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User manual

SeekOne® DD Single Cell 3’ Transcriptome-seq Kit

V2.2

K00202-0201 & K00202-0202 & K00202-0203 & K00202-0204 & K00202-0205

K00202-0801 & K00202-0802 & K00202-0803 & K00202-0804 & K00202-0805

Envision the Future

Beijing SeekGene BioSciences Co.,Ltd

Product Introduction

SeekOne® DD Single Cell 3’ Transcriptome-seq Kit (Digital Droplet) is a commercial single cell transcriptome library construction kit independently developed by Beijing SeekGene BioSciences Co.,Ltd., which uses microfluidic digital droplets and Barcoded Beads technology. This reagent kit needs to be used with our independently developed SeekOne® Digital Droplet System (abbreviated as SeekOne® DD) to complete the entire process from single cell nucleic acid labeling to transcriptome library construction. When equipped with single cell data analysis software SeekSoul Tools, we provide you with one-stop-shop single cell transcriptome solutions.

SeekOne® DD Single Cell 3’Transcriptome-seq Kit includes: chip (SeekOne® DD Chip S3, referred to as Chip S3), Gasket, Carrier Oil, gel Beads (SeekOne® DD 3 'Barcoded Beads, abbreviated as Barcoded Beads), amplification reagents, library construction reagents, and single cell data analysis software (SeekSoul® Tools).

Intended Use

SeekOne® DD Single Cell 3’ Transcriptome-seq Kit is based on the principle of microfluidic technology. It realizes the separation and capture of single cells through water-in-oil droplets, and uses nucleic acid modified Barcoded Beads to mark the RNA from different cell sources, finally obtains a high-throughput single-cell 3' transcriptome library. The kit can achieve the analysis of single-cell gene expression. It is applicable to tumor, immunity, cell development, virus infection, drug guidance and target screening.

Intended User

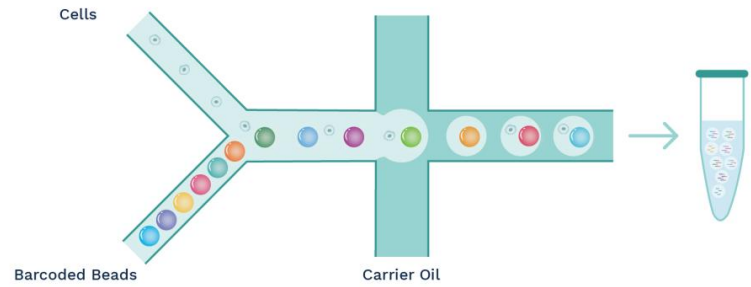
The operators of this reagent kit are mainly laboratory technicians.

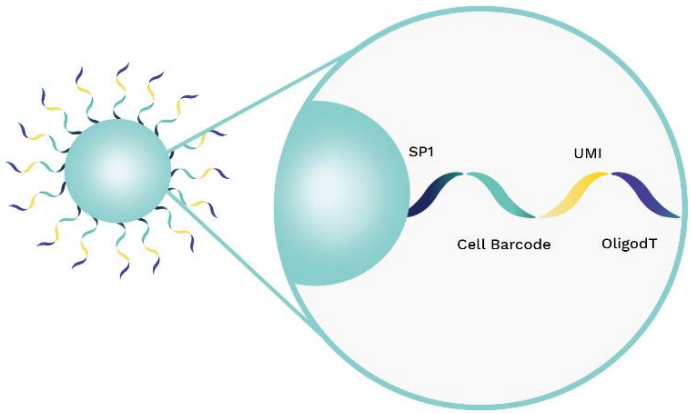
They should have a certain level of theoretical knowledge and operational skills in molecular biology. After training and qualification, they are capable to operate this reagent kit.

Limitation of Test Methods

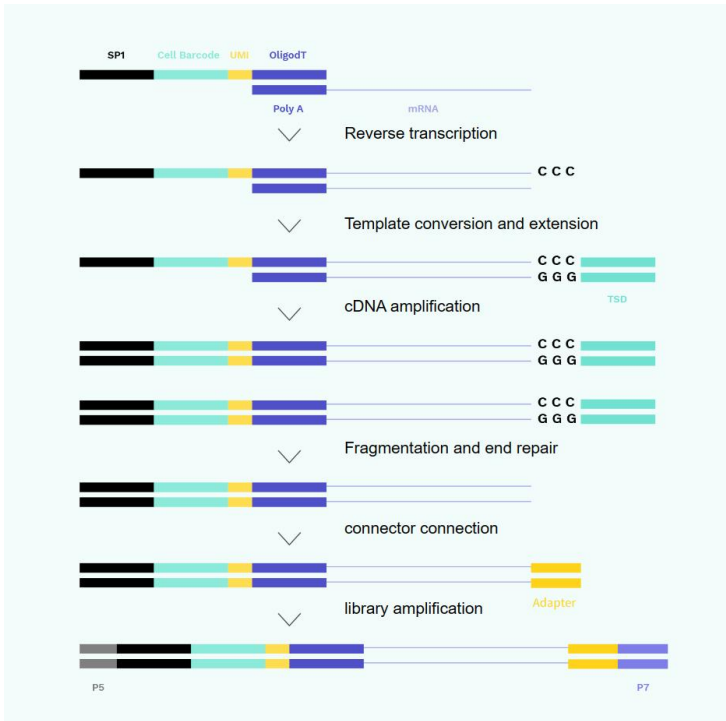
This reagent kit is only used for the pre-treatment of tissue samples and is intended for use in other in vitro diagnostic tests. It is NOT intended to be used directly as a result of an in vitro diagnostic test.

Experimental Principles





Library building process



Sample Requirements

1. Sample type requirements

   ☐Single cell suspension: Large particle precipitation should not appear. If there are large particle precipitation, please filter with a 40 μm cell strainer.

   ☐Cell diameter: 5-40 μm;

2. Sample quality requirements

   ☐Cell count: the minimum input cell count shall not be less than 1000;

   ☐Cell viability: The best analysis results are achieved when the cell viability is＞90% (counting by cell counter) For single-nucleus experiments, cell viability should be below 5% and nuclear staining with DAPI (40X magnification) should show intact and undamaged nuclear membranes (confirmed by cell counter).

   ☐Cell aggregation rate＜10%, Nucleated cell rate＞70%. For single-nucleus experiments, the percentage of nuclear impurities should be below 30% and impurity diameter should not exceed 40 μm.

3. Sample storage requirements

   For fresh single-cell suspensions, it is optimal to perform cell lysis and labeling experiments within 2 hours of placing the sample on ice.

   ***Note**: Before the experiment starts, it is necessary to use a cell counter to count the cells and calculate the live cell rate of the single cell suspension.*

Load Recommendations

Cells are resuspended using RPMI 1640 culture medium, with a recommended concentration range of 700~1200 cells for live cells/μL。

Product components and storage conditions

SeekOne® DD Single Cell 3' Transcriptome-seq Kit is divided based on their reagent functions and storage conditions, including SeekOne® DD Chip S3 Kit, SeekOne® DD Single Cell 3' Barcoded Beads Kit, SeekOne® DD Single Cell 3' Reverse Transcription Kit, SeekOne® DD Single Cell Reverse Transcription Kit-A, SeekOne® DD Library Construction Kit and SeekOne® DD Single Cell Cleanup Kit.

Name		Tube color	Component	REF	K00202-0201 K00202-0202 K00202-0203 K00202-0204	K00202-0801 K00202-0802 K00202-0803 K00202-0804
SeekOne® DD Chip S3 Kit		-	SeekOne® DD Chip S3	R0003001	2pieces	8pieces
		-	Gasket	R0003101	2pieces	8pieces
		●	Carrier Oil	R0003201	0.6 mL	1.2 mL ×2tube
		○	Demulsion Agent	R0003302	0.24 mL	1.0 mL
SeekOne® DD Single Cell Cleanup Kit		○	Cleanup Beads	R0003401	0.5 mL	1.75 mL
SeekOne® DD Single Cell 3' Barcoded Beads Kit		○	Single Cell 3' Barcoded Beads	R0003502	45 μL ×2tube	45 μL ×8tube
		●	TSO	R0003602	10 μL	20 μL
SeekOne® DD Single Cell 3' Reverse Transcription Kit	-	●	3x RT Buffer	R0008401	80 μL	280 μL
		●	RT Enzyme	R0003801	15 μL	50 μL
		○	Reducing Buffer	R0003901	180 μL	180 μL
	SeekOne® DD Single Cell Reverse Kit-A	●	2×PCR Master Mix	R0002101	60 μL	240 μL
		●	cDNA Primers	R0004002	10 μL	20 μL
SeekOne® DD Library Construction Kit		●	Fragmentation Buffer	R0004102	15 μL	50 μL
		●	Fragmentation Enzyme	R0004202	24 μL	100 μL
		●	Ligation Buffer	R0004302	60 μL	240 μL
		●	DNA Ligase	R0004402	15 μL	50 μL
		●	Adaptor	R0004501	15 μL	50 μL
		●	2×PCR Master Mix	R0002101	60 μL	240 μL
		●	N501	R0004601	25 μL	25 μL
		●	N502	R0004701	25 μL	25 μL
		●	N503	R0004801	-	25 μL
		●	N504	R0004901	-	25 μL
		●	N701	R0005001	25 μL	25 μL
		●	N702	R0005101	25 μL	25 μL
		●	N703	R0005201	-	25 μL
		●	N704	R0005301	-	25 μL

Storage condition

SeekOne® DD Chip S3 Kit: **Room temperature** storage and transportation;

SeekOne® DD Single Cell Cleanup Kit: Store at **4** ℃ and transport in ice bags (ice bags must not directly contact the reagent kit);

SeekOne® DD Single Cell 3' Barcoded Beads Kit: Store at **-80** ℃ and transport on dry ice;

SeekOne® DD Single Cell 3' Reverse Transcription Kit: Store at **-20** ℃ and transport on dry ice;

SeekOne® DD Library Construction Kit: Store at **-20** ℃ and transport on dry ice.

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 joint sequence of the library is as follows:*

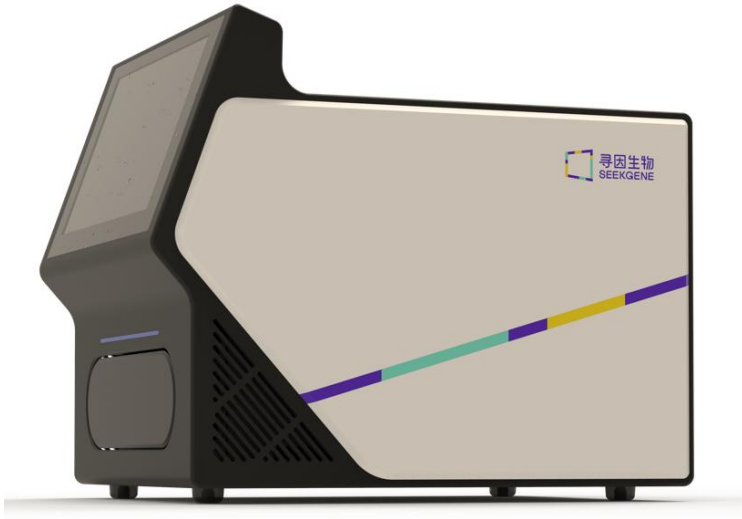
N5	5' AATGATACGGCGACCACCGAGATCTACAC**[N5]**ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
N7	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**[N7]**]ATCTCGTATGCCGTCTTCTGCTTG 3'

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 500-12000 cells;
3. Water-in-oil generation rate: 150,000 water-in-oil droplets generated within 3 minutes;
4. Doublet rate: Approximately 0.3% per 1000 cells.

Compatible instruments and consumables

1. SeekOne® Digital Droplet System



2. SeekOne® DD Accessories：Each 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 Chip S3;

2) 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 used to be placed at the position where no sample is added.

Instruments and additional reagents to be provided by the user.

1. Self-provided instruments and consumables

Name	Models	Recommended Manufacturer and Item No.
Cell counter equipment	CountStar Rigel S2	Countstar, IN030101
DynaMag-2 magnetic frame	-	Thermo Fisher Scientific , 12321D
pipette	2.5 μL; 20 μL; 200 μL; 1,000 μL	Eppendorf, -;RAININ, -
Support 100 μ L deep hole PCR instrument	C1000 Eppendorf, Article number International 6321 000.019 Thermo Fisher Scientific, Article number 4375786 Long Gene, model A300 Deep	BioRad, 1851196
Nucleic acid fragment analyzer	Agilent 4200 TapeStation system Agilent 2100 Bioanalyzer Agilent 2100 Biological Analyzer	Agilent, G2991AA Agilent, G2939BA
	Qsep400	Bioptic, Qsep400
0.2 mL PCR tube	0.2mL	Axygen, PCR-02-L-C
0.2 mL 8-way manifold	0.2mL	Axygen, PCR-2CP-RT-C
Qubit 4.0	Qubit fluorometer	Thermo Fisher Scientific, Q33238
Micro centrifuge	-	Biochemistry, OSE-MP25
Oscillator	MS3(MS3.4/MS3.5)	IKA, -
DNase/RNase-free low adsorption EP pipe	-	Axygen, MCT-150-L-C
Low adsorption filter cartridge gun head	0.5-10μL/ 200μL/ 1000μL	Axygen, T-300-L-R-S; T-200-C-L-R-S; T-1000-C-L-R-S

2. Self-provided reagent/testing kit

Name	Manufacturer and article number
RPMI medium 1640	Gibco, 11875093
Anhydrous ethanol (analytically pure)	Millipore Sigma, E7023-500ML
Nuclease-free Water	Thermo Fisher Scientific, AM9937
DNA sorting magnetic beads(AmPure® XP magnetic beads or VAHTH DNA clean beads)	Beckman Coulter, B23318 or A63882 Vazyme, H411-03
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, Q32854
Reagents for fragment analyzer	Agilent
High Sensitivity D1000 ScreenTape/Reagents	5067-5592/ 5067-5593
High Sensitivity D5000 ScreenTape/Reagents	5067-5584/ 5067-5585

Experimental operation steps

Step 1 Water-in-Oil Generation and Barcode Labeling

Step 1-0 Preparation before experiment

- ☐ Prepare the ice box in advance;
- ☐ Take out the 3x RT Buffer and Reducing Buffer from -20℃ in advance for thawing, vortex them thoroughly, centrifuge them instantly, and place them on an ice box for backup;
- ☐ RT Enzymes should be taken out from -20℃ before use and be immediately used after instant centrifugation;
- ☐ Remove Barcoded Beads from the -80℃ refrigerator in advance and balance at room temperature for 30 minutes for backup;
- ☐ Take out TSO from -80℃ in advance to thaw, vortex thoroughly, and centrifuge immediately, then place it on an ice box for backup.

Step 1-1 Prepare the single-cell mixture

1. Prepare Mix on ice according to the table below, mix 15 times, and centrifuge instantaneously (be sure to prepare the reaction Mix according to the table below before use);

Component	Volume/sample
3x RT Buffer	26.6μL
RT Enzyme	5.2 μL
TSO	2 μL
Reducing Buffer	1.6 μL
Total	35.4 μL

2. To determine the number of captured cells, first add the corresponding volume (44.6 (μL)-single-cell suspension volume (μL)) of water to Mix and mix well; Then add the single-cell suspension corresponding to the table below (pipetting and mixing before adding the single-cell suspension); The total volume of the final single-cell mix is 80 μL.

Cell Stock Concentration (Cells/μL)	Targeted Cell Recovery												
	500	1,000	2,000	3,000	4,000	5,000	6,000	7,000	8,000	9,000	10,000	11,000	12,000
300	3.3	6.7	13.3	20.0	26.7	n	n	n	n	n	n	n	n
400	2.5	5.0	10.0	15.0	20.0	25.0	30.0	n/a	n	n	n	n	n
500	2.0	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	n	n	n	n
600	1.7	3.3	6.7	10.0	13.3	16.7	20.0	23.3	26.7	30.0	n	n	n
700	1.4	2.9	5.7	8.6	11.4	14.3	17.1	20.0	22.9	25.7	28.6	31.4	n
800	1.3	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0
900	1.1	2.2	4.4	6.7	8.9	11.1	13.3	15.6	17.8	20.0	22.2	24.4	26.7
1,000	1.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0
1,100	0.9	1.8	3.6	5.5	7.3	9.1	10.9	12.7	14.5	16.4	18.2	20.0	21.8
1,200	0.8	1.7	3.3	5.0	6.7	8.3	10.0	11.7	13.3	15.0	16.7	18.3	20.0
1,300	0.8	1.5	3.1	4.6	6.2	7.7	9.2	10.8	12.3	13.8	15.4	16.9	18.5
1,400	0.7	1.4	2.9	4.3	5.7	7.1	8.6	10.0	11.4	12.9	14.3	15.7	17.1
1,500	0.7	1.3	2.7	4.0	5.3	6.7	8.0	9.3	10.7	12.0	13.3	14.7	16.0
1,600	0.6	1.3	2.5	3.8	5.0	6.3	7.5	8.8	10.0	11.3	12.5	13.8	15.0
1,700	0.6	1.2	2.4	3.5	4.7	5.9	7.1	8.2	9.4	10.6	11.8	12.9	14.1
1,800	0.6	1.1	2.2	3.3	4.4	5.6	6.7	7.8	8.9	10.0	11.1	12.2	13.3
1,900	0.5	1.1	2.1	3.2	4.2	5.3	6.3	7.4	8.4	9.5	10.5	11.6	12.6
2,000	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0

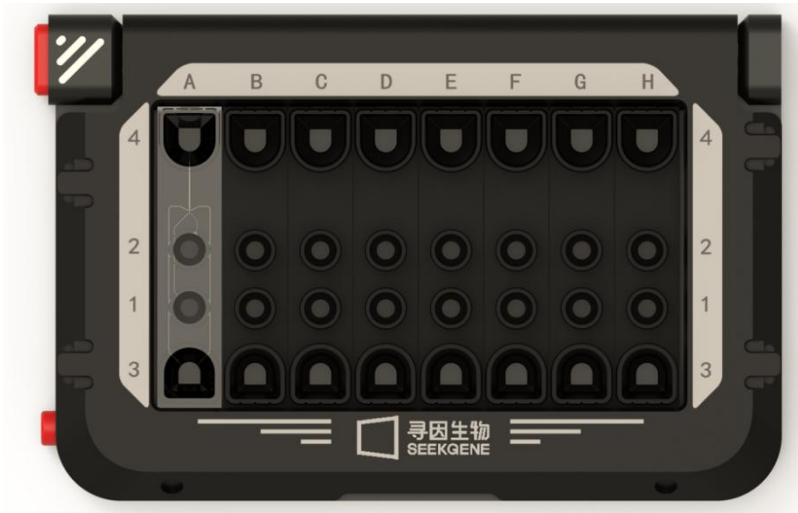
***Note 1: Nuclease-free Water must not be added directly to the cell suspension, it has to be added to the Mix**; Then add the corresponding volume of single-cell suspension.*

***Note 2:** The blue background is marked as the optimal single-cell suspension stock concentration range.*

***Note 3**: The sample volume for loading = Targeted Cell Recovery / 50% / Final Suspension Concentration. The volume of water to be added = 44.6 µl - sample loading volume.*

Step 1-2 Add reagent to Chip S3

1. Take out the Chip S3 with the corresponding sample quantity, replace the Chip P with prepared Chip S3, and then cover the chip holder (as shown in the figure below);



***Note 1**: Be sure to set Chip P in the unfilled position.*

***Note 2**: Take out Chip S3 corresponding to the number of samples from the bag and use it within 24H after opening*

2. Pipette and mix the single-cell mix by pipetting it up and down 15 times with a pipette. Take 78 µL of single-cell mix and insert the tip of the tip vertically into the bottom center of the hole corresponding to label 1 slightly above the bottom plane position, inject slowly without bubbles, and let stand for 30 sec;

3. Vortex well the Barcoded Beads at room temperature for 30 sec, briefly centrifuge for 2 sec, ensure that there are no air bubbles in the Barcoded Beads liquid, and pipette 38 µL. The tip should be inserted vertically into the bottom center of the hole corresponding to label 2 slightly above the bottom plane position, and inject slowly without generating air bubbles;

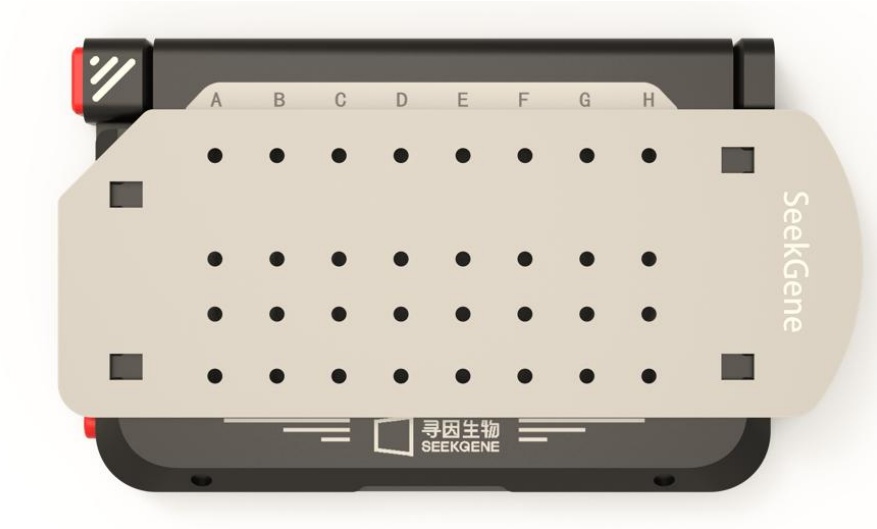
***Note 1:** When adding reagents, keep the pipette tip moving with the liquid level and always keep only one point of the pipette tip below the liquid level to avoid generating bubbles.;*

***Note 2:** 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.*

4. Aspirate 120 μL of Carrier Oil with a 200 μL pipette, insert the corresponding well position of label 3, lean against the inner wall, and inject slowly without generating air bubbles; Repeat this step once to add a total of 240 μL of Carrier Oil.

***Note**: Adding Carrier Oil improperly may result in the failure of water-in-oil droplet generation or damage to the instrument.*

5. Attach the Gasket to the upper layer of the chip holder as shown, ensuring that the Gasket hole and the chip hole are aligned.



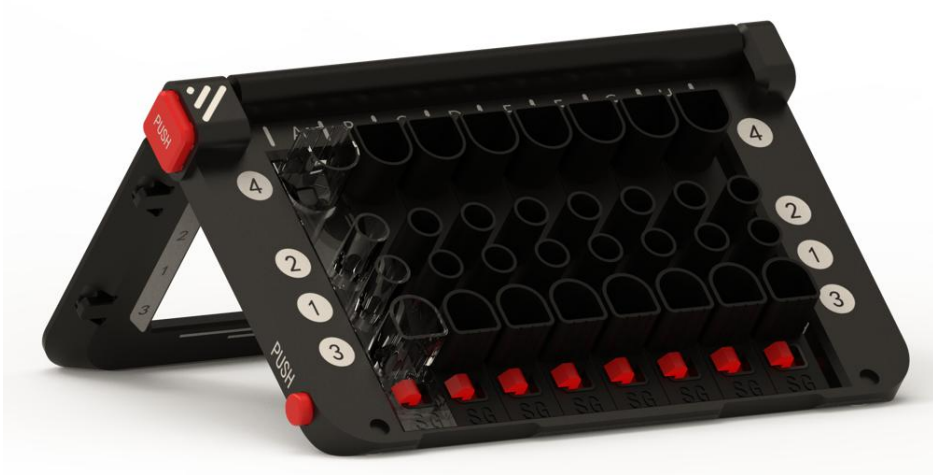
***Note**: Do not touch the smooth surface of Gasket.*

Step 1-3 Run SeekOne® DD (Do not shake/move the device while it is running)

1. Click the "Open Chip Compartment" button on the SeekOne® DD to eject the tray;
2. Put the chip holder covering Gasket into the tray according to the illustration, make sure the holder is placed horizontally, click the "Close Chip Compartment" button to retract the holder tray;
3. Click the "3' 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 to remove the holder. Proceed to the next step of the experiment now

Step 1-4 Transfer the resulting water-in-oil

1. Place a new 0.2 mL PCR tube on ice;
2. Remove the Gasket, press and hold the PUSH button according to the illustration, open the holder cover, and place the chip at 45° horizontal;



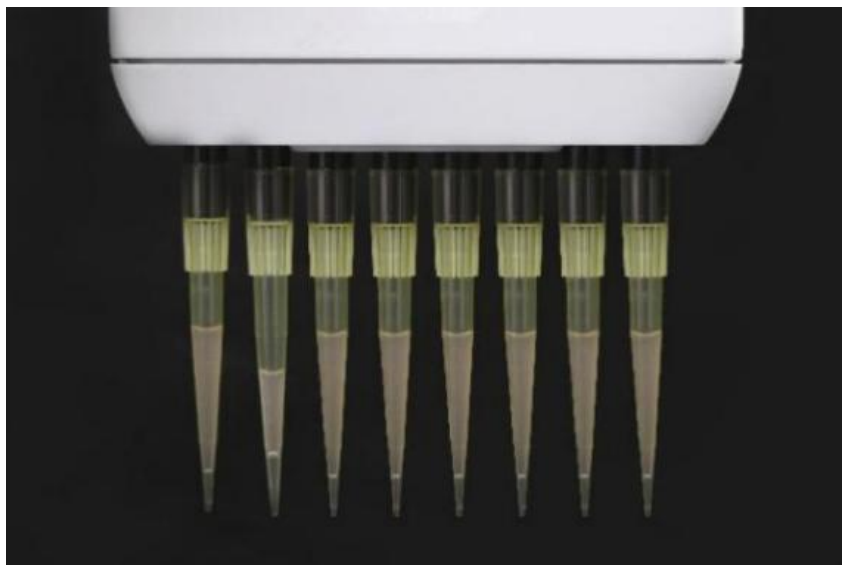
3. Labels 1 and 2 had the single-cell mixture and Barcoded Beads solution volumes in the corresponding wells, and abnormal remaining volumes in either well indicate that the chip is blocked;

***Note**: The remaining volume of hole 1 (aqueous phase) is greater than 10 μL. The remaining volume of hole 2 (adhesive bead phase) is greater than 15 μL indicates that the chip is blocked .*

4. Use a pipette to **slowly** aspirate all (at least 120 uL) the water-in-oil liquid from the well corresponding to labeled 4;

***Note:** When sucking taking out water-in-oil, the tip of the tip should not be in close contact with the bottom of the chip.*

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



***Note**: If the tube appears like the left second tube in figure, it means that there is a blockage in the chip.*

6. Slowly (~20 sec) inject the water-in-oil inside the tip into the PCR tube along the wall of the PCR tube placed on ice.

STOPPING POINT：*After the water-in-oil solution is covered with the tube cap, it should not be placed on ice for more than 1 h.*

Step 1-5 Water-in-oil reverse transcription reaction

1. Run the following procedure by placing the PCR tube containing water-in-oil from the previous step into the PCR machine, PCR hot lid at 85 °C and volume 100 μL. If you have more than 100 μL, use a different tube to incorporate additional volume and run PCR reaction

Steps	Temperature	Time
1	42℃	90 min
2	85℃	5 min
3	4℃	Hold

STOPPING POINT：*Reverse transcription of water-in-oil can be stored at 4°C for up to 72 h or -20°C for up to 1 week.*

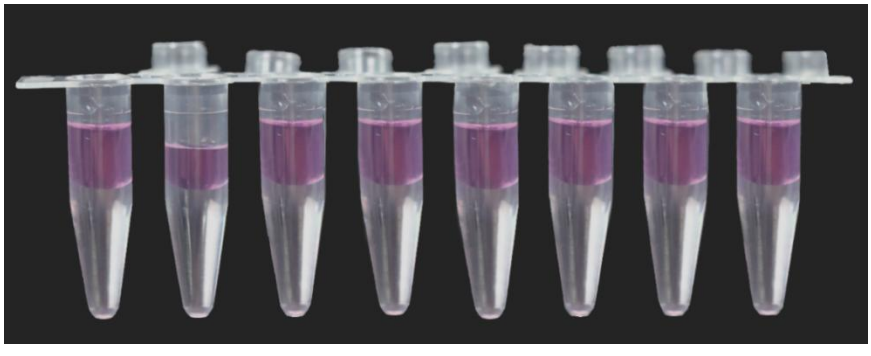
Step 2 Sample recovery and cDNA amplification

Step 2-0 Prepare before the experiment

- ☐ Prepare an ice box in advance;
- ☐ Remove 2× PCR Master Mix, cDNA Primers, and Reducing Buffer from -20 °C on ice in advance Thaw, vortex thoroughly, centrifuge instantaneously, and place on ice box for later use;
- ☐ Prepare 10 mL of 80% ethanol (prepare it just before use, and keep it for ＜ 24 hours).;
- ☐ Equilibrate the Cleanup Beads and DNA sorting beads to room temperature in advance.

Step 2-1 Oil-in-water demulsification

1. Add 100 μL of Demulsion Agent to each tube of water-in-oil liquid at room temperature and let stand for 2 min at room temperature;



***Note 1**: The resulting mixture (shown above) includes Demulsion Agent/Carrier Oil (transparent) and water-phase reaction solution (Pink).*

***Note 2**: If there is too little water phase solution, it indicates that the chip may be blocked (as shown in the second left of the figure above).*

2. Formulate the Cleanup Mix according to the table below

Component	Volume/Sample
Cleanup Beads	175.5 µL
Reducing Buffer	4.5 µL
Total	180 µL

3. Remove 130 µL Demulsion Agent/Carrier Oil mixture by slowly pipetting from the bottom of the PCR tube, without sucking up the pink water phase reaction solution;

***Note:** If a cloudy upper aqueous phase is still observed after the oil break step 1, you can repeat steps 1-3 for a second oil break.*

4. Add 180 µL of vortex-mixed Cleanup Mix to each tube sample. Gently pipette up and down for at least 15 times, making sure to avoid introducing bubbles. Incubate the tubes at room temperature for 10 minutes with the lid open. During the middle 5 minutes, gently pipette up and down for 10 times.

***Note**: When adding liquid, gently mix by moving the pipette tip up and down with the liquid level to prevent overfilling and contamination.*

5. After the incubation, the PCR tube is placed on a magnetic rack to adsorb until the solution is clear and the supernatant is removed.

***Note:** During adsorption, use a pipette tip with the opposite orientation of the adsorbed magnetic beads to gently pipette up and down 5 times to mix and enhance magnetic bead adhesion.*

6. Keep adding 300 µL of 80% ethanol on a magnetic rack for about 30s and carefully remove the supernatant; **Repeat this step once.**

7. Briefly Centrifuge to remove all residual supernatant with a 10 µL pipette;

8. Allow the ethanol to evaporate by leaving the mixture at room temperature for 2 minutes. Add 23.5 µL of Nuclease-free Water to fully suspend the magnetic beads and leave at room temperature for another 2 minutes.

***Note:** The magnetic beads should dry to a dull matte color without cracking. If the temperature in the room is too high or too low, adjust the drying time according to the state of the magnetic beads.*

9. Place on a magnetic rack to adsorb until the solution appears clear, transfer the supernatant 23 µL to a new 0.2 mL PCR tube.

Step 2-2 cDNA amplification

1. Preparation of cDNA amplification mixes：

Component	Volume/Sample

2×PCR Master Mix	25 μL
cDNA Primers	2 μL
Total	27 μL

2. Add the prepared 27 μ L cDNA Amplification Mix to the 23 μL cDNA product purified in Step 2-1, pipetting and mixing 10 times, briefly centrifuge, and then proceed with PCR. Set up the PCR program as follows, hot lid temperature 105 ° C, volume 50 μL:

Number of	Temperature	Time
	98℃	3 min
10-13	98℃	10 sec
	63℃	15 sec
(See table below)	72℃	3 min
	72℃	5 min
	4℃	Hold

Cell diameter	Number of loaded cells	Recommended number of amplification cycles
≤10 μm	500-5,000	13
	5,000-15,000	12
	15,000-24,000	11
>10 μm	500-5,000	12
	5,000-15,000	11
	15,000-24,000	10

STOPPING POINT：*The reaction products can be stored at 4 °C for up to 72 h or -20 °C for up to 1 week.*

Step 2-3 cDNA enrichment product purification

1. Pipette 30 μL (0.6×) of DNA sorting beads into step 2-2 PCR products, mix by pipetting 10 times or vortex to mix well, and centrifuge briefly.

2. After incubating standing at room temperature for 5 min, the mixed product should be placed on a magnetic rack and adsorbed until the solution appears clear and the supernatant should be removed.

Note: *During adsorption, pipette the solution against the surface of the magnetic beads in the opposite direction for gentle aspiration and mix for 5 times. Apply magnetic force to enhance beads capture.*

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

4. Briefly Centrifuge and remove all residual supernatant with a 10 μL pipette;

5. Allow the ethanol to completely volatilize (the beads are dark and matte, about 3-5 min) by incubating at room temperature, add 40.5 μL of Nuclease-free Water to fully suspend the beads, and let stand at room temperature for 2 min;

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

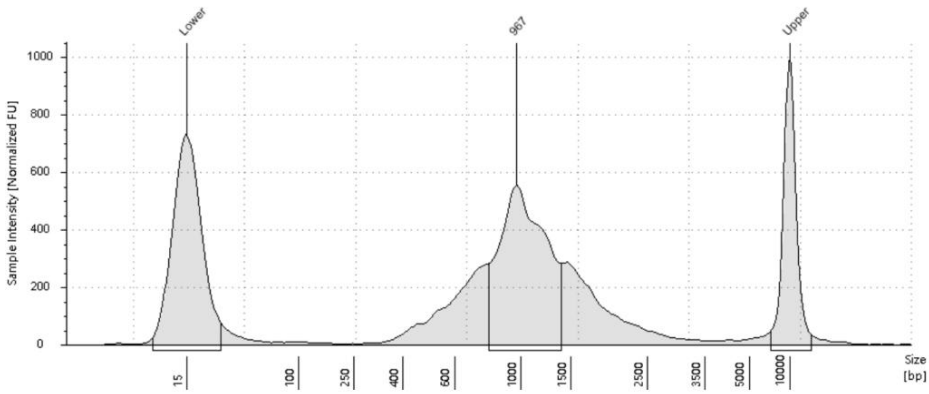
***Note 1**: 0.6X refers to the volume ratio of added DNA sorting magnetic beads to PCR products, which is 30μL / 50μ L=0.6X。*

***Note 2**: During adsorption, pipette the solution against the surface of the magnetic beads in the opposite direction for gentle aspiration and mix for 5 times. Apply magnetic force to enhance beads capture.*

***STOPPING POINT**：After purification, cDNA enrichment products can be stored at -20°C for no more than 1 month or 4°C for no more than 72 h.*

Step 2-4 cDNA enrichment production material test

1. Qualified enrichment product sizes range from 250-5,000 bp with major peaks in the 750-2,000 bp range without small fragments. If there are small fragments, perform another 0.6 × purification until there are no small fragments (Agilent 4200 TapeStation);



2. Qubit 4.0 measures cDNA enrichment product concentration.

cDNA Quality Control Criteria:

1. For capturing 500-5000 cells, the concentration of cDNA (Qubit) should be ≥1 ng/μL. The fragment range should be between 250 bp and 5000 bp, with the main peak falling within the 750-2500 bp range, indicating a pass.

2. For capturing 6000-10000 cells, the concentration of cDNA (Qubit) should be ≥3 ng/μL. The fragment range should be between 250 bp and 5000 bp, with the main peak falling within the 750-2500 bp range, indicating a pass.

3. If the concentration (Qubit) is between 0.5 ng/μL and <1 ng/μL, or if the concentration (Qubit) is ≥1 ng/μL but the detected peak range is between 250 bp and 5000 bp, and the main peak does not fall within the 750-2500 bp range, it suggests a potential risk.

4. If the concentration (Qubit) is <0.5 ng/μL, or if there are no target fragments detected within the 250-5000 bp range, or if there is no apparent main peak, it is considered a fail.

Quality Control Result: The result shows that the majority of fragments are concentrated within the range of 250-5000 bp, with the main peak falling within the 750-2500 bp range, indicating a pass.

Step 3 Library construction

Step 3-0 Prepare before the experiment

- ☐ Prepare an ice box in advance;
- ☐ Take out in advance the Fragmentation Buffer, Ligation Buffer, Adaptor, 2×PCR Master Mix from the ice at -20 °C to thaw, vortex it fully and centrifuge it instantaneously, and put it on the ice box for later use;
- ☐ Fragmentation Enzyme, DNA Ligase are then removed from -20 °C before use, and used immediately after instantaneous centrifugation;
- ☐ Prepare 10 mL of 80% ethanol (prepare it just before use, and keep it for ＜ 24 hours);
- ☐ Equilibrate the DNA sorting beads to room temperature in advance.

Step 3-1 DNA fragmentation and end repair

1. Set up the program according to the table below and run the thermal cycler with a hot lid temperature at 105 °C and a volume of 50 μL;

Steps	Temperature	Time
1	4℃	Hold
2	37℃	5 min
3	65℃	30 min
4	4℃	Hold

2. Prepare the reaction system according to the following table. Mix thoroughly by shaking, centrifuge briefly, and keep on ice for later use. For each sample, take 50-100 ng total cDNA as the template. Calculate the template volume to be added and supplement with the corresponding volume of nuclease-free water to complete the system configuration.

***Note**: For example, if the cDNA concentration is 5 ng/μl, the volume of the library template for 50 ng would be 50 ng divided by 5 ng/μl, which equals 10 μL.*

Component	Volume/Sample
cDNA enrichment products	10 μL
Nuclease-free Water	25 μL
Fragmentation Buffer	5 μL
Total	40 μL

3. Add 10 μL of Fragmentation Enzyme to each reaction system, pipette up and down 15 times on ice, and centrifuge instantaneously;

4. Immediately place the mixed reaction reagent into the running thermal cycler and click "Next" to continue running the PCR program.

Step 3-2 Fragment sorting

1. Immediately after the reaction, centrifuge briefly and add 30 μL of DNA selection magnetic beads (0.6×) to the mixture. Mix by pipetting up and down 10 times or by gentle shaking, then centrifuge briefly.

***Note**: 0.6× refers to the volume ratio of the added DNA selection magnetic beads to the PCR product, i.e., 30 μL / 50 μL = 0.6×.*

2. Allow the mixture to stand at room temperature for 5 minutes, then place it on a magnetic stand until the solution becomes clear. Transfer the supernatant to another PCR tube containing 10 μL of DNA selection magnetic beads (0.8×). Mix by pipetting up and down 10 times.

***Note 1**: When performing the magnetic bead adsorption, pipette the mixture gently in a reverse direction against the surface of the magnetic beads, perform light aspiration and mixing five times to enhance magnetic adsorption.*

***Note 2**: 0.8× refers to the volume ratio of the total added DNA selection magnetic beads to the PCR product, i.e., (30 μL from the first addition + 10 μL from the second addition) / 50 μL = 0.8×.*

3. Allow the well-mixed product to stand at room temperature for 5 minutes, then place it on a magnetic stand until the solution becomes clear. Remove the supernatant.

***Note:** When performing the magnetic bead adsorption, pipette the mixture gently in a reverse direction against the surface of the magnetic beads, perform light aspiration and mixing five times to enhance magnetic adsorption.*

4. Add 200 μL of 80% ethanol (keep placed on a magnetic rack) and carefully remove the supernatant after 30 sec; **Repeat this step once;**

5. Centrifuge the PCR tube briefly, use a 10 μL pipette to remove any remaining ethanol supernatant. During centrifugation, ensure that the magnetic beads face outward to prevent the beads from being flung onto the tube walls.

6. Let them stand at room temperature for 3-5 min to completely evaporate the ethanol (the magnetic beads appear dull and no crack);

7. Add 50.5 μL of Nuclease-free Water to fully resuspend the beads and let stand for 2 min at room temperature;

8. Place the tube on a magnetic rack until the solution becomes clear, then transfer 50 μL of the supernatant to a new 0.2 mL PCR tube.

***Note**: When performing the magnetic bead adsorption, pipette the mixture gently in a reverse direction against the surface of the magnetic beads, perform light aspiration and mixing five times to enhance magnetic adsorption.*

Step 3-3 Adaptor ligation

1. Prepare reaction system according to the following table, fully vortexn and mixed, and briefly centrifuge;

Component	Volume/Sample
Ligation Buffer	25 μL
DNA Ligase	5 μL
Adaptor	5 μL
Nuclease-free Water	15 μL
Total	50 μL

2. Add 50 μL of the reaction system to the fragmentation product, mix 15 times with a pipette, and briefly centrifuge;

3. Set up the PCR program according to the table below and perform the reaction with a thermal cycler hot lid at 30 °C and a volume of 100 μL;

Steps	Temperature	Time
1	20℃	15 min
2	4℃	Hold

Step 3-4 Ligation product purification

1. After the end of the reaction, centrifuge instantaneously and add 80 μL of DNA sorting beads (0.8×), mix 10 times with a pipette or mix with shaking and centrifuge instantaneously;

***Note**: 0.8× refers to the volume ratio of added DNA selection beads to PCR products, i.e., 80 μL / 100 μL = 0.8×.*

2. After incubating at room temperature for 10 min, place the tube on a magnetic rack to allow the solution to clarify. Remove the supernatant;

   ***Note:** During adsorption, use a pipette tip to gently mix by pipetting up and down against the surface of the adsorption beads, repeating this process 5 times to enhance magnetic adsorption.*

3. Add 200 μL of 80% ethanol (keep on magnetic rack) for approximately 30s. Carefully remove the supernatant; Repeat this step once;
4. Briefly centrifuge the PCR tube to remove all residual supernatant with a 10 μL pipette;
5. Let it stand at room temperature for 3-5 min to completely volatilize the ethanol (the magnetic beads appear dull and no crack);
6. Add 23.5 μL of Nuclease-free Water to fully resuspend the beads and let it stand for 2 min at room temperature;
7. Place on a magnetic rack to adsorb until the solution appears clear, transfer the supernatant 23 μL to a new 0.2 mL PCR tube.

   ***Note:** During adsorption, use a pipette tip to gently mix by pipetting up and down against the surface of the adsorption beads, repeating this process 5 times to enhance magnetic adsorption.*

Step 3-5 Library amplification

1. Prepare reaction system according to the following table, fully vortex and mixed, and centrifuged instantaneously;

Component	Volume/Sample
2×PCR Master Mix	25 μL
N5	1 μL
N7	1 μL
Total	27 μL

   ***Note:** If there are multiple samples, it is recommended to purchase the 96-well plate index kit. This kit offers a wider variety of indices and consists of I5 and I7 dual-indexes in each well. It is suitable for high-throughput sequencing strategies with large sample volumes and lane pooling. After thorough mixing, directly pipette 2 μL of the well-mixed index into the amplification system.*

2. Add 27 μL of the reaction system to the ligated purification product, mix 15 times with a pipette, and briefly centrifuge.
3. Set up the PCR program with hot lid at 105 °C according to the following conditions and perform the reaction.

Number of	Temperature	Time
	98℃	3 min
6-16	98℃	20 sec
	54℃	30 sec
(See table below)	72℃	20 sec
	72℃	5 min
	4℃	Hold

cDNA input amount	Recommended number of amplification
1-25 ng	14-16
25-150 ng	12-14
150-500 ng	10-12
500-1,000 ng	8-10
1,000-1,500 ng	6-8

Step 3-6 Fragment sorting

1. After the reaction is completed, centrifuge briefly to ensure proper settling. Add 25 μL (0.5×) DNA selection magnetic beads and pipette up and down 10 times or mix by gentle shaking. Centrifuge briefly again to facilitate bead separation.

   ***Note**: 0.5× refers to the volume ratio of added DNA selection beads to PCR products, i.e., 25 μL / 50 μL = 0.5×.*

2. After incubating at room temperature for 5 minutes, place the tube on a magnetic rack to allow the solution to clarify. Transfer the supernatant to another PCR tube containing 15 μL (0.8×) DNA selection magnetic beads. Pipette up and down 10 times to mix thoroughly.

   ***Note:** 0.8× refers to the volume ratio of added DNA selection beads to PCR products, i.e., (first time 25 μL + second time 15 μL) / 50 μL = 0.8×.*

3. After incubating at room temperature for 10 min, place the tube on a magnetic rack to allow the solution to clarify. Remove the supernatant.

   ***Note:** During adsorption, use a pipette tip to gently mix by pipetting up and down against the surface of the adsorption beads, repeating this process 5 times to enhance magnetic adsorption.*

4. Add 200 μL of 80% ethanol (keep on magnetic rack) for approximately 30 s. Carefully remove the supernatant; Repeat this step once;

5. Centrifuge the PCR tube briefly and use a 10 μL pipette to remove all remaining supernatant.;

6. Let stand at room temperature for 3-5 min to completely volatilize the ethanol (the magnetic beads appear dull);

7. Add 30.5 μL of Nuclease-free Water to fully resuspend the beads and let stand for 2 min at room temperature;
8. Place the tube on a magnetic rack to allow the magnetic beads to attract and clarify the solution. Transfer 30 μL of the supernatant to a new 0.2 mL PCR tube.

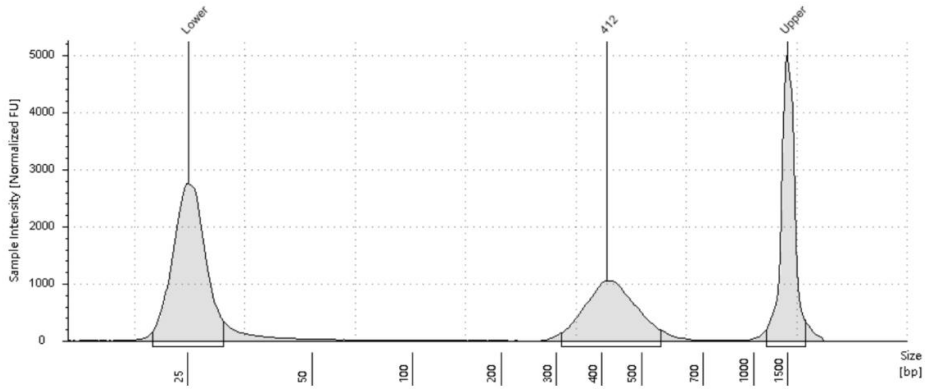
***Note 1**: During adsorption, use a pipette tip to gently mix by pipetting up and down against the surface of the adsorption beads, repeating this process 5 times to enhance magnetic adsorption.*

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

***STOPPING POINT**: Library products can be stored at -20 °C for no more than 6 months after purification.*

Step 3-7 Library quality control

1. The qualified library has a main peak fragment size ranging from 350-750 bp, with no small fragments present. If small fragments are present, perform an additional 0.75x purification until no small fragments are present (Agilent 4200 TapeStation).



2. Qubit 4.0 measures library concentration.

Library Quality Control Standards:

(1) Library concentration ≥ 5 ng/μL, with product fragment size ranging from 250 bp to 1500 bp. No contamination by small fragments is allowed, and the main peak should fall within the range of 350 bp to 750 bp for qualification.

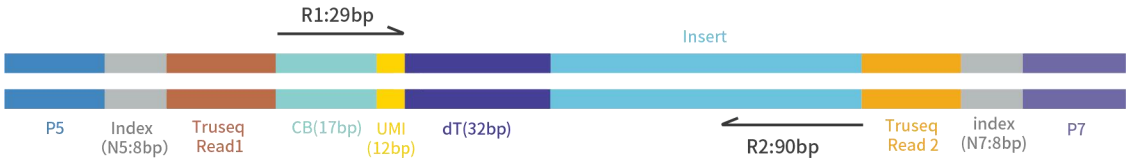
(2) Library concentration ranging from 1 ng/μL to < 5 ng/μL, with product fragment size ranging from 250 bp to 1500 bp. No contamination by small fragments is allowed, and the main peak should fall within the range of 350 bp to 750 bp. This is considered a risk for sequencing.

(3) Library concentration ≥ 5 ng/μL, with product fragment size ranging from 250 bp to 1500 bp. The main peak should fall within the range of 350 bp to 750 bp, but there is contamination by small fragments. The height of the small fragments should be lower than that of the target fragments. This is considered a risk for sequencing.

(4) Library concentration < 1 ng/μL, or the product fragment analysis shows no target fragments in the 250-1500 bp range, no prominent main peak, or the height of small fragments is higher than that of the target fragments. This is considered as not meeting the quality standards.

Step 4 High-throughput sequencing

1. Sequencing Library: SeekOne® DD Single-Cell 3' 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, and MGI sequencing platforms (MGI platform sequencing requires circularization).

- GeneMind sequencing platforms: SURFSeq 5000

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

- MGI platform: DNBSEQ-T7RS

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 sequencing

- Read Length: Read1: 29 bp, N7 Index: 8 bp, N5 Index: 8 bp, Read2: 90 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 for paired-end sequencing; Read1 should be at least 29 bp to capture complete Cell Barcode and UMI sequences, and Read2 should be at least 90 bp for downstream single-cell transcriptome data analysis.*

4. Library Loading Concentration:

Platform	Instrument	Loading concentration(pM)	PhiX(%)
GeneMind	SURFSeq 5000	200	30
Illumina	MiSeq	11	1
	NextSeq 500/550	1.8	1
	HiSeq 2500(RR)	11	1
	HiSeq 4000	240	1
	NovaSeq	150*/300	1
MGI	DNBSEQ-T7RS	1 (circularization)	5

***Note**: Use a loading concentration of 150 pM for Illumina XP workflow.*

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 split for subsequent data analysis.

6. Bioinformatics Analysis:

Analysis Software: Single-cell data analysis employs SeekSoul® Tools (http://seeksoul.seekgene.com/en/v1.2.0/index.html), developed independently by Beijing SeekGene Bioinformatics. SeekSoul® Tools can identify cell barcode labels, perform alignment quantification, and generate downstream analysis of cell expression matrices for subsequent cell clustering and differential analysis.

Input Files: FASTQ

Output Files: BAM, HTML, CSV, matrices filtered_feature_bc_matrix, raw_feature_bc_matrix

Operating System: Linux

【Manufacturer/After-sales service unit】

Manufacturer: Beijing SeekGene BioSciences Co.,Ltd

Address: Room 201, Floor 2, Tower A Building 9, Zone 1, 8 Life Science Parkway, Changping District, Beijing, China

Zip code: 102206

Tel: +86- (0) 10 56918048

【Information on EU representatives】

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EU Address: Mies-van-der-Rohe-Strasse 8,80807 Munich, Germany

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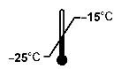
Tel: +49 (0) 89 189174474

Fax: +49 (0) 89 5485 8884

Email: info@medpath.pro

【Explanations for Symbols】

	Manufacture		European union representative
	In vitro diagnostic medical device		Use-by date
	Batch code		Catalogue number
	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
	Store at -25~-15℃		CE mark
	Store at 2~8℃		Store at -80℃

【Edition】

V2.2

【Revised date】

2023/11/3

Appendix 1：SeekOne® Digital Droplet System User Manual

Refer to《SeekOne® Digital Droplet System User Manual》

Appendix 2：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
Controller Conmmunication Failure	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 (support@seekgene.com) for further assistance.
Tray Exit Failure	The operation in and out of the warehouse may be blocked. Please confirm that there are no objects 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 (support@seekgene.com) for further assistance.
Tray Entry Failure	The operation in and out of the warehouse may be blocked. Please confirm that there are no objects 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 (support@seekgene.com) for further assistance.
Lifting Failure	Please try again or restart the operation. If the message appears repeatedly, please contact (support@seekgene.com) for further assistance.
Plate Setting Down Failure	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 (support@seekgene.com) for further assistance.
Temperature Regulation Failure	Restart the device, if it recurs, contact (support@seekgene.com) for further assistance.

Error Detected: Row 1 Flow	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 (support@seekgene.com) for further assistance.
Error Detected: Row 1 Pressure	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 (support@seekgene.com) for further assistance.