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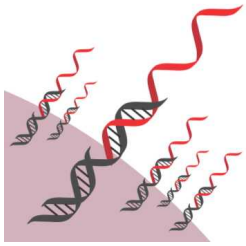
MULTI-seq sample multiplexing for 10x Genomics RNA+ATAC multiomic profiling



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We use this protocol and it's working

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Abstract

This protocol describes the application of the MULTI-seq sample multiplexing technology (McGinnis, Patterson, et al., 2019, Nature Methods) for single-cell RNA+ATAC multiomic profiling using the 10x Genomics platform. This protocol is demonstrated on nuclei isolated using the 'Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing' protocol from 10x Genomics. Users should test for effective temperature-stable MULTI-seq labeling using fluorophore-conjugated oligonucleotides prior to using alternative nuclei isolation workflows (see McGinnis, Patterson, et al., 2019, Nature Methods for details).

Attachments

CSV multiseq_barcodes.cs... 7KB

CSV multiseq_library_oli... 1KB

Materials

• PBS (GIBCO, cat. no. 10010-023)
• Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) (Thermo Fisher Scientific, cat. no. 12090-015)
• Nuclease-free water (Thermo Fisher Scientific, cat. no. AM9937)
• Tris-HCl (pH 7.4, 1 M) (Millipore-Sigma, cat. no. T2194)
• NaCl (5 M) (Millipore-Sigma, cat. no. 59222C)
• MgCl2 solution (1 M) (Millipore-Sigma, cat. no. M1028)
• Tween 20, 10% (wt/vol) (Bio-Rad, cat. no. 1662404)
• DTT (1 M) (Millipore-Sigma cat. no. 646563)
• Nonidet P40 Substitute, 10% (wt/vol) (Millipore-Sigma cat. no. 74385)
• Digitonin, 5% (wt/vol) (Thermo Fisher Scientific, cat. no. AM9937)
• Protector RNase Inhibitor (40 U/µl) (Millipore-Sigma cat. no. 3335402001)
• MULTI-seq Lipid-Modified Oligos (50 µM) (Millipore-Sigma cat. no. LMO0001)
• MULTI-seq sample barcode oligonucleotides (see 'multiseq_barcodes.csv' attachment for sequences). Oligonucleotides are ordered as standard desalting purified and lyophilized (Integrated DNA Technologies), then dissolved in nuclease-free water and stored as 10 µM stock solutions at −20 °C.
• BSA, 10% (wt/vol) (Miltenyi Biotech, cat. no. 130-091-376)
• EB buffer (10 mM Tris-HCl, pH 8.5) (Qiagen, cat. no. 19086)
• Trypan blue solution (Thermo Fisher Scientific, cat. no. 15250061)
• Glycerin (glycerol), 50% (v/v) Aqueous Solution (Ricca Chemical Company, cat. no. 3290-32)
• Ethanol, Pure (200 Proof, anhydrous) (Millipore-Sigma, cat. no. E7023-500ML)
• Chromium Next GEM Single Cell Multiome Reagent Kit A, 16 or 4 reactions (10x Genomics, cat. no. PN-1000282 or PN-1000284).
• Chromium Next GEM Chip J Single Cell Kit, 48 or 16 reactions (10x Genomics, cat. nos. PN-1000230 and PN-1000234).
• Single Index Kit N, Set A, 96 reactions (10x Genomics, cat. no. PN-1000212)
• Dual Index Kit TT Set A, 96 reactions (10x Genomics, cat. no. PN-1000215)
• MULTI-seq library preparation primers:Universal I5 primer and TruSeq RPI indices (see 'multiseq_library_oligos.csv' attachment for sequences). Oligonucleotides are ordered as standard desalting purified and lyophilized (Integrated DNA Technologies), then dissolved in nuclease-free water and stored as 10 µM stock solutions at −20 °C.
• SPRIselect reagent kit (Beckman Coulter, cat. no. B23318)
• Isopropyl Alcohol (Thermo Fisher Scientific, cat. no. MPX18304)
• Kapa HiFi HotStart ReadyMix (2X) (Roche, cat. no. 07958927001)
• Qubit dsDNA high-sensitivity assay kit (Thermo Fisher Scientific, cat. no. Q32854)
• Bioanalyzer high-sensitivity DNA analysis kit (Agilent, cat. no. 5067-4626)

Troubleshooting

Preparation

1 Make 1µM Anchor LMO + MULTI-seq BC solution (n=10)
- Add 4.8uL PBS to strip tubes
- Add 6uL 2uM anchor LMO to each tube
- Add 1.2uL 10uM BC to tubes

2 Make 1uM CoA LMO (n=10 → make 110uL)
- 107.8uL PBS
- 2.2uL 50uM CoA LMO

3 Prep cold PBS (5ml), 0.04% BSA in PBS (50mL), and 2% BSA in PBS (15mL)

4 Make Diluted Nuclei Buffer (200uL)
- 10uL 20X Nuclei Buffer
- 2uL 0.1M DTT
- 5uL 40U/uL RNase inhibitor
- 183uL nuclease-free water

5 Make Wash Buffer w/BSA (21mL)
- 210uL 1M Tris-HCL (pH 7.4)
- 42uL 5M NaCl
- 63uL 1M MgCl2
- 2.1mL 10% BSA
- 210uL 10% Tween-20
- 21uL 1M DTT
- 525uL 40U/uL RNase Inhibitor
- 17.83mL nuclease-free water

6 Make Wash Buffer w/o BSA (11mL)
- 110uL 1M Tris-HCL (pH 7.4)
- 22uL 5M NaCl
- 33uL 1M MgCl2
- 110uL 10% Tween-20
- 11uL 1M DTT
- 275uL 40U/uL RNase Inhibitor
- 10.44mL nuclease-free water

7 Make Lysis buffer (1.1mL)
- 11uL 1M Tris-HCl (pH 7.4)
- 2.2uL 5M NaCl
- 3.3uL 1M MgCl2
- 110uL 10% BSA

- 11uL 10% Tween-20
- 11uL 10% Nonidet P40 Substitute
- 2.2uL 5% digitonin
- 1.1uL 1M DTT
- 27.5uL 40U/uL RNase inhibitor
- 921uL nuclease-free water

Sample Preparation

8 Prepare a single-cell suspension with ideally >5e5 total viable cells that is suitable for nuclei lysis (i.e., minimal debris and resuspended in PBS w/ 0.04% BSA)

9 Spin @ 300xg, 5', 4C and aspirate supernatant
(NOTE: use optimal centrifugation conditions for your particular samples)

10 Add 100 uL chilled Lysis Buffer. Pipette mix 10x, incubate on ice for 2′
(NOTE: Lysis time must be optimized per sample, see the "Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing" protocol from 10x Genomics for more details)

11 Add 1 mL chilled Wash Buffer (w/ BSA) to the lysed cells

12 Spin (300xg, 5', 4C), aspirate supernatant

13 Resuspend in 1 mL chilled Wash Buffer (NO BSA), count nuclei
(NOTE: Adjust nuclei numbers to mirror desired pooling proportions; e.g., for evenly-pooled samples, ensure that nuclei counts for each sample are the same at this step)

14 Spin (500xg, 4′, 4C), aspirate supernatant

15 Resuspend in 180 uL chilled PBS

16 Add 10 µL of 1uM LMO-BC solution to nuclei, pipette mix, incubate on ice, 5′
(NOTE: Each sample receives a unique MULTI-seq barcode)

17 Add 10 µL of 1uM CoA LMO to nuclei, pipette mix, incubate on ice, 5′

18 Quench labeling reactions with 1 mL chilled 2% BSA in PBS, pipette mix

19 Spin (500xg, 4', 4C), aspirate supernatant

20 Resuspend in 100uL chilled 2% BSA in PBS, pool samples into one 1.5mL lo-bind eppie, top off with 2% BSA in PBS
(NOTE: Consider counting again at this step to ensure samples are pooled according to desired experimental design)

21 Spin (500xg, 4', 4C), aspirate supernatant

22 Resuspend in 50 uL Diluted Nuclei Buffer, count
(NOTE: Dilute 1uL cells in 9uL nuclei buffer before trypan mixing to avoid inaccurate counting of highly concentrated nuclei pool).

23 Adjust concentration to 1e4 nuclei/uL with Diluted Nuclei Buffer
(NOTE: 1e4 nuclei/uL results in 5e4 cells loaded per 10x lane → 1.5e4-2e4 singlets per lane after QC and doublet removal; adjust according to your desired experimental design)
(NOTE: Make sure count is very precise here → repeat if A/B are variable)

24 Mix 5uL and 10 µl Transposition Mix into PCR strip tube, run tn5:
- 50C lid, 15ul volume
- 37C 1hr
- 4C hold
(NOTE: Number of tn5 reactions = Number of 10x lanes loaded)

25 Process with rest of 10x protocol until Step 4: Pre-Amplification PCR

Multiseq Library Preparation

26 Transfer 10 µL of the product from 'Pre-Amplification SPRI Cleanup' (Step 4.3p) portion of the 10x Multiome protocol into a fresh PCR strip tube

27 Add 40 µL of Buffer EB to bring to a total volume of 50 µL

28 Add 30 µL SPRI (0.6X), mix, incubate at room temperature for 5'

29 Place strip tubes on magnetic rack (HIGH) and wait for solution to clear → DO NOT DISCARD SUPERNATANT (MULTI-seq barcodes are in the supernatant, cDNA and gDNA fragments are bound to the SPRI beads)

30 Transfer supernatant to fresh 1.5 mL microcentrifuge tubes

31 Add 130 µL SPRI (3.2X) and 90 µL isopropanol (1.8X), mix, incubate at room temperature for 5′

32 Place strip tubes on magnetic rack and wait for solution to clear

33 Remove and discard supernatant

34 Wash beads with 500 µL of 80% ethanol twice for 30″

35 After second wash, briefly centrifuge beads and place on magnetic rack

36 Remove remaining ethanol with P20 pipette

37 Air-dry beads on magnet for 2′ (do NOT exceed 2′)

38 Remove from magnet, resuspend beads in 50 µL Buffer EB

39 Incubate at room temperature for 2′

40 Return to magnet and wait for solution to clear

41 Transfer supernatant to PCR strip tube (avoid transferring beads)

42 Quantify barcode DNA concentration using Qubit

43 Make the following library preparation PCR reactions:
- 26.25 µL Kapa HiFi HotStart ReadyMix (2X)
- 2.5 µL – 10uM Universal I5 primer
- 2.5 µL – 10uM RPI primer *(choose unique RPI for each sample from 10X lane)*
- 3.5 ng barcode DNA *(volume based on concentration from Qubit)*
- Nuclease-free water to 50 µL final volume

44 Perform library preparation PCR:
- 95˚C, 5:00
- 98˚C, 0:15
- 60˚C, 0:30
- 72˚C, 0:30
- Repeat Steps 2-4 ×10
- 72˚C, 1:00
- 4˚C, Hold

45 Add 80 µL SPRI (1.6X) to PCR product, mix, incubate at room temperature for 5′

46 Place tube on magnet (HIGH), wait for solution to clear

47 Remove and discard supernatant

48 Wash beads with 200 µL of 80% ethanol twice for 30″

49 After second wash, briefly centrifuge beads and place on magnetic rack (LOW)

50 Remove remaining ethanol with P20 pipette.

51 Air-dry beads on magnet for 2′ (do NOT exceed 2′)

52 Remove from magnet, resuspend beads in 25 µL Buffer EB

53 Incubate at room temperature for 2′

54 Return to magnet and wait for solution to clear

55 Transfer supernatant to PCR strip tube (avoid transferring beads)

56 Quantify barcode library concentration (1:5) using Bioanalyzer High Sensitivity DNA analysis
- Secondary peaks are from carryover of small MW gDNA fragments and do not affect sequencing

57 Final Library Structure: We suggest sequencing the MULTI-seq library at a depth of 3,000 reads/cell, MULTI-seq libraries can be sequenced in a pool with GEX libraries

Protocol references

1. McGinnis CS*, Patterson DP*, et al. (2019). MULTI-seq: Scalable sample multiplexing for single-cell RNA sequencing using lipid tagged indices. Nature Methods 16, 619-626.
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4. Sheth MU, et al. (2025). Mapping enhancer-gene regulatory interactions from single-cell data. BioRxiv, **biorxiv.org/content/10.1101/2024.11.23.624931v1**.