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UDA-5'RNA-protocol V.2

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We use this protocol and it's working

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Abstract

Droplet microfluidics-based single-cell combinatorial indexing sequencing represents an attractive way to balance cost, scalability,
robustness, and accessibility. However, current methods need a tailored protocol for specific modality respectively, which may limit their potential for automation. We introduce UDA-seq, universal droplet microfluidics-based combinatorial indexing for massive-scale single-cell multimodal sequencing. We demonstrate that when necessary, UDA-seq enables effectively generating more than 100,000 single-cell data in a single-channel experiment of droplet microfluidics. Meanwhile, UDA-seq provides a universal workflow for
accomplishing several multimodal tasks, including single-cell co-assay of RNA and VDJ, RNA and ATAC, and RNA and CRISPR guide RNA.

Materials

Reagent

Nuclease-free Water(Ambion #AM9932)
DPBS(GIBCO #14190144)
BSA(sigma #A1933-25G)
RNasin Plus RNase Inhibitor(promega #N2615)
SUPERase•In RNase Inhibitor(Invitrogen #AM2694)
methanol (Fisher Scientific #M/4000/17)
Chromium Next GEM Single Cell 5’ GEM Kit v2(10X genomics #1000244)
Single Cell VDJ 5' Gel Bead (i10x Genomics #1000264/1000267)
Dynabeads MyOne SILANE(10x Genomics #2000048)
Chromium Next GEM Chip K Single Cell Kit(10x Genomics #1000286)
Buffer EB(Qiagen #19086)
10% Tween 20(yuanye Bio #R21998)
KAPA HiFi HotStart 2X ReadyMix(Kapa #KK2602)
Trueprep tagment enzyme(vazyme #S601-01)
5x Reaction Buffer (vazyme L buffer)
Agencourt AMPure XP SPRI beads(Beckman Coulter #A63881)
NEBNex High-Fidelity 2X PCR Master Mix (NEB#M0541S)
Amp Mix (10x Genomics #2000047/2000103)

Plastics

0.22um filter(Millipore #SLGPR33RS)
Syringes(BD #301947)
Pre-Separation Filters, 20 μm(Miltenyi #130-101-812)
Flowmi Cell Strainer, 40 μm(Bel-Art H13680-0040)
PCR Tubes, 0.2 mL(Eppendorf #0030124359)
DNA LoBind Tubes, 2.0 mL(Eppendorf #0030108078)
PCR Plates LoBind(Eppendorf #30129504)
DNA LoBind tube, 1.5ml(Eppendorf #30108051)
DNA LoBind tube, 5ml(Eppendorf #0030108310)
Conical Sterile Polypropylene Centrifuge Tubes,15mL(Nunc #339650)
Conical Sterile Polypropylene Centrifuge Tubes,50mL(Nunc #339652)
10 μL Microvolume（Axygen #TF-300-R-S)
10 μL Maxymum Recovery（Axygen #TF-400-L-R-S)
100 μL Microvolume（Axygen #TF-100-R-S)
200 μL Microvolume（Axygen #TF-200-R-S)
1000 μL Microvolume（Axygen #TF-1000-R-S)

Equipment

VORTEX-6(kylinbell)
10x Magnetic Separator(10x Genomics #120250)
Chromium Controller(10X genomics)
C1000 Touch Thermal Cycler(bio-rad #1851197)

Fluorescence Cell Counter(LUNA #LUNA-FL)
Micro-plate Centrifuge(#SB-YH0461)
Centrifuge(Thermo Fisher Scientific #Micro21R)

GEM Generation & Barcoding. 45m

1 Prepare Master Mix & Load Chromium Next GEM Chip K

1.1 A certain number of cells or nuclei were added to Master Mix (18.8ul RT Reagent B (2000165); 7.3ul 1.1ul Template Switch Oligo (3000228); 1.9ul Reducing Agent B (2000087) and 8.3ul RT Enzyme C(2000085/2000102) each sample).

1.2 The microfluidic Chromium Next GEM Chip K (2000182) was loaded with 70 µl of cells or nuclei in thermoligation mix (inlet 1), 50 µl of Single Cell VDJ 5' Gel Bead (inlet 2, 10x Genomics catalog no. 1000264/1000267) and 45 µl of Partitioning Oil (inlet 3, 10x Genomics catalog no. 2000190) and run on the Chromium system.

1.3 The PCR mix was incubate in a thermomixer to perform enrichment PCR as follows: 53˚C for 45min, 4˚C hold.

GEM clean-up 2h

2 Cleanup – Dynabeads

2.1 Add 125 µl Recovery Agent to each sample (post GEM-RT incubation) at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 2 min.

2.2 Slowly remove and discard 125 µl Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.

2.3 Add 150ul PBS to the remaining aqueous phase, mix well then dispense the liquid evenly into 96-well plate, each well add 2ul.
After brief centrifugation, the products can be stored at −80 °C for at least 2 weeks.

2.4 Incubate 85˚C for 5min.

2.5 Add 6ul EB.

2.6 Pure the samples with 16ul Dynabeads Cleanup Mix(40ul Nuclease-free Water; 1465ul Cleanup Buffer(2000088); 64ul Dynabeads MyOne SILANE(2000048) and 40ul Reducing Agent B(2000087), vortex for 15 sec to mix thoroughly.

2.7 Incubate 10 min at room temperature.

2.8 Prepare Elution Solution I(1960ul Buffer EB; 20ul 10% Tween 20 and 20ul Reducing Agent B(2000087)). Vortex and centrifuge briefly.

2.9 At the end of 10 min incubation, place on a on a 96-well plate magnet until the solution clears.

2.10 Remove the supernatant.

2.11 Add 300 µl 80% ethanol to the pellet while on the magnet. Wait 30 sec.

2.12 Remove the ethanol.

2.13 Add 200 µl 80% ethanol to pellet. Wait 30 sec.

2.14 Remove the ethanol.

2.15 Centrifuge briefly. Remove remaining ethanol. Air dry for 2 min.

2.16 Remove from the magnet. Immediately add 17.5 µl Elution Solution I.

2.17 Vortex for 15 sec to mix thoroughly.
If beads still appear clumpy, continue pipette mixing until fully resuspended.

2.18 Incubate 1 min at room temperature.

2.19 Place on the magnet•Low until the solution clears.

2.20 Transfer 17 µl sample to a Pre-Amplification PCR.

Pre-Amplification PCR

2h

3 cDNA index amplification

3.1 Prepare linear amplification mix(2X KAPA HiFi HotStart Ready Mix; 0.5uM Truseq-i5 index primer; 0.5uM Partial TSO/IS and 0.5uM P5 primer).

3.2 Pipette 10X to mix thoroughly. Centrifuge briefly.

3.3 PCR mix was incubate in a thermomixer to perform enrichment PCR as follows: 98˚C for 45s , and then 14 cycles of [98˚C for 20s, 63 °C for 30s, 72˚C for 1min]; 72˚C for 1min.

3.4 cDNA PCR product were purified with 0.6x XP beads and elute in 300 ul EB.

3.5 Purified with 0.7x XP beads and elute in 55 ul EB.

5′ Gene Expression (GEX) Library Construction

1h 30m

4 Fragmentation

4.1 50 ng mass of cDNA products (35ul) were mixed with 15 µl of i7-only TN5 Tagmentation Mix(10ul 5x Reaction Buffer (vazyme L buffer) and 5ul Self-i7 TN5).

4.2 Pipette mix 15x (pipette set to 30 µl) on ice. Centrifuge briefly.

4.3 Incubate in a thermal cycler with the following protocol. 55 ˚C for 10min.

4.4 PCR product were purified with 0.8x XP beads and elute in 40.5 ul EB.

5 GEX Sample Index PCR

5.1 Prepare and add Sample Index PCR Mix(NEBNex High-Fidelity 2X PCR Master Mix (NEB#M0541S); 0.5uM Partial P5 and 0.5uM Nextera P7-index).

5.2 Pipette mix and centrifuge briefly.

5.3 Incubate in a thermal cycler with the following protocol. 72 ˚C for 5 min, 98 ˚C for 45 s, 8-9 cycles of [98 ˚C for 20 s, 60 ˚C for 30 s, 72 ˚C for 1min], 72 ˚C for 5 min in thermocycler, storage at 4 ˚C.

5.4 PCR product were selected size with 0.6-0.8x XP beads and elute in 25.5 ul EB.

VDJ Capture

2h

6 VDJ capture 1

6.1 Place a tube strip on ice and transfer 5 µl cDNA product.

6.2 Prepare V(D)J Amplification 1 Reaction Mix(Amp Mix (2000047/2000103); 0.5uM Partial P5 and 0.5uM T/B VDJ outer primer in nuclease-free water) on ice. Vortex and centrifuge briefly.

6.3 Add 75 µl V(D)J Amplification 1 Reaction Mix to each tube containing 5 µl sample.

6.4 Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

6.5 Incubate in a thermal cycler with the following protocol. 98 ˚C for 45 s, 10 cycles for T cells /8 cycles for B cells of [98 ˚C for 20 s, 62 ˚C for 30 s, 72 ˚C for 1min], 72 ˚C for 1 min in thermocycler, storage at 4 ˚C.

6.6 Store at 4°C for up to 72 h or proceed to the next step.

6.7 PCR product were selected size with 0.5X-0.8x XP beads and elute in 30.5 ul EB.

7 VDJ capture 2

7.1 Prepare and add 50ul V(D)J Amplification 2 Reaction Mix(Amp Mix (2000047/2000103); 0.5uM Partial P5 and 0.5uM T/B VDJ inner primer) .

7.2 Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

7.3 Incubate in a thermal cycler with the following protocol. 98 ˚C for 45 s, 8 cycles for T cells /8 cycles for B cells of [98 ˚C for 20 s, 62 ˚C for 30 s, 72 ˚C for 1min], 72 ˚C for 1 min in thermocycler, storage at 4 ˚C.

7.4 Store at 4°C for up to 72 h or proceed to the next step.

7.5 PCR product were selected size with 0.5X-0.8x XP beads and elute in 30.5 ul EB.

VDJ (GEX) LibraryConstruction

1h 30m

8 Fragmentation

8.1 50 ng mass of VDJ capture products (35ul) were mixed with 15 µl of i7-only TN5 Tagmentation Mix(10ul 5x Reaction Buffer (vazyme L buffer) and 5ul Self-i7 TN5).

8.2 Pipette mix 15x (pipette set to 30 µl) on ice. Centrifuge briefly.

8.3 Incubate in a thermal cycler with the following protocol. 55 ˚C for 5min.

8.4 PCR product were purified with 0.8x XP beads and elute in 40.5 ul EB.

9 GEX Sample Index PCR

9.1 Prepare and add Sample Index PCR Mix(NEBNex High-Fidelity 2X PCR Master Mix (NEB#M0541S); 0.5uM Partial P5 and 0.5uM Nextera P7-index).

9.2 Pipette mix and centrifuge briefly.

9.3 Incubate in a thermal cycler with the following protocol. 72 ˚C for 5 min, 98 ˚C for 45 s, 8 cycles of [98 ˚C for 20 s, 60 ˚C for 30 s, 72 ˚C for 1min], 72 ˚C for 5 min in thermocycler, storage at 4 ˚C.

9.4 PCR product were selected size with 0.8x XP beads and elute in 25.5 ul EB.