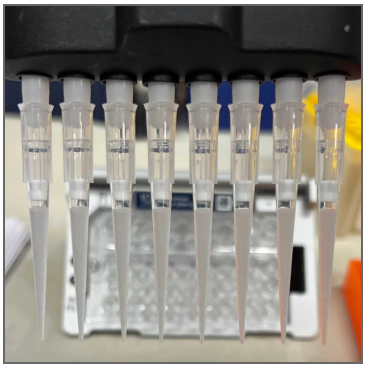

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PBMCs Processing for Single Cell Multiome ATAC + Gene Expression Sequencing

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ABSTRACT

This protocol was designed for the workflow of the Multiome assay on 16 PBMC samples using the 10X Next GEM Single Cell Multiome ATAC + Gene Expression Kit. We adapted Jimmy Ye Lab's pooling and demultiplexing strategy, overloading cells in each well. For the demultiplexing step using this protocol, you may need genotyping information of your samples.

BEFORE START INSTRUCTIONS

1. This protocol is designed for sample pooling and overloading strategy and needs sample genotyping information.
2.Double-check if your samples have neutrophil contamination; if so, you should consider removing them to get good quality of your ATAC libraries.

Protocol status: Working
We use this protocol and it's working

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1. Thawing PBMCs(~ 2 hrs, Benzonase treatment)

1 Remove cryovials from liquid nitrogen storage and place them on dry ice.

2 Warm The medium to **37°C** in a water bath and prepare the washing Medium.

3 Thaw frozen vials in the water bath at **37°C** for **1-2** min.

4 Pour the thawed cells gently into a **15 mL conical tube** containing 10 mL pre-warmed **Complete medium**.

5 Rinse the cryovial with **1 mL** pre-warmed **Complete medium** and add the rinse to the 15 mL tube.

6 Centrifuge at **300g** for **5 min** at room temperature and aspirate the supernatant.

7 Resuspend cells with **1 mL** pre-warmed **Washing medium** and add 9 mL more **Washing medium** to each tube.

8 Incubate for **10 min** at room temperature to ensure the free-floating DNA is depleted.

9 Centrifuge at **300g** for **5 min** at room temperature and aspirate the supernatant.

10 Prepare samples for Cell counting.

10.1 Resuspend thecell pellet in **1 mL** of pre-warmed Complete medium (dropped slowly along the side of the tube) by gently tapping.

10.2 Add **4 mL** of pre-warmed Complete medium.

10.3 Invert the tube gently several times to homogenize the cell suspension.

10.4 Take 10 uL for cell counting.

10.5 Add another **5 mL** of pre-warmed **Complete medium** and centrifuge at **350g** for **5 min** at room temperature.

11 Count cells and determine viability.

Note

Caution:

Filter the trypan blue solution with 0.45 µm filter before using each time.

11.1 Count the cells by adding 10 µL of cells to 10 µL of trypan blue and pipetting up and down to mix.

11.2 Apply 10 uL of the mixture to the hemacytometer or cell counter.

11.3 Calculate the cell number.

12 Resuspend cell pellet in **1 mL** of pre-warmed Complete medium by gently tapping. Add another **9 mL** of pre-warmed Complete medium. Briefly vortex (optional). Centrifuge at **350g** for **5 min** at **room temperature**.

Note

Using Complete medium from now on is to wash away benzonase.

13 Resuspend cells in Complete medium and adjust to **1 x 10^6 cells per mL**. Gently tap tubes.

14 Seed cells into a 24-well plate (low attachment plate) at 1 x 10^6 cells per well in 1 mL Complete medium, using a wide-bore 1 mL pipet tip.

Note

Tips:

1. If there are more than 4 million cells per individual, seed four wells (1 million cells per well): two for the control (Ctr) and the other two for lipopolysaccharide (LPS).
2. Pellet, wash once with PBS, and freeze all the leftover cells at -80°C.

15 Transfer the cell culture plate to the incubator (37°C, 5% CO_2) for 16-24 hrs, and leave the cells for recovery.

2. Stimulation of the PBMCs with ligands

16 Prepare LPS (200ng/mL, 20x) in Complete medium.

Note

Tips:

- Prepare these 20x solutions in Complete medium and can be stored in 4°C overnight.
- To prepare 1000 µL of 200 ng/mL LPS, add **10 µL** of 20 µg/mL LPS (-80°C stock) into **990 µL** Complete medium.

17 Add **50 µL** of **LPS** (200ng/mL, 20x) in one well (with 1 mL medium) at a final concentration of **10 ng/mL**.

18 Tap the plate 10 times (at each side of the plate) to resuspend cells and mix the medium.

19 Transfer the cell culture plate to the incubator (37°C, 5% CO2) for **6 hrs**.

3. Harvest PBMCs

8m

20 Preparation and check the following list before starting this section

Note

Checking List:

1. Set centrifuge for 5 mL tubes at 4°C. Prepare/label FACS tubes.
2. Bring Dead Cell Removal beads(0.5 ml aliquot) and buffer(1 tube with 0.5 ml aliquot 20x buffer, @ 4°C) to room temperature.
3. Bring 4% BSA(1 tube with 1ml aliquot, @ -20°C box) to room temperature.
4. Bring TrypLE Express to RT.

21 Move the cell culture plate out of the incubator at the end of the incubation (6 hr after adding LPS).

21.1 Check cells under the microscope to see if there are any cell clusters.

21.2 Transfer cells with medium into a 5 mL FACS tube using a 1 mL wide-bore pipette. Rinse the well with 1 mL PBS and add the rinse to the FACS tube.

21.3 Check the cell plate under a microscope to see if any cells are left over.

21.4 Add 200 µL Tryple E, incubate at 37°C for 5 min, and rinse with another 1 mL PBS.

21.5 Pipette a few times and transfer all the cells to the FACS tubes by wide-bore pipette.

22 350 x g, 4°C, 00:08:00 Centrifuge at 350g for 8 min at 4°C. 8m

22.1 Pour off the supernatant into the sink (usually will have 50 uL liquid leftover).

22.2 Invert the tube and dry the tube top with a Kim wipe.

23 Wash cells with Staining Buffer (from BioLegend)

23.1 Add 0.5 mL Staining Buffer to each sample.

23.2 Tap on the white columns several times to resuspend cells.

23.3 Add another 1.5 mL Staining Buffer to each sample.

23.4 Centrifuge at 350g for 5 min at 4°C.

23.5 Pour off the supernatant.

23.6 Dry the tube top.

24 Count cells.

24.1 Add 500 µL Staining Buffer (now total volume around 550 uL). Tap on the white columns to resuspend cells.

24.2 Take 10 µL for Trypan Blue staining and count cells using cell counter Countess.

24.3 Calculate the cell number and volumes (will be used in step 26).

25 Wash cells with Staining Buffer.

25.1 Add 2 mL Staining Buffer.

25.2 Centrifuge at 350g for 5 min at 4°C.

25.3 Pour off the supernatant.

25.4 Dry the tube top.

26 Pool cells

Note

Examples:

- we thawed PBMCs from 8 individuals, and each received 2 treatments (Ctrl, LPS); thus we had 16 cell samples;
- We pool the Ctr and LPS treated samples into two separated tubes and label them as P1(Ctr) and P2(LPS).
- If we do 16 individuals and we plate the samples to two groups and each group has 8 individuals and we pool samples the same as 8 individuals, but pool the other group to P3(Ctr) and P4(LPS).
- For each cell sample, we collect as many cells as possible and pool the same amount of cells from each sample.

27 Place extra cells at 4°C (in case).

Note

Caution:

- Total cell number after pooling should be better higher than 2 million, at least higher than 1 million;
- If less than 2 million, the cell number after dead cell removal and filtration will be too low due to cell loss in each step.
- Use low-binding Eppendorf tubes for all steps below.

4. Dead cell removal(~1.5 hrs)

28 Check the list below before this section:

28.1 Prepare Binding buffer (from Dead cell removal kit, 4°C), and add 0.5 mL Binding buffer into 9.5 mL distilled water. Bring to RT before use.

28.2 Prepare 10 mL 0.4% BSA in PBS, add 400 µL 10% BSA (-20°C aliquots) into 9.6 mL PBS (no Ca^{2+}, no Mg^{2+}). Keep on ice.

28.3 Prepare 10 mL 0.04% BSA in PBS, and add 1 mL 0.4% BSA into 9 mL PBS (no Ca^{2+}, no Mg^{2+}). Keep on ice.

29 Filter cells with 70 µm cell strainer using a regular 1 mL tip. Centrifuge at 350g for 5 min at 4°C. Remove the supernatant.

30 Add 100 µL dead cell removal microbeads (well-vortexed). Resuspend by pipetting using P100 pipet. Leave at room temperature for 15 min.

Note

Tips:

During the 15 min incubation, bring the following reagents out:
- 50% Glycerol(2 aliquot tubes) from -80 °C to room temperature.
- TSO (template switch oligo) from -80 °C to room temperature.

31 During the last 1 min, rinse the MS column with 500 µL binding buffer (room temperature). Keep the MS column wet all the time.

32 Add 400 µL binding buffer to the pooled cells.

33 Transfer the cell suspension into the column and leave them to pass through. Collect the follow-through in a low-binding 1.5 mL Eppendorf tube.

34 Rinse the column with another 500 µL binding buffer. Collect the follow-through in the same tube from the last step.

35 Centrifuge at 350g for 5 min at 4°C and discard the supernatant.

36 Rinse the column with 500 µL binding buffer twice. Save the flowthrough in another 1.5 mL tube and place on ice (in case more cells can be collected).

37 Resuspend the cell pellet with 1 mL 0.4% BSA in PBS, slowly pipette using a wide-bore tip.

38 Centrifuge at 350g for 5 min at 4°C. Use a pipet to remove SPN.

39 Resuspend the cell pellet with 1000 µL 0.04% BSA in PBS. Pass through the 40 µm Flowmi Cell Strainer using P1000.

40 Take 10 µL for Trypan Blue staining and count cells using cell counter Countess, record cell viability.

41 Calculate each pool's total cell number and ensure each pool has around **1M** cells (this is 10x Genomics suggestion).

42 Centrifuge at **300g** for **10min** at **4 °C**.

43 Remove **ALL** the supernatant without disrupting the cell pellet and put cells **ON ICE**.

Note

CRITICAL STEP:

- Get rid of all the supernatant.
- Make sure to avoid the visible cell pellet when pipetting. Optimal removal of supernatant and minimal disruption of the cell pellet is attained when the final 100 µl is removed in a consistent and fluid motion without starting and stopping.
- **Two pipetting steps:**
  1. Aspirate down to 100 µl with a p1000 pipette.
  2. Then, remove the final 100 µl with a p200 pipette.

5. Neutrophils depletion(Optional, CD66abce MicroBead Kit, human)

44

Note

Attention:

- There is no need for the overnight recovered PBMCs since most of the neutrophils will be broken.
- Kit: CD66abce MicroBead Kit
  - Miltenyi Biotec: Order no. 130-092-393
- Column:
  - Miltenyi Biotec: MS(Order no. 130-042-201)

6 Nuclei isolation(pool cells, ~ 70 min)

45 Add 100 µL of chilled Lysis Buffer for every 1 million cells. You need to scale up or down the volume of the lysis Buffer based on the cell numbers of each sample. Pipette mix 10 times and incubate for 3 min on ice.

Note

Tips:

- Use an **interval timer** for this step.
- For 2 pools, set a **75-second** timer for each pool and a **30-second** break at the end. The total time will be 180 seconds(3min).
- For 3 pools, set a **50-second** timer for each pool and a **30-second** break at the end. The total time will be 180 seconds(3min).
- For 4 pools, set a **40-second** timer for each pool and a **20-second** break at the end. The total time will be 180 seconds(3min).
- Run the Interval timer script in the terminal: *swift IntervalTimergTTS4FourSamples.swift*
- Start timer, add LSB to sample 1, and pipet mix 10x. Follow the timer to add LSB to sample 2, and pipet mix 10x. Continue the step to add LSB to all samples.

Command

Interval Timer by Swift language (MacOS 14.3.1)

```
import Foundation
import AVFoundation

func intervalTimer(intervals: [(TimeInterval, () -> Void, String)]) {
    for interval in intervals {
        let (duration, action, intervalName) = interval
        print("Interval name: \(intervalName)")

        // Convert the interval name to an AVSpeechUtterance
        let utterance = AVSpeechUtterance(string: intervalName)
        utterance.voice = AVSpeechSynthesisVoice(language: "en")

        // Use AVSpeechSynthesizer to speak the utterance
        let synthesizer = AVSpeechSynthesizer()
        synthesizer.speak(utterance)

        // Wait for the specified duration
        Thread.sleep(forTimeInterval: duration)

        // Perform the action
        action()
    }
}

// Define the intervals and corresponding actions
let intervals: [(TimeInterval, () -> Void, String)] = [
    (10, { print("Interval 0 action") }, "Be ready for nuclei isolation"),
    (40, { print("Interval 1 action") }, "Sample 1"),
    (40, { print("Interval 2 action") }, "Sample 2"),
    (40, { print("Interval 3 action") }, "Sample 3"),
    (40, { print("Interval 4 action") }, "Sample 4"),
    (20, { print("Interval 5 action") }, "Break time and prepare for adding
washing buffer"),
    (40, { print("Interval 6 action") }, "Sample 1"),
    (40, { print("Interval 7 action") }, "Sample 2"),
```

```
    (40, { print("Interval 8 action") }, "Sample 3"),
    (40, { print("Interval 9 action") }, "Sample 4"),
    (10, { print("Interval 10 action") }, "Nuclei isolation is done")
]

// Run the interval timer with the specified intervals
intervalTimer(intervals: intervals)
```

46 Add 500 µL chilled Wash Buffer(WSB) for every 1 million cells to the lysed cells. Invert the tube 5 times to mix. You need to scale up or down the volume of the lysis Buffer based on the cell numbers of each sample.

Note

Tips:

- Use the same **interval timer** used in the lysis step(Step 45) for this step.
- Add 500 µl of WSB for every 1 M cells.

47 Centrifuge at **500g** for **5 min** at **4 °C**.

48 Remove the supernatant without disrupting the nuclei pellet.

49 Resuspend nuclei pellet with 1000 µl **chilled Wash Buffer(WSB)** and pipette **5 times** to mix. There is no need for an interval timer for washing.(If the volume is more than 1.5 ml, transfer the nuclei to a 2-ml microcentrifuge tube)

50 Centrifuge at **500g** for **5 min** at **4 °C**.

51 Resuspend nuclei pellet with 1000 µl **chilled Wash Buffer(WSB)** and pipette **5 times** to mix for a total of **3 washes**.

52 Pass nuclei suspension through a **40 µm Flowmi Cell Strainer**.

Note

Attention:

- 1. Use the Flowmi to remove cell nuclei aggregates and cellular debris.
- 2. Since the recommendation for samples having a maximum concentration of 2 million cells/ml(40 µm), I moved the filter step here from step 55.
- 3. The Dead volume of Flowmi is around 20 to 30 µL(for 50µl to 1000µl in total).

53 Centrifuge at **500g** for **5 min** at **4 °C**.

54 Remove ALL supernatant, taking care not to disturb the pellet.

55 Resuspend pooled nuclei in **~50µL** chilled **Diluted Nuclei Buffer (DNB)** to reach the target **15M/ml** concentration. Maintain on ice.

Note

Critical：

The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell ATAC assay performance.

56 Take **2µL** of nuclei suspension for counting, and record counts in the attached template.

Note

Critical :

Prepare sample slides one by one for counting because the nuclei will be broken in Trypan blue buffer for a short time.

56.1 Make 1:5 dilution by adding 8µL DNB to 2µL nuclei.

56.2 Count on a Countess II in duplicates.

56.3 The desired concentration is 2.5-4M/mL for diluted nuclei and 12-20M/mL for nuclei in Transposition.

56.4 If observe a clump of nuclei, filter through a 40µm strainer and count again.

57 Proceed immediately to Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide(CG000338).

7. Transposition(~ 75 min)

58 During Cell count in **Step 56**, bring out the transposition reagents.

58.1

	Action	Item	10x PN	Storage
	Equilibrate to RT	ATAC Buffer B	2000193	-20 °C
	Equilibrate to RT	20X Nuclei Buffer	2000207	-20 °C
	Place on Ice	ATAC Enzyme B	2000265/2000272	-20 °C
	Place on Ice	Nuclei	-	Ice

59 Based on the nuclei count from **step 6.15**, calculate the volume of nuclei resuspension and DNB needed to load **50k single nuclei/well** to **4 wells**(for 16 samples) or 6 wells (for 24 samples) using the attached template.

60 Refer to *Single Cell Multiome User Guide* for Transposition Mix and Isothermal Incubation(**Page 30**).

8. GEM Generation & Barcoding(~ 130 min, Move forward to 10X Single Ce…

61 Prepare single-cell master mix.

62 Bring following items to the Room 540

Note

Checking list:

- Transposed Nuclei
- Master Mixture
- Ice box
- PCR stripe(8-well)
- Kit Box(Chip J and Gasket)
- 50% Glycerol
- Partition oil
- Gel Beads
- 100 µl pipet tips
- 100 µl pipet
- 200 µl multi-channel pipet
- PCR tube rack
- Gloves
- 10X Single Cell Multiome user guide

63 Load chromium next GEM Chip J (cells are used in this step).

64 Run the chromium controller.

65 Transfer GEMs.

66 GEM incubation.

67 Quenching Reaction.

9. Buffers

68 Preparation before the assay:

Note

Caution:

- Add **DTT** to each buffer during cell counting at the end of Part 1 because the DTT is unstable in the solution.
- For Digitonin in **LYB**, incubate at 65 °C to dissolve it entirely and cool down to RT before using. Otherwise, the Digitonin solution shows a backflow in the P10 tip.

68.1 Prepare the following buffers and keep all at 4 °C.

68.2 Cool down the centrifuge at 4 °C.

68.3 Prepare a total of 550 µl(11 vials) of RNase inhibitor.

69 DNB(Diluted Nuclei Buffer)

69.1

	1X Nuclei Buffer	Stock	Final	975µL
	Nuclei Buffer (20X)	20X	1X	50µL
	DTT	1000mM	1mM	1µL

	1X Nuclei Buffer	Stock	Final	975µL
	Nuclease-free Water	-	-	924µL

69.2

	DNB	Stock	Final	400µL
	1X Nuclei Buffer	1X	1X	390µL
	RNase inhibitor	40U/µL	1U/µL	10µL

70 WSB(Wash Buffer)

70.1

	WSB	Stock	Final	2.5mL(1M)	10mL(4M)	15mL(6M)	20mL(8M)
	Tris-HCl (pH 7.4)	1M	10mM	25µL	100µL	150µL	200µL
	NaCl	5M	10mM	5µL	20µL	30µL	40µL
	MgCl2	1M	3mM	7.5µL	30µL	45µL	60µL
	BSA	10%	1%	250µL	1000µL	1.5mL	2.0mL
	Tween-20	10%	0.1%	25µL	100µL	150µL	200µL
	DTT	1000mM	1mM	2.5µL	10µL	15µL	20µL
	RNase Inhibitor	40U/µL	1U/µL	62.5µL	250µL	375µL	500µL
	Nuclease-free Water	-	-	2.1225mL	8.49mL	12.735mL	16.98mL

71 LSB(Lysis Buffer)

71.1

	LSB	Stock	Final	0.45ml(4M)	0.9ml(8M)	1.8ml（16M）
	Tris-HCl (pH 7.4)	1M	10mM	4.5µl	9µL	18µL
	NaCl	5M	10mM	0.9µl	1.8µL	3.6µL

	LSB	Stock	Final	0.45ml(4M)	0.9ml(8M)	1.8ml（16M）
	MgCl2	1M	3mM	1.35μl	2.7μL	5.4μL
	Tween-20	10%	0.1%	4.5μl	9μL	18μL
	NP40	10%	0.1%	4.5μl	9μL	18μL
	Digitonin	5%	0.01%	0.9μl	1.8μL	3.6μL
	BSA	10%	1%	45μl	90μL	180μL
	DTT	1000mM	1mM	0.45μl	0.9μL	1.8μL
	RNase Inhibitor	40U/μL	1U/μL	11.25μl	22.5μL	45μL
	Nuclease-free Water	-	-	376.65μl	753.3μL	1.5066mL

10. Reagents Details

72 Complete medium (for PBMC culture)

Note

Recipe:

RPMI-1640 + 10% FBS + 100 U/mL penicillin + 100 U/mL streptomycin + 1 mM sodium pyruvate + 2 mM L-glutamine

73 Washing medium

Note

Recipe:

50% Complete medium + 50% X-vivo 15 medium + 25 U/mL benzonase (3.1 uL of 400 U/mL benzonzase into 50 mL Washing medium)

74 Benzonase (Millipore, Cat: 71205-3)

75 Lipopolysaccharide (LPS). Ultrapure lipopolysaccharide from E. coli K12 (InvivoGen, Cat. tlrl-peklps)

76 5% Digitonin: Invitrogen, BN2006.

77 1X D-PBS

78 Trypan blue 0.4% (filtered)

79 Staining Buffer. Cell staining buffer (BioLegend #420201)

80 Dead cell removal kit (Miltenyi Biotec, Cat. 130-090-101)

81 Distilled water. UltraPure distilled water (Invitrogen, Cat. 10977-015)

82 10% BSA: MACS BSA stock solution(Miltenyi Biotec, # 130-091-376)

83 Bleach