size Selection) VAHTS DNA Clean Beads	Beckman Coulter, B23318 or A63882; Vazyme, N411-03
Qubit dsDNA Quantification Assay Kits	Thermo Fisher Scientific, Q32854
High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents S2-Standard Cartridge Kit, S1-High Resolution Cartridge Kit ***Choose the kits based on the available equipment**	Agilent,5067-5592/ 5067-5593 Agilent, 5067-5584/ 5067-5585, Bioptic, C105201/C105801/C405101 Bioptic, C105202/C105802/C405102

2. Experimental Operation Steps

Protocol Steps and Time

Steps		Timing
Sample Preparation		
~5 h	1 Deparaffinization & Nuclei isolation	1-1.25 h
	2 RNA extraction and QC	1.75 h
	3 Pre-decrosslinking	2 h
Step 1 In-situ RT		
~2.3 h	1-1 Preparation for In-situ RT	10 min
	1-2 In-situ RT	130 min
Step 2 Emulsion Droplets Generation and Barcode Labeling		
~ 1.5 h	2-1 Prepare the Single-nucleus Mixture	10 min
	2-2 Add Reagent to Chip S3	5 min
	2-3 Run SeekOne™ DD	5 min
	2-4 Transfer the Resulting Water-in-oil	3 min
	2-5 Water-in-oil Ligation Reaction	70 min
	○ **STOPPING POINT: 4 ℃ ≤ *24 hours, or -80 ℃ ≤ 4 days***	
Step 3 Decrosslinking and Purification		
~ 1.3 h	3-1 Water-in-Oil Demulsification	5 min
	3-2 Decrosslinking	30 min
	○ **STOPPING POINT: -80℃ ≤ 18 hours**	
	3-3 Purification	45 min
Step 4 Secondary RT and Purification		
~ 3.6 h	4-1 Secondary RT	90 min
	○ **STOPPING POINT: 4°C ≤72 h or −20°C ≤1 week**	
	4-2 Purification of post secondary RT products	30 min
	○ **STOPPING POINT: 4°C ≤72 h or −20°C ≤1 week**	
	4-3 cDNA Amplification	40 min
	4-4 cDNA Enrichment Products Purification	30 min
	○ **STOPPING POINT: 4°C ≤72 h or −20°C ≤1 month**	
	4-5 Quality Control of cDNA Enrichment Products	30 min
Step 5 Library Construction		
~ 1.8 h	7-1 Library Amplification	50 min
	7-2 Library Purification	30 min
	○ **STOPPING POINT: -20℃ ≤ 6 months**	
	7-3 Library Quality Control	30 min

Step 1 In-situ RT

Step 1-1 Preparation for In-situ RT

1. Please review the table below to prepare reagents before starting Step 1.

Source	Material	Cap color	Take from:	Thaw at:	Action
SeekOne™ DD scFAST-seq Reverse Transcription Kit	3× RT Buffer	●	-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
	Reducing Buffer	●	-20℃	Ice	
	RT Enzyme	●	-20℃	——	Take out directly before use, centrifuge briefly , and use immediately.
SeekOne™ DD Single Cell Barcode Ligation Kit	RNase Inhibitor	●	-20℃	Ice	Once thawed, vortex thoroughly, centrifuge briefly, place on ice until use.
	FFPE RT Primers	●	-20℃	Ice	
SeekOne™ DD scFAST-seq Barcoded Beads Kit	TSO	○	-80℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use. Put back to -80 ℃ refrigerator promptly after use.
SeekOne™ DD Single Cell Decrosslinking Kit	Buffer T	●	Ambient	Ambient	
Self-supply	1× PBS solution		Ambient	Ambient	

2. Pre-program the thermal cycler (with deep-well) to ramp at 1.5℃/s.

Step 1-2 In-situ RT

1. Prepare the Wash Buffer and 1× PBS-RI according to the table below.

Components	Wash Buffer	1× PBS-RI
1× PBS	3 mL	0.5 mL
● RNase Inhibitor	7.5 µL	1.25 µL
● Buffer T	30 µL	/
Total	**3 mL**	**0.5 mL**

2. Prepare the Master Mix on ice according to the table below, mix 15 times with a pipette, and centrifuge briefly (be sure to prepare the Master Mix according to the table below before use). Keep on ice until use.

Components	Volume/Sample
● 3× RT Buffer	26.6 µL
● RT Enzyme	5.2 µL
○ TSO	2 µL
● FFPE RT Primers	3 µL
● Reducing Buffer	1.6 µL
Total	**38.4 µL**

Note 1: 3 × RT Buffer should be pink in color. Discard it if the color changes or if there is precipitation.

Note 2: RT Enzyme has high viscosity. When pipetting, avoid inserting the pipette tip too deep into the liquid, and aspirate slowly to prevent insufficient reagent due to wall adhesion.

3. Prepare the single-nucleus mixture (total 80 μL).

a. Master Mix (38.4 μL): Obtained from above. Consisting of 3 × RT Buffer, RT Enzyme, TSO, FFPE RT Primers and Reducing Buffer.

b. 1× PBS-RI: Add 41.6 μL **minus** the volume of single-nucleus suspension to the Master Mix. Pipette and mix well.

c. Single-Nucleus Suspension: Determine the number of nuclei to load (recommended: 50,000-100,000). Calculate the required volume based on the concentration. **Mix the suspension well** and add it to the prepared mixture to **reach a final volume of 80 μL**.

Note 1: Maintain the order of pipetting. Do not add 1× PBS-RI directly to the single-nucleus suspension.

Note 2: For multiple samples, add 1× PBS-RI first, then mix in the single-nucleus suspension to minimize nuclei exposure time and maintain viability.

4. Pipette and mix the single-nucleus mixture from above (total of 80 μL) by pipetting it up and down 15 times with a pipette. Run the following reverse transcription procedure by placing the PCR tube containing single-nucleus mixture into the thermal cycler.

	Lid Temp.	Reaction Volume	Run Time
	85℃	80 μL	~83 min
Total cycles	**Temperat**	**Time**	**Ramp Rate**
15 cycles	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	

5. After RT reaction, transfer the sample to a new 1.5 mL centrifuge tube, add 1.3 mL of Wash Buffer, pipette and mix well, centrifuge at 1000g for 5 min at 4℃, and discard supernatant. Repeat wash step once.

Note: When pipetting the supernatant, leave 50 μL of liquid in the bottom of the tube to avoid aspiration of nuclei precipitates, which may result in loss of nuclei volume.

6. Resuspend by adding 1× PBS-RI, and then use a cell counter for quality control.

***Note**: Resuspension volume can be determined by target nuclei number, recommended nuclei concentration is in the range of **500-1200 cells/μL**. For example, if the nuclei number to be load is 100,000 at a concentration of 1,000 cells/μL, the theoretical volume of 1×PBS-RI to be added is: 100,000/1,000 = 100 μL. However, considering a potential loss of cell nuclei during the above washing steps, estimated at approximately 20%, the volume to be added should be adjusted to 100,000*80%/1000 = 80 μL.*

Step 2 Emulsion Droplets Generation and Barcode Labeling

Step 2-0 Preparation Before Experiment

1. Please review the table below to prepare reagents before starting Step 2.

Source	Material	Cap color	Take from:	Thaw at:	Action
SeekOne™ DD scFAST-seq Reverse Transcription Kit	Reducing Buffer	○	-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
SeekOne™ DD Single Cell Barcode Ligation Kit	10× Ligation Buffer	○	-20℃	Ice	
	Primer R	🟢	-20℃	Ice	
	DNA Ligase Mix	🔴	-20℃	Ice	Take out directly before use, centrifuge briefly and use immediately.
SeekOne™ DD scFAST-seq Barcoded Beads Kit	Barcoded Beads	○	-80℃	Ice	Equilibrate to room temperature in advance (at least 30 minutes).
SeekOne™ DD Chip S3 Kit	SeekOne™ DD Chip S3	-	Ambient	-	
	Gasket	-	Ambient	-	
	Carrier Oil	●	Ambient	-	

***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.*

2. Ensure the SeekOne™ Digital Droplet System is properly positioned, operating at room temperature, and free from vibration or collisions. Turn on the SeekOne™ Digital Droplet System, 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.

Step 2-1 Prepare the Single-nucleus Mixture

1. Prepare the Mix on ice according to the table below. Mix by pipetting up and down 15 times, and then centrifuge briefly. (Make sure to prepare the Mix according to the table below before use). Keep on ice until use.

Component	Volume/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: **DNA Ligase Mix** has high viscosity. When pipetting, avoid inserting the pipette tip too deep into the liquid, and aspirate slowly to prevent insufficient reagent due to wall adhesion.*

2. Prepare the single-nucleus mix to be added to the Chip S3. It contains 3 parts:

a. **Mix from above (17.2 μL)**: Consisting of 10×Ligation Buffer, DNA Ligase Mix, Primer R, and Reducing Buffer.

b. **1× PBS-RI (62.8 μL minus single-nucleus suspension volume)**: Add to the Mix and mix well.

c. **Single-nucleus suspension**. Determine the target number of nuclei. Calculate the volume based on the concentration of the nucleus suspension and the number of target nuclei. **Mix well** and add to above mixture for a **final 80 μL** single-nucleus mix.

*Note 1: **Maintain pipetting order.** For multiple samples, add 1×PBS-RI to all samples before adding and mixing the single-nucleus suspension. This helps reduce the exposure time of nuclei in the mix, preventing RNA diffusion in the mix.*

Note 2: Calculate nuclei loading based on expected capture rate (25% ± 10%). Ensure maximum loading doesn't exceed 24,000.

For example, if the target number of nuclei is 5,000, the calculation based on a capture rate of 25% yields: (5,000 cells / 25%) = 20,000 nuclei. Calculating at 35% would result in 5,000 / 35% ≈ 14,286 nuclei. Load the nuclei accordingly. After determining the number of nuclei, calculate the corresponding volume based on the concentration. For instance, if the concentration is 1000 cells/μL and the loading quantity is 20,000 nuclei, the volume for loading would be 20 μL.

Note 3: The following may result in a lower nucleus capture rate in FFPE samples:

Poor membrane integrity of the cell nucleus.

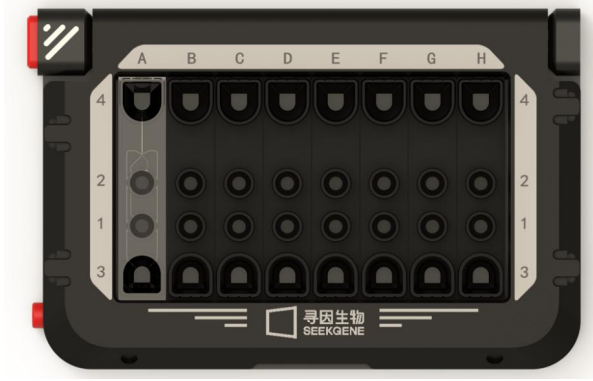
Poor quality and low RNA content of the nucleus suspension.

Heterogeneity in RNA content among cell nuclei.

Poor nucleated cell rate of the nucleus suspension.

Step 2-2 Add Reagent to Chip S3

1. Take out the necessary amount of Chip S3 from the package, one Chip S3 is needed for each sample (e.g. 4 samples, take out 4 Chip S3). Place them into the slots of the Chip Holder by pressing the red button on the left to allow loading of the Chip S3. For all remaining places, insert the Chip P (if there are only 4 samples, 4 positions are free, fill these with Chip P). Then close the cover of the Chip Holder (as shown in the figure below). Make sure that all positions contain a Chip.



Note 1: If there are less than 8 samples, the empty chip positions must be replaced with Chip P. The 8 chip positions in the chip holder should not be left empty. ***But nothing is pipetted into Chip P!***

Note 2: Take out the amount of Chip S3 according to the number of samples from the plastic bags and use them within 24 hours after opening to avoid dust or other contamination.

2. Pipette and mix the single-nucleus mix from above (total of 80 μL) by pipetting it up and down 15 times with a pipette. Take 78 μL of the single-nucleus mix and insert the tip of the pipette tip into the Well 1, hold it slightly tilted into the bottom center of the well, slightly above the very bottom of the well, pipette slowly without bubbles, and let the mix stand for 30 seconds.

3. Vortex the Barcoded Beads well at room temperature for 30 seconds, briefly centrifuge for 2 seconds, ensure that there are no air bubbles in the Barcoded Beads liquid, and pipette 38 μL into the Well 2. The tip should be inserted slightly tilted into the bottom center and slightly above the very bottom of the well, and pipette slowly without generating air bubbles.

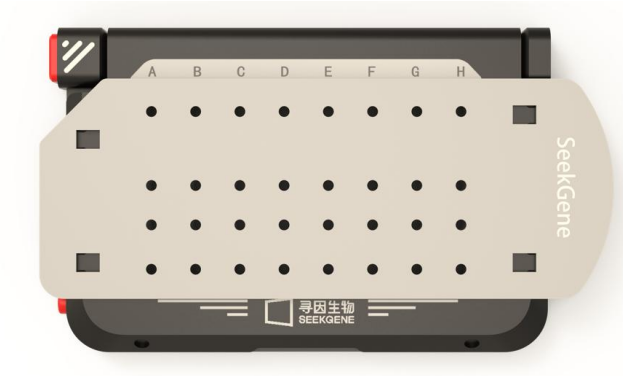
Note 1: When adding reagents, keep the pipette tip moving with the liquid level and always ensure that only the tip of the pipette is below the liquid surface by 3 mm to avoid generating bubbles.

Note 2: The Barcoded Beads liquid is viscous. After pipetting the designated volume, leave the pipette tip in the reagent tube for 5 seconds before removing it for sample addition.

4. Pipette 120 μL of Carrier Oil with a 200 μL pipette into the Well 3, lean the tip against the inner wall, and pipette slowly without generating air bubbles. Repeat this step for a total of 240 μL of Carrier Oil in Well 3.

Note: Adding Carrier Oil improperly may fail water-in-oil droplet generation or damage the instrument.

5. Attach the Gasket over the Chip Holder as shown in the illustration below, ensuring that the Gasket holes and the chip wells are aligned. The cut-off corner should be on the left upper side.



Note: Do not touch the smooth surface of Gasket.

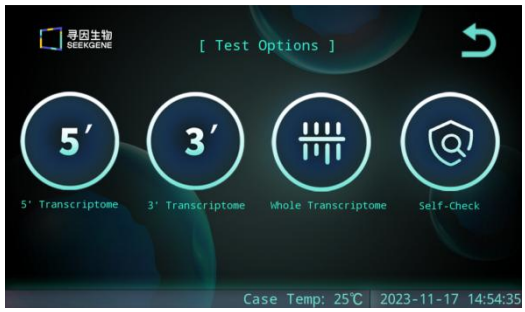
Step 2-3 Run SeekOne™ DD

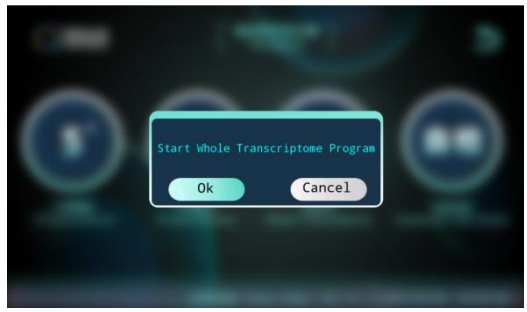
Note: Do not shake or move the instrument while it is running.

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

2. Put the Chip Holder with the covering Gasket into the tray according to the illustration, make sure the Chip Holder is placed horizontally, click the "Close Chip Compartment" button to retract the holder tray.

3. Click the "Whole Transcriptome" program and the "OK" button on the instrument screen to start the program.



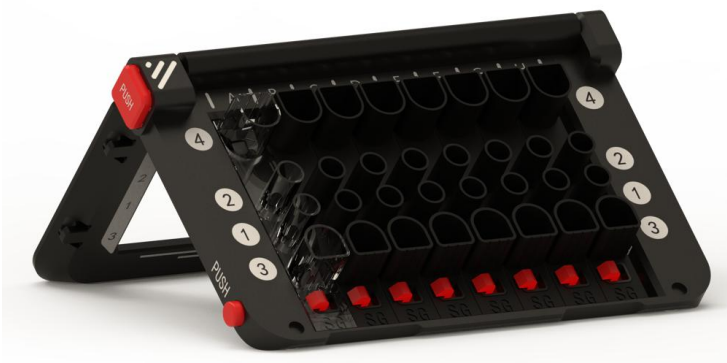


4. After the program is finished, click the "Run Completed" button and remove the Chip Holder. Immediately proceed to the next step.

Step 2-4 Transfer the Resulting Water-in-oil

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

2. Discard the Gasket, press and hold the square **PUSH** button, and open the Chip Holder cover all the way, until the cover is at a 45° horizontal angle shown in the picture:



3. Check the volume of the single-cell mixture and Barcoded Beads solution in both Well 1 and Well 2. Abnormally high volume in either well indicates a clog.

Note: The remaining volume of well 1 (aqueous phase) should be <10 ***μL,*** *and in Well 2 (beads phase), it should be <15 μL. The total product volume is approximately 120 μL.*

4. Use a pipette to **slowly** aspirate all (≥ 120 μL) the water-in-oil emulsion from Well 4.

Note: When pipetting out water-in-oil emulsion, the pipette tip should be kept suspended in the liquid without touching the bottom of the chip well. If there is any excess carrier oil (clear) at the ***bottom****, use a 0.5 - 10 μL pipette to aspirate and discard it, making sure not to carry over the upper emulsion layer.*

5. Observe the water-in-oil emulsion inside the pipette tip. The liquid should appear homogeneous and opaque, indicating a properly formed emulsion.

6. **Slowly** (~20 sec) pipette the water-in-oil emulsion from the pipette tip into the 0.2 mL PCR tube placed on ice, by pipetting it along the wall of the tube. **Do not vortex or centrifuge** the emulsion. The water-in-oil product should immediately proceed to the next step of the ligation reaction.

Note：For each sample, use 2 PCR tubes if needed, ensuring you transfer at least 120 μL total. Do not exceed 100 μL per tube to remain within the volume limits set in the PCR program, which ensures uniform heating of the liquid within the thermal cycler's heated block. If the volume limit set in the PCR program allows for more than 120 μL, you may transfer the entire sample into a single tube and proceed with the PCR program without splitting into two tubes.

Step 2-5 Water-in-oil Ligation Reaction

1. Use a thermal cycler that can accommodate at least 100 μL volume. Run the following procedure by placing the PCR tubes containing water-in-oil emulsion from the previous step into a thermal cycler. If the water-in-oil emulsion is split into two tubes for reverse transcription, combine the contents of both tubes after the PCR program is complete to achieve approximately 120 μL for each sample. Briefly centrifuge to ensure proper mixing.

Lid Temperature	Reaction Volume	Run Time
85℃	100 μL	~70 min
Step	**Temperature**	**Time**
1	20℃	60 min
2	65℃	10 min
3	4℃	Hold

STOPPING POINT： *The products generated in step 2-5 can be stored at 4 ℃ for 16-18 hours or at -80 ℃ for up to 4 days.*

Step 3 Decrosslinking and Purification

Step 3-0 Prepare Before the Experiment

1.Please review the table below to prepare reagents before starting Step 3.

Source	Material	Cap color	Take from:	Thaw at:	Action
SeekOne™ DD scFAST-seq Reverse Transcription Kit	Reducing Buffer		-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
SeekOne™ DD Single Cell Decrosslinking Kit	DCL Buffer		Ambient	-	If the DCL buffer appears white or crystalline, heat it at 55 ℃ until the solution becomes clear. Do not overheat or leave it at this temperature for extended periods.
	Enzyme K1		Ambient	-	
SeekOne™ DD Chip S3 Kit	Demulsion Agent		Ambient	-	
SeekOne™ DD Single Cell Cleanup Kit	Cleanup Beads		4℃	Ambient	Equilibrate to room temperature in advance (at least 30 minutes).
Self-supply	80% ethanol		Ambient	-	Prepare just before use. 3 mL per sample.
	DNA selection beads		4℃	Ambient	Equilibrate to room temperature in advance (at least 30 minutes).
	Nuclease-free Water		Ambient	-	

Step 3-1 Water-in-Oil Demulsification

1. Add 100 μL of Demulsion Agent to each tube of water-in-oil liquid at room temperature and let the tubes stand for 2 minutes at room temperature. During this time, the liquid in the tube will separate into two layers. The upper clear aqueous phase is the sample that needs to be collected, while the lower oil phase is a mixture of Demulsion Agent and Carrier Oil that should be discarded.

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 clear mixture at the bottom of the tube to avoid taking any of the pink aqueous phase.

Note 1: If the upper aqueous phase remains turbid after the de-emulsification step, repeat Step 3-1-1 and Step 3-1-2 for a second de-emulsification.

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

Step 3-2 Decrosslinking

1.Transfer all the reaction solution obtained from Step 3-1 (after oil break) to a new 1.5 mL centrifuge tube, add 90 µL of DCL Buffer and 18 µL of Enzyme K1, vortex thoroughly to mix.

STOPPING POINT：*The products generated in above can be stored at -80℃ for 16-18 hours.*

2. Place the sample in a thermal metal bath and run the following program:

55°C at 500 rpm for 15 minutes, followed by 80°C at 500 rpm for 30 minutes.

STOPPING POINT：*After this step, the sample can be stored at -80℃ for 16-18 hours.*

The decrosslinked product stored at -80℃ can be thawed in a 55℃ metal bath before proceeding with Cleanup Beads purification.

Step 3-3 Purification

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

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

Note: Vortex the Cleanup Beads for 2 minutes and mix well before use.

2. Add 396 µL of vortex-mixed Cleanup Mix to each sample tube. Gently pipette up and down at least 15 times, making sure to avoid introducing bubbles. Incubate the tubes at 37℃ for a total of 10 minutes. After the first 5 minutes, gently pipette up and down 10-15 times and then leave to incubate for the last 5 minutes.

Note: During mixing, move the pipette tip up and down with the liquid surface.

3. After the incubation, the centrifuge tube is placed on the magnetic rack to adsorb until the beads are stuck to the magnet and the solution is clear (1-2 minutes). **Remove and discard the supernatant.**

Note 1: If a milky cloudiness or solidification occurs during this step, place the tube in a 55℃ metal bath for approximately 1 minute to clear. Do not leave it at 55℃ for too long.

Note 2: During adsorption, gently pipette 5 times, with the pipette tip positioned on the opposite side of the magnetic beads, to enhance magnetic bead adsorption.

4. Close the lids and briefly centrifuge them. Put the tubes back onto the magnetic stand, and **remove any residual supernatant with a 10 μL pipette**. During centrifugation, ensure that the side of the tubes with the beads is facing away from the central axis.

5. Add 50 μL of Nuclease-free Water, followed by 60 μL (1.2×) of DNA selection beads. Mix thoroughly by pipetting.

6. After incubating at room temperature for 5 minutes, briefly centrifuge and place the tubes onto the magnetic rack. The beads will stick to the magnet and the solution will appear clear after 1-2 minutes. Then **remove and discard the supernatant**.

7. Keep the sample on the magnetic rack and add 800 μL of 80% ethanol. After 30 seconds, **carefully remove the ethanol supernatant and discard it. Repeat this step once**.

8. Close the lids and briefly centrifuge them. Put the tubes back onto the magnetic stand, and **remove any residual supernatant with a 10 μL pipette.** During centrifugation, ensure that the side of the tubes with the beads is facing away from the central axis.

9. Keep the tubes open and allow the ethanol to evaporate for about 3-5 minutes (depending on the environmental conditions, the beads will be dark and matte without drying or cracking, do NOT wait too long) by incubating at room temperature. Add 46 μL of nuclease-free water to fully suspend the beads, vortex for 10-15 seconds, then briefly centrifuge before pipetting up and down 15 times. Incubate at room temperature for 5 minutes on a normal rack (not on the magnetic stand) and then briefly centrifuge.

10. Place the tubes onto the magnetic rack until the beads are stuck to the magnet and the solution appears clear, **transfer 44.6 μL of supernatant to a new 0.2 mL PCR tube**. This now contains nucleic acid, put the tubes on ice and pipette the next steps on ice. The tube with the beads can be discarded.

Note: During adsorption, gently pipette 5 times, with the pipette tip positioned on the opposite side of the magnetic beads, to enhance magnetic bead adsorption.

Step 4 Secondary RT and cDNA Amplification

Step 4-0 Prepare Before the Experiment

1. Please review the table below to prepare reagents before starting Step 4.

Source	Material	Cap color	Take from:	Thaw at:	Action
SeekOne™ DD scFAST-seq Reverse Transcription Kit	Reducing Buffer		-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
	3×RT Buffer		-20℃	Ice	
	RT Enzyme		-20℃	Ice	Take out directly before use, immediately briefly centrifuged
SeekOne™ DD scFAST-seq Barcoded Beads Kit	TSO		-80℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use. Put back to -80 ℃ refrigerator promptly after use.
SeekOne™ DD Single Cell Library Amplification Kit	2×PCR Master Mix		-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
	Post-Primers		-20℃	Ice	
Self-supply	Nuclease-free Water		Ambient	-	
	DNA selection beads		-20℃	Ambient	Equilibrate to room temperature in advance (at least 30 minutes).
	80% ethanol		Ambient	-	Prepare just before use. 3 mL per sample (for total experiment).

Step 4-1 Secondary RT

1. After thawing the TSO, vortex thoroughly and briefly centrifuge. Dilute TSO according to the table below. The remaining diluted TSO can be stored at -80℃.

Component	Volume/Sample
TSO	1 μL
Nuclease-free Water	8 μL
Total	**9 μL**

2. Prepare the Mix on ice according to the table below. Mix by pipetting up and down 15 times, and then centrifuge briefly. (Make sure to prepare the Master Mix according to the table below before use). Keep on ice until use.

Component	Volume/sample
3 × RT Buffer	26.6 μL
RT Enzyme	5.2 μL
Diluted TSO	2.0 μL
Reducing Buffer	1.6 μL
Total	**35.4 μL**

3. Add the prepared 35.4 μL of Mix to the 44.6 μL cDNA sample purified in Step 3-3. Mix by pipetting 10 times, briefly centrifuge, and then proceed with the reverse transcription reaction. Set up the PCR program as follows:

Lid Temperature	Reaction Volume	Run Time
85℃	80 μL	90 min
Temperature		**Time**
42℃		90 min
4℃		Hold

Centrifuge the samples after the PCR run is completed.

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

Step 4-2 Purification of post-secondary RT products

1. Vortex to resuspend the DNA selection beads. Pipette 96 μL (1.2×) of DNA selection beads into each sample, mix by pipetting 10 times or vortex to mix well, and centrifuge briefly.

> ***Note****: 1.2× refers to the volume ratio of added DNA sorting magnetic beads to PCR products, which is 96 μL / 80 μL=1.2×.*

2. After incubating at room temperature for 5 minutes, briefly centrifuge and place the tubes onto the magnetic rack. The beads will stick to the magnet and the solution will appear clear after 1-2 minutes. **Then the supernatant should be removed and discarded.**

> *Note: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.*

3. Keep the sample on the magnetic rack and add 300 μL of 80% ethanol. After 30 seconds, carefully **remove the ethanol supernatant and discard it. Repeat this wash step once.**

4. Close the lids and briefly centrifuge them. Put the tubes back onto the magnetic stand, and **remove any residual supernatant with a 10 μL pipette**. During centrifugation, ensure that the side of the tubes with the beads is facing away from the central axis.

5. Keep the tubes open and allow the ethanol to evaporate for about 3-5 minutes (depending on the environmental conditions, the beads will be dark and matte without drying or cracking, do NOT wait too long) by incubating at room temperature. Add 24 μL of nuclease-free water to fully suspend the beads, vortex for 10-15 seconds. Incubate at room temperature for 5 minutes on a normal rack (not on the magnetic stand) and then briefly centrifuge.

6. Place the sample on the magnetic rack until the solution becomes clear (1-2 minutes). **Transfer 23 μL of the supernatant to a new 0.2 mL tube.**

Note: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.

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

Step 4-3 cDNA Amplification

1. Preparation of cDNA amplification mix:

Component	Volume/Sample
2× PCR Master Mix	25 μL
Post-Primers	2 μL
Total	**27 μL**

2. Add the prepared 27 μL cDNA amplification mix to the 23 μL cDNA sample purified in Step 4-2, pipetting and mixing 10 times, briefly centrifugate, and then proceed with PCR. Set up the PCR program as follows:

Lid Temperature	Reaction Volume	Run Time
105℃	50 μL	~25 min
Total Cycles	**Temperature**	**Time**
	98℃	3 min
12	98℃	10 sec
	63℃	15 sec
	72℃	1 min
	72℃	5 min
	4℃	Hold

Centrifuge the samples after the PCR run is completed.

Step 4-4 cDNA Enrichment Product Purification

1. Vortex to resuspend the DNA selection beads. Pipette 50 μL (1.0×) of DNA selection beads into each sample, mix by pipetting 10 times or vortex to mix well, and centrifuge briefly.

Note 1*: 1.0× refers to the volume ratio of added DNA selection beads to PCR products, which is 50 μL / 50 μL=1.0×*

2. After incubating at room temperature for 5 minutes, briefly centrifuge and place the tubes onto the magnetic rack. The beads will stick to the magnet and the solution will appear clear after 1-2 minutes. **Then the supernatant should be removed and discarded.**

Note: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.

3. Keep the sample on the magnetic rack and add 200 µL of 80% ethanol. After 30 seconds, **carefully remove the ethanol supernatant and discard it. Repeat this wash step once**.

4. Close the lids and briefly centrifuge them. Put the tubes back onto the magnetic stand, and **remove any residual supernatant with a 10 µL pipette.** During centrifugation, ensure that the side of the tubes with the beads is facing away from the central axis.

5. Keep the tubes open and allow the ethanol to evaporate for about 3-5 minutes (depending on the environmental conditions, the beads will be dark and matte without drying or cracking, do NOT wait too long) by incubating at room temperature. Add 41 µL of nuclease-free water to fully suspend the beads, vortex for 10-15 seconds. Incubate at room temperature for 5 minutes on a normal rack (not on the magnetic stand) and then briefly centrifuge.

6. Place on the magnetic rack until the solution appears clear (1~2 minutes). **Transfer 40 µL of the supernatant to a new 0.2 mL tube.**

***Note**: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.*

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

Step 4-5 Quality Control of cDNA Enrichment Products

1. Quantification of cDNA enrichment products is performed using the Qubit 4.0 system. The Agilent 4200 TapeStation is employed for molecular size and integrity assessment.

cDNA Quality Control Criteria:

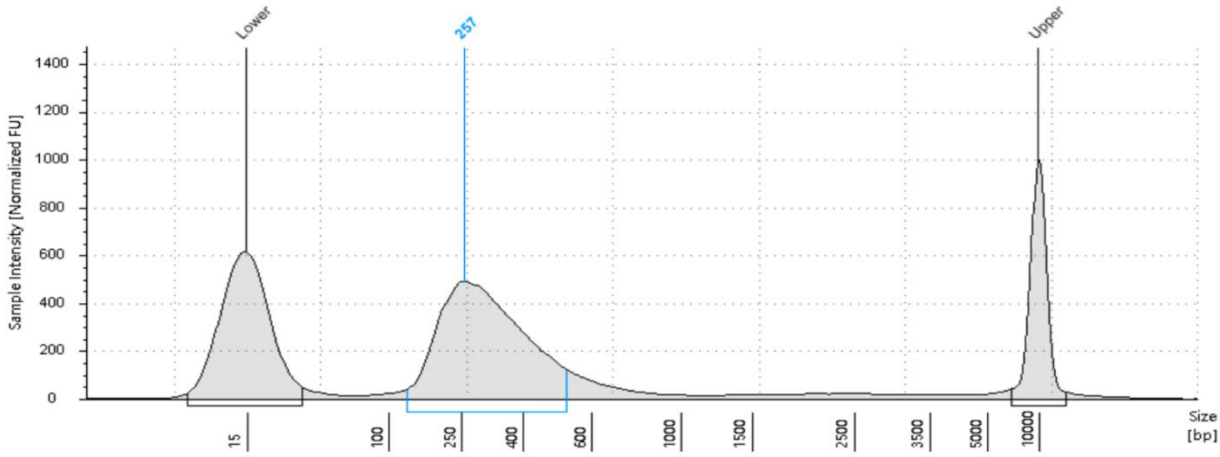
1. For input sample nuclei ranging from 3,000 to 6,000, the concentration of cDNA enrichment products (Qubit) should be ≥ 0.2 ng/µL, and the molecular size distribution should fall within the range of 180-800 bp (Agilent 4200 TapeStation) to be deemed qualified.

2. For input sample nuclei ranging from 6,000 to 12,000, the concentration of cDNA enrichment products should be ≥ 0.4 ng/µL, and the molecular size distribution should fall within the range of 180-800 bp to be deemed qualified.

3. For input sample nuclei ranging from 12,000 to 24,000, the concentration of cDNA enrichment products should be ≥ 0.6 ng/μL, and the molecular size distribution should fall within the range of 180-800 bp to be deemed qualified.

4. For cDNA enrichment products with a concentration of 0.1 ng/μL ≤ concentration < 0.6 ng/μL, and the molecular size distribution should fall within the range of 180-800 bp, it suggests a risk.

5. If the cDNA enrichment product is <0.1 ng/μL, it is deemed unacceptable.



Step 5 Library Construction

Step 5-0 Prepare Before the Experiment

1.Please review the table below to prepare reagents before starting Step 5.

Source	Material	Cap color	Take from:	Thaw at:	Action
SeekOne™ DD Single Cell Library Amplification Kit	2×PCR Master Mix	🔴	-20℃	Ice	Thaw 30 min in advance, vortex thoroughly, centrifuge briefly, place on ice until use.
	N5	🟠	-20℃	Ice	
	N7	🟠	-20℃	Ice	
Self-supply	Nuclease-free Water		Ambient	-	
	DNA selection beads		4℃	Ambient	Equilibrate to room temperature in advance (at least 30 minutes).
	80% ethanol		Ambient	-	Prepare just before use. 3 mL per sample (for total experiment).

Step 5-1 Library Amplification

1. Prepare reaction system according to the following table, fully vortex and centrifuge briefly.

Component	Volume/Sample
2× PCR Master Mix	25 μL
N5	1 μL
N7	1 μL
Nuclease-free Water	13 μL
Total	**40 μL**

2. Pipette 10 μL of purified cDNA enrichment product into the prepared reaction system. Mix 15 times with a pipette, centrifuge briefly. Set up the PCR program as follows and perform the reaction.

Lid Temperature	Reaction Volume	Run Time
105℃	50 μL	~21-32 min
Total Cycles	**Temperature**	**Time**
	98℃	3 min
7-13 (See table below)	98℃	20 sec
	54℃	30 sec
	72℃	1 min
	72℃	5 min
	4℃	Hold

cDNA input amount	Recommended Cycles
10-50 ng	7-9
1-10 ng	9-13

Note: *If the cDNA concentration exceeds 5 ng/μL, directly use 50 ng of cDNA for the amplification.*

Step 5-2 Library Purification

1. After the reaction is completed, centrifuge briefly to ensure proper settling. Add 45 μL of the well-mixed DNA selection beads (0.9×) and pipette up and down 10 times or mix by vortexing.

Note: 0.9× refers to the volume ratio of added DNA selection beads to PCR products, i.e., 45 μL / 50 μL = 0.5×.

2. Incubate the mixture at room temperature for 5 minutes, then centrifuge briefly. Place the tube on the magnetic rack until the solution appears clear. **Remove the supernatant and discard it.**

Note: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.

3. Add 200 μL of 80% ethanol (keep placed on the magnetic rack), wait for about 30 seconds. **Carefully remove the supernatant ethanol and discard it. Repeat this step once.**

4. Close the lids and briefly centrifuge them. Put the tubes back onto the magnetic rack, and **remove any residual supernatant with a 10 μL pipette.** During centrifugation, ensure that the side of the tubes with the beads is facing away from the central axis.

5. Leave them standing on the magnetic rack at room temperature for 3-5 minutes to let the ethanol evaporate completely (the magnetic beads should appear dull and no crack).

6. Add 31 μL of nuclease-free water to fully resuspend the beads and let the tubes stand for 5 minutes at room temperature on a normal rack (not a magnetic rack), then centrifuge briefly.

7. Place the tube on the magnetic rack to allow the beads to attach and the solution to become clear. **Transfer 30 μL of the supernatant to a new tube**. Take 1 μL for Quibit measurement.

Note 1: During adsorption, use a pipette tip to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion, tilt the tip away from the beads.

Note 2: Record the library concentration, sample name, and index number on the tube wall simultaneously.

***STOPPING POINT：** The products generated in step 5-2 can be stored at -20℃ for up to 6 months.*

Step 5-3 Library Quality Control

1. The qualified library has a primary peak fragment size ranging from 200-900 bp, with absence of significant contamination by high molecular weight fragments. If high molecular weight fragments are detected, perform additional selection steps with 0.5× and 0.4× until the library is free of such fragments (Agilent 4200 TapeStation/ Qsep 400).

2. Qubit 4.0 measures library concentration.

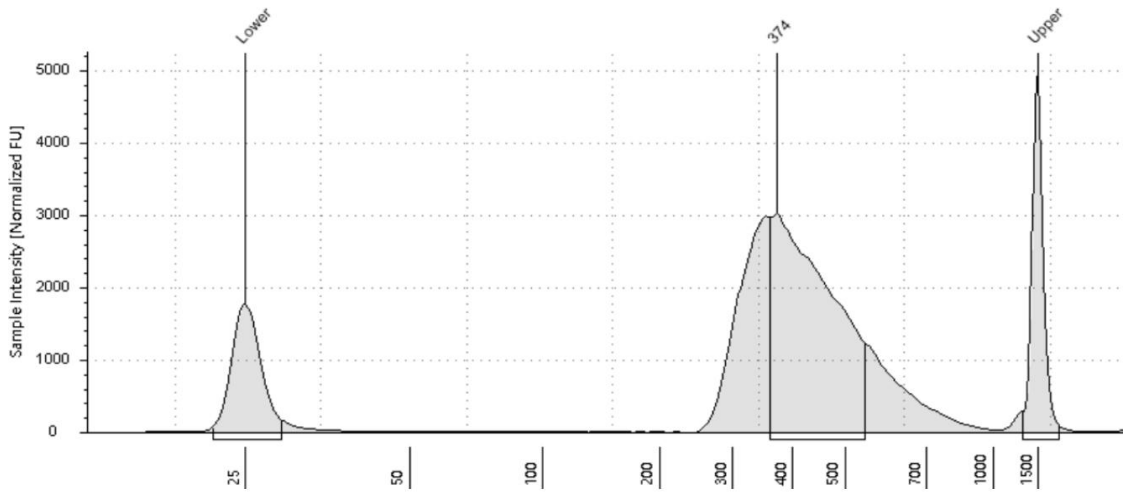
Library Quality Control Criteria:

(1) Library concentration (Qubit): ≥5 ng/μL, with the main peak between 200-900 bp and minimal high molecular weight fragment contamination (Agilent 4200 TapeStation/ Qsep 400). Acceptable.

(2) Library concentration: ≥ 5 ng/μL, main peak between 200-900 bp, but high molecular weight fragments present. If their intensity is lower than target fragments, perform additional selection steps. Risky for sequencing.

(3) Library concentration: 1 ng/μL ≤ concentration < 5 ng/μL, main peak between 200-900 bp, minimal high molecular weight fragment contamination. Risky for sequencing.

(4) Library concentration: < 1 ng/μL, no target fragments within 200-900 bp, no distinct main peak, or high molecular weight fragment intensity surpasses target fragments. Unacceptable.



Manufacturer/After-sales service unit

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Explanations for Symbols

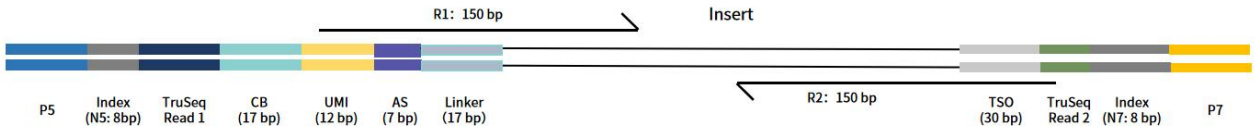
	Manufacture	EC REP	European union representative
IVD	In vitro diagnostic medical device		Use-by date
LOT	Batch code	REF	Catalogue number
UDI	Unique device identifier		Consult instructions for use
	Keep dry		Keep away from sunlight
	Do not use if package is damaged and consult instructions for use		Cautions
	Biological risks		Fragile, handle with care
-25℃ -15℃	Store at -25~-15℃	CE	CE mark
2℃ 8℃	Store at 2~8℃	-80℃	Store at -80℃

Edition: V1.0

Revised date: 2024/12/26

Appendix 1 High-throughput Sequencing

1. Sequencing Library: SeekOne™ DD Single-Cell FFPE Transcriptome sequencing library starts with P5 and ends with P7 sequences. The cell barcode (CB) contains 17 bp, UMI is 12 bp, and sample dual-end indexes are N5 (8 bp) and N7 (8 bp). Sequencing the library will yield basic FASTQ data for standard single-cell analysis.



2. Sequencing Platforms: Single-cell libraries constructed with this kit are compatible with GeneMind sequencing platforms, Illumina sequencing platforms.

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

3. Library Sequencing Depth and Run Parameters:

Sequencing Depth	Minimum 20,000 reads per cell, recommended ≥50,000 reads per cell
Sequencing Type	Paired-end, dual indexing
Read Length	Read 1：150 bp N7 Index：8 bp N5 Index：8 bp Read 2：150 bp

***Note 1**: Recommended sequencing depth is ≥50,000 reads per cell to ensure the accuracy of single-cell sequencing data analysis.*

***Note 2**: Recommended read length : Paired-end sequencing, with Read1 and Read2 both sequenced at 150 bp.*

4. **Library Loading Concentration**:

Platform	Instrument	Loading Concentration (pM)	PhiX（%）
Illumina	MiSeq	11	1
	NextSeq500/550	1.8	1
	HiSeq2500(RR)	11	1
	HiSeq 4000	240	1
	NovaSeq	150*/300	1

***Note**: Use a loading concentration of 150 pM for Illumina XP workflow. For additional sequencing platform information please contact us (info@seekgene.com) for further assistance.*

5. Library Pooling: Considering that gene expression libraries may be pooled into a single lane for sequencing, make sure that the sequencing libraries used for pooling do not have the same indexes, as samples with the same index cannot be demultiplexed for subsequent data analysis.

Appendix 2. Bioinformatics Analysis

Analysis Software: Single-cell data analysis utilizes SeekSoul Tools, a proprietary software developed independently by Beijing SeekGene BioSciences Co., Ltd. SeekSoul Tools can identify cell barcode labels, perform quantitative alignment, and generate downstream analysis results in the form of a cell expression matrix, which is used for subsequent cell clustering and differential analysis.

1) Input Files: FASTQ

2) Output Files: BAM, HTML, CSV, Matrix (filtered_feature_bc_matrix, raw_feature_bc_matrix)

3) Operating System: Linux

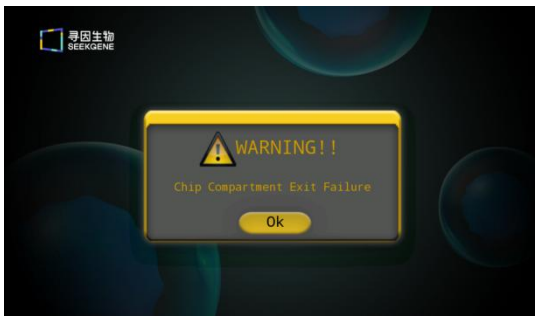
Software Access: To obtain the software package and installation instructions, please visit http://seeksoul.seekgene.com/en/index.html

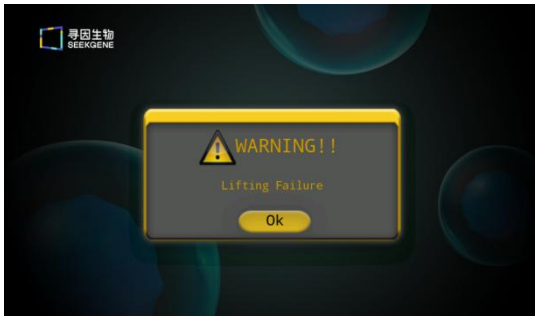
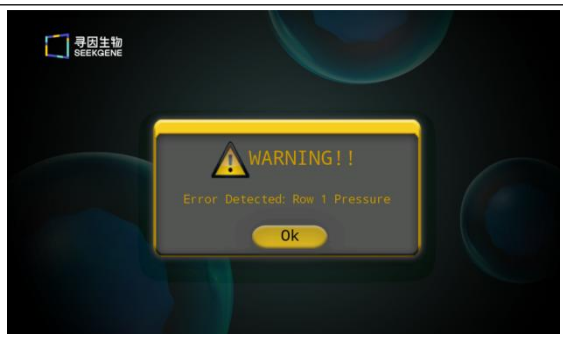
Appendix 3: SeekOne™ Digital Droplet System User Manual

Refer to *SeekOne™ Digital Droplet System User Manual*

Appendix 4: SeekOne™ Digital Droplet System Troubleshooting

Problems may occur during the operation of the equipment. The following table describes the fault types and how to deal with them. When the equipment malfunctions occur, the user can first troubleshoot and deal with it according to the following table, if the issue cannot be solved, please contact our company in time.

Failure type	Solution
	Please make sure the device is installed correctly, Click “OK” to perform a self-check, or restart the device. If this message appears repeatedly, it may indicate an internal hardware issue. Continued use under these circumstances can result in damage to the instrument. Please contact (info@seekgene.com) for further assistance.
	The operation in and out of the warehouse may be blocked. Please confirm that no objects are blocking the running path and click the "OK" button on the prompt window. The instrument will proceed to the next step. If the message appears repeatedly, please contact (info@seekgene.com) for further assistance.
	The operation in and out of the warehouse may be blocked. Please confirm that no objects are blocking the running path and click the "OK" button on the prompt window. The instrument will proceed to the next step. If the message appears repeatedly, please contact (info@seekgene.com) for further assistance.

	Please try again or restart the operation. If the message appears repeatedly, please contact (info@seekgene.com) for further assistance.
	Please verify if the gasket is properly seated on the Chip Holder and reposition the Chip Holder. Check if there are any foreign objects on the surface of the chip compartment and clean the surface. If the message appears repeatedly, please contact (info@seekgene.com) for further assistance.
	Restart the device, if it recurs, contact (info@seekgene.com) for further assistance.
	Please check if the sealing gasket is clean, if the chip has any damage on its surface, and if the Chip Holder is installed correctly. If there is dirt in the sealing gasket or damage on the chip's surface, please replace the gasket or chip and try again. If the error message recurs, contact (info@seekgene.com) for further assistance.
	Please check if the sealing gasket is clean, if the chip has any damage on its surface, and if the Chip Holder is installed correctly. If there is dirt in the sealing gasket or damage on the chip's surface, please replace the gasket or chip and try again. If the error message recurs, contact (info@seekgene.com) for further assistance.

Appendix 5: Revision

No.	Revision	Modified Content	Effective Date
1	New Creation	New file	2024/04/01
2	Revision	All 'Nuclease-free Water' in Step1 and Step2 was changed to '1x PBS-RI', and the volume of ethanol used in Step 4-2 was adjusted from 200 ul to 300 ul. Added some notes.	2024/06/14
3	Revision	Added a few handling tips, revised sequencing recommendations.	2024/12/26