uCoTargetX

Single-cell version-2025

Reagents

- STE buffer: 10 mM Tris-HCl pH 8.0, 50 mM NaCl, and 1mM EDTA
- 0.1% (m/v) BSA-PBS
- 36.5% formaldehyde (sigma)
- 2.5 M Glycine (do not adjust pH)
- 100% methanol (pre-chill methanol at -20°C before use)
- Wash buffer: 1 ml 1 M HEPES pH 7.5, 1.5 ml 5 M NaCl, 12.5 µl 2 M spermidine, 10 mM sodium butyrate, and the final volume to 50 ml with ddH_2O.
- Wash-buffer-Dig: Wash buffer adding 0.01% Digitonin.
- Wash-buffer-TX: Wash buffer adding 0.01% Digitonin, 0.05% TX-100
- Wash-buffer-TX-high salt: Wash buffer adding 0.01% Digitonin, 0.05% TX-100 and final 300 mM NaCl
- Reaction buffer: 10 mM TAPS pH 8.3, 10 mM $MgCl_2$, 10 mM sodium butyrate, 0.01% Digitonin.
- NSB (nuclei suspension buffer) buffer: 10mM Tris-HCl, pH 7.5, 10mM NaCl, 3mM MgCl2, 0.01% TX-100
- Lysis buffer: 10 mM Tris-HCl pH 8.5, 0.05% SDS and 0.1 mg/ml Proteinase K.
- 10×T4 ligation buffer (NEB)
- 400 U/µl T4 Ligase (NEB)
- 10 mM PMSF (Sigma): dissolve PSMF powder to 100 mM with isopropanol. Dilute 100 mM PMSF to 10 mM with ddH_2O before use.

Preparing oligonucleotides for ligations

1. Dissolve the round 1 linker with STE buffer to 90 µM. Dissolve the round 1 barcodes with STE buffer to 100 µM. Dissolve the round 2 linker with STE buffer to 110 µM. Dissolve the round 2 barcodes with STE buffer to 120 µM.

2. Mix 20 µl round 1 linker and 20 µl round 1 barcodes and place them at a thermal cycler for 5 min at 95°C followed by programmed temperature decrease at 0.1°C/s to 25°C. The resulting round 2 hybridization adaptor concentration is 45 µM linker and 50 µM barcodes. Store the annealed round 1 hybridization adaptors at 4°C before use.

3. Mix 20 µl round 2 linker and 20 µl round 2 barcodes and place them at a thermal cycler for 5 min at 95°C followed by programmed temperature decrease at 0.1°C/s to 25°C. The resulting round 2 hybridization adaptor concentration is 55 µM linker and 60 µM barcodes. Store the annealed round 2 hybridization adaptors at 4°C before use.

4. Dilute the hybridization adaptors to working solution: take 2 µl annealed round 1 or 2 hybridization adaptor to 38 µl STE buffer to make a 40 µl mix (20-fold dilution). The working solution is suggested to be used within 1 week.

Samples fixation

1. Harvest cells and wash cells once with 0.1% BSA-PBS.

2. Resuspend cells with 1 ml cold 0.1% BSA-PBS and add 7 μl 36.5% formaldehyde to the system (working solution of formaldehyde is 0.25%), quickly turn upside down to mix well. Sit the system on ice for 5 minutes for fixation. Other fixation conditions are also available and practical, such as 0.1%-0.25% FA at room-temperature for 3 min, 1%-4% PFA on ice 3 min, methanol only, etc.

3. Add 14 μl 2.5 M Glycine to the system, quickly turn upside down to mix well. Sit the system on ice for another 5 minutes to quench free formaldehyde.

4. Wash cells twice with 0.1% BSA-PBS.

5. Resuspend cells with 100 μl 0.1% BSA-PBS and add 900 μl pre-chilled -20°C methanol dropwise to the system. The fixed samples can be stored at -80°C for one year.

Antibody-PAT-T7 complex assembly

1. Set up the following assembly system at minimal volume. Incubate the system at room temperature for 1 hour.

Antibody------0.5 μg (3.33 pmol)

37.5 μM PAT-T7------0.22 μl (8.25 pmol)

Wash buffer------5 μl

2. The Antibody-PAT-T7 complex is suggested to be used in uCoTarget experiments within 24 hours of assembly.

Antibody-tethered tagmentation

1. Wash cells twice with 0.1% BSA-PBS.

2. Incubate cells with 1st kind of Antibody-PAT-T7 complex in 100 μl Wash-buffer-TX-high salt plus 2 mM EDTA at room temperature for 1 hour.

3. Wash cells 3 times with Wash-buffer-TX-high salt to remove free unbound complex.

4. Resuspend cells with 50 μl Reaction buffer and incubate the reaction at 37°C for 1 hour at a thermal cycler. Hot lid is set 40°C.

5. Centrifuge the cells and remove the Reaction buffer. Resuspend cells with 180 μl Wash-buffer-TX-high salt with 5 mM EDTA and rotate at room temperature for 5 minutes.

6. Repeat the above 2-5 steps for the other kinds of Antibody-PAT-T7 complex.

Free PAT tagmentation

1. Incubate cells with secondary antibodies corresponding to the species in which the primary antibody conjugates at 1:1000 dilution factor in 100 μl Wash-buffer-TX at 4°C for 30 minutes.

2. Wash cells 3 times with Wash-buffer-Dig.

3. Distribute cells to 8 wells with different PAT-T5 (dilute 37.5 μM PAT-T5 at 450 fold in the system) in each well in 100 μl Wash-buffer-TX-high salt. Incubate the system at 4°C for 1 hour.

4. Wash cells twice with Wash-buffer-TX-high salt.
5. Resuspend cells with 50 μl reaction buffer and incubate the reaction at 30°C for 1 hour at a thermal cycler. Hot lid is set 40°C.
6. Remove the reaction buffer by centrifugation. Resuspend cells with 180 μl Wash-buffer-TX-high salt with 5 mM EDTA and rotate at room temperature for 5 minutes.

Reverse transcription

1. Wash cells twice with 0.1% BSA-PBS for rehydration.
2. Resuspend cells with 50 μl reverse transcription mix with different RT barcode. Incubate the system at 50°C for 10 minutes then 3 cycles of 8 °C for 12 s, 15 °C for 45 s, 20 °C for 45 s, 30 °C for 30 s, 42 °C for 2 min and 50 °C for 3 min followed by a final step at 50 °C for 5 min.
1 M Tris-HCl pH=8.3------1.25 μl (final concentration=25 mM)
5 M NaCl------0.3 μl (final concentration=30 mM)
10 mM GTP------2.5 μl (final concentration=0.5 mM)
1 M $MgCl_2$------0.125 μl (final concentration=2.5 mM)
0.1 M DTT------4 μl (final concentration=8 mM)
10 mM dNTPs------2.5 μl (final concentration=0.5 mM)
200 U/μl Maxima H minus------2.5 μl (final concentration=10 U/μl)
Takara RNase inhibitor------0.5 μl
5 μM RT barcode------5 μl (final concentration=0.5 μM)
100 μM TSO------1 μl (final concentration=2 μM)
ddH_2O------to 50 μl
3. After reverse transcription, combine all cells and wash cells twice with NSB.

2 round Ligation

1. Resuspend cells with ~1 ml NSB and distribute cells to 96 wells at 10 μl each well. The 96 wells have contained 40 μl 1st round ligation mix. Incubate the system at room temperature for 30 minutes with gentle shaking (300 rpm).
10×T4 ligation buffer------5 μl
10% TX-100------0.2 μl
NSB------10 μl
ddH_2O------22.8 μl
Round 1 hybridization adaptor------2 μl
cells in NSB buffer------10 μl
2. Add the following 10 μl round 1 blocking mix to each well. Incubate the system at room temperature for 30 minutes with gentle shaking (300 rpm).
100 μM round 1 blocking------0.11 μl
10×T4 ligation buffer------2 μl
ddH_2O------7.89 μl
3. Combine all wells and distribute cells to a new plate of 96 wells at 50 μl each well. The new 96 wells have contained the following 10 μl round 2 hybridization mix in each well. Incubate

the system at room temperature for 30 minutes with gentle shaking (300 rpm).
Round 2 hybridization adaptor------2 μl
ddH_2O------8 μl
4. Add the following 10 μl round 2 blocking mix to each well. Incubate the system at room temperature for 30 minutes with gentle shaking (300 rpm).
100 μM round 2 blocking------0.132 μl
1% TX-100------1 μl
ddH_2O------8.868 μl
5. Combine all wells and wash cells twice with NSB. Resuspend cells with the following 200 μl final ligation system. Incubate the system at room temperature for 30 minutes with gentle shaking (300 rpm).
10×T4 ligation buffer------20 μl
400 U/μl T4 Ligase------10 μl
1% TX-100------10 μl
NSB------40 μl
ddH2O------120 μl
• Note: final ligation system can be scaled down to 20 μl for each sample in pilot experiments.
6. Wash cells twice with NSB. Count cells density and dilute cells to 2,000-5,000/μl with 0.1% BSA-PBS.
7. Aliquot cells to as much as possible wells at 1 μl/well. The wells have contained 4 μl lysis buffer. Incubate the mix at 55°C for 30 minutes to lyse cells.
8. Add 1 μl 1.8% TX-100 and 1 μl 10 mM PMSF to each well and incubate the system at 37°C for 15 minutes to quench SDS and deactivate proteinase K.

Pre-amplification

1. Prepare 1st round PCR mix (50 μl) as follows. Perform PCR as: 72°C 5 min; 95°C 2 min; 6 cycles of 98°C 20s, 65°C 30s, 72°C 2 min 30 s; 72°C 5 min.
cell lysate------7 μl
5xHiFi buffer------10 μl
10 mM dNTP------1 μl
10 μM Biotin-IS primer------2 μl
Truseq-i5-connector (10 μM) ------4 μl
Truseq-i7-connector (10 μM) ------2 μl
25 mM $MgCl_2$------1 μl
KAPA enzyme------0.5 μl
ddH_2O------to 50 μl
2. Purify the PCR product with 0.9× AMPure XP beads and eluted in 25 μl ddH2O.
3. Prepare C1 streptavidin beads: Pipette 1 μl original C1 beads to a new tube, wash beads twice with 2X B&W buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA and 0.05% Tween 20), and resuspend the beads with 25 μl 2X B&W buffer. Add 25 μl prepared C1 beads to the 25 μl pre-amplified DNA/RNA products and incubate at room temperature for 30 min with

rotation.

4. Briefly spin the PCR tube and put onto a magnetic stand. Carefully transfer the liquid to a new PCR tube for DNA part library preparation. From now on, the beads will be processed as RNA part library preparation.

DNA-part library preparation

1. Purify the DNA part liquid with 0.45× + 0.45× AMPure beads for size selection and eluted in 10 μl ddH2O.

2. Prepare index PCR mix as follows. Perform PCR as: 95°C 2 min; 9 cycles of 98°C 20s, 65°C 30s, 72°C 1 min; 72°C 5 min.

5xHiFi buffer------10 μl
10 mM dNTP------1 μl
Truseq P5 (25 μM) ------1 μl
Truseq P7(25 μM) ------1 μl
25 mM MgCl2------1 μl
KAPA enzyme------0.5 μl
ddH2O------to 50 μl

3. Purify the PCR product with 0.9× AMPure XP beads followed by 0.45× + 0.45× for size selection. Elute the final library product in 20 μl ddH_2O.

4. Measure the library concentration with Qubit.

5. Libraries can be sequenced with the standard recipe on the Nova-seq platform (Illumina).

RNA-part library preparation

1. Briefly wash C1 beads (bound with cDNA) by 100 μl Wash Buffer (+0.05% TX-100) twice, and pull off all the liquid. Mix 50 μL PCR mix with beads, transfer the sample to the PCR tube. Perform PCR as: 72°C 5 min; 95°C 2 min; 9 cycles of 98°C 20s, 65°C 30s, 72°C 2 min 30 s; 72°C 5 min.

5xHiFi buffer------10 μl
10 mM dNTP------1 μl
10 μM IS primer (no need biotin) ------2.5 μl
Truseq-i5-connector (10 μM) ------2.5 μl
25 mM $MgCl_2$------1 μl
KAPA enzyme------0.5 μl
ddH_2O------to 50 μl

2. Purify each sample with 0.9×AmPure XP beads. Elute cDNA to 16 μl ddH2O.

3. Quantify cDNA concentration by Qubit using 1 μl cDNA. Take 50 ng for MEB-PAT tagmentation.

cDNA (50 ng) ------x μl
MEB-PAT------1 μl
5 ×TAPS-$MgCl_2$-DMF------1.5 μl
ddH_2O------to 7.5 μl

4. Prepare index PCR mix as follows. Perform PCR as: 95°C 2 min; 7 cycles of 98°C 20s, 65°C 30s, 72°C 1 min; 72°C 5 min.

Tagmented cDNA------7.5 μl
5xHiFi buffer------10 μl
10 mM dNTP------1 μl
Truseq P5 (25 μM) ------1 μl
Nextera i7(25 μM) ------1 μl
25 mM $MgCl_2$------1 μl
KAPA enzyme------0.5 μl
ddH_2O------to 50 μl

5. Purify the PCR product with 0.9× AMPure XP beads. Elute the final library product in 20 μl ddH_2O.

6. Measure the library concentration with Qubit.

7. Libraries can be sequenced with the standard recipe on the Nova-seq platform (Illumina).

Table 1. The sequences of primers (RNA part) used in uCoTargetX experiments (Xiong et al., Science Advances, 2024).

Name	Sequence	Modification	Notes
TSO	AAGCAGTGGTATCAACGCAGAGTACATG(r)G(r)G(+)	G(r):RNA; G(+):XNA (LNA)	reverse transcription
RT_1-oligodT25VN	TCGTCGGCAGCGTCTAAAGAADDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_2-oligodT25VN	TCGTCGGCAGCGTCTAACAGCDDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_3-oligodT25VN	TCGTCGGCAGCGTCTCAACCGDDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_4-oligodT25VN	TCGTCGGCAGCGTCTCAAGTCDDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_5-oligodT25VN	TCGTCGGCAGCGTCTGAAATADDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_6-oligodT25VN	TCGTCGGCAGCGTCTGAAGGGDDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_7-oligodT25VN	TCGTCGGCAGCGTCTACTGCADDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
RT_8-oligodT25VN	TCGTCGGCAGCGTCTCGTTATDDDDDDDDTTTTTTTTTTTTTTTTTTTTTTTTTVN	5'P	
biotin-IS	AAGCAGTGGTATCAACGCAGAGTACAT	5'biotin	pre-amplification
Truseq-i5-connector	ACACTCTTTCCCTACACGACGCTCTTCCGATCT		
Truseq-i7-connector	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCTCGTGGGCTCGGCTGTCCC		
IS	AAGCAGTGGTATCAACGCAGAGTACAT		re-amplification