11/06/2023 Version – John Blair

Phospho-seq with Multiome

This protocol will take you through performing Phospho-seq with a 10x single-cell Multiome (scATAC + scRNA) kit. It is almost identical to the Phospho-seq protocol with the ATAC-seq kit, but with some added primers and RNAse Inhibitor. Make all solutions fresh on the day of the experiment. The pre-processing steps are contained here with the necessary modifications to the 10x protocol attached at the end. This protocol goes up to the sequencing of the libraries, please refer to the other protocols at Phospho-seq.com for preparing conjugated antibody pools and data processing and analysis.

Materials

16% FA – 30 ul/sample – 500 ul

- 219 µl 36.5% Formaldehyde (Sigma: F8775)
- 281 µl 1X PBS (Cytiva: SH30256)

1M Glycine – 68.5 ul/sample – 10 ml

- 750.7 mg Glycine (BioBasic: GB0235)
- 10 ml H_2O

1X PBS

Permeabilization buffer (100 ul/ sample - 1 ml)

- 1M Tris-HCl (Thermo: 15567027)- 10 µl
- 5M NaCl (Thermo: AM9759) - 2 µl
- 1M MgCl2 (Sigma: M1787) - 3.33 µl
- 10% NP40 (Thermo: 28324) - 10 µl
- 10% BSA in H_2O (Fisher: BP9706100) - 100 µl
- H_2O - 874 µl
- RNAse Inhibitor (Lucigen: 30281-1) – 50 µl
- 0.1M DTT (Invitrogen: y00147) - 10 µl

Wash Buffer (#1) (1 ml/ sample - 10 ml)

- 1M Tris-HCl - 100 µl
- 5M Nacl - 20 µl
- 1M MgCl2 - 33.3 µl
- 10% BSA in H_2O - 1 ml
- H_2O -8850 µl

- RNAse Inhibitor – 250 µl
- 0.1M DTT - 100 µl

Blocking Buffer (100 ul/ sample - 1 ml)

- PBS w/ 3% BSA - 909 µl

Note: Make a 500 ml bottle of this (15 g BSA in 500 ml PBS), filter and store at 4C

- ssDNA - 1 mg (50 µl at 20 mg/ml)

Note: Use a 30 nt oligo that is blocked at the end to reduce random amplification - `NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN/3ddC/`

- 3.75 nMol of Total-Seq-A oligo total - use laddered concentrations – 20 ul,10µl ,5 µl and 2.5 µl of four different 100 uM TSA oligos. TSA oligo format for ordering: `/5AmMC12/CCTTGGCACCCGAGAATTCCAXXXXXXXXXXXXBAAAAAAAAAAAAAAAAA AAAAAAAAAAAA*A*A` where Xs are the indexing barcode

Note: This helps with additional blocking and also can be captured and sequenced to use during processing as a metric for how well the cell is permeabilized, subsequently regressing ADT counts against them (see data analysis protocol)

- RNAse Inhibitor – 25 µl
- 0.1M DTT - 10 µl

Staining buffer (100 µl/ sample – 1 ml)

- PBS w/ 3% BSA – 940 µl
- RNAse Inhibitor – 50 µl
- 0.1M DTT - 10 µl

Wash buffer #2 – 6 ml/ sample - 25 ml

- PBS w/ 3% BSA – 24.75 ml
- 10% Tween (BioVision: 2109-100) - 250 µl
- RNAse Inhibitor – 625 µl
- 0.1M DTT - 250 µl

Note: RNase Inhibitor is expensive, so you should scale down this volume as much as you'd like – also, washing in 300 µl vs 500 µl later in the protocol is unlikely to be detrimental to the results.

10X NEB Buffer 4 (NEB: B7004S) – 10 µl

E.Coli Single-stranded binding protein (SSB) (Promega: M3011) - 8 ug/ug of ab

9% BSA and 3X PBS – 50 ul/ experiment – 10 ml

- 10X PBS (Thermo: 70011-044) – 3 ml
- BSA – 900 mg
- 7 ml H_2O
- RNAse Inhibitor (Lucigen: 30281-1) – 750 µl
- 0.1M DTT (Invitrogen: y00147) - 100 µl

Note: Make the mixture without the RNAse Inhibitor and then only take the 50 µl you need and add the DTT and RNAse Inhibitor to that part only to save money.

1x Nuclei Buffer – 1 ml/ experiment

- 20X Nuclei Buffer (10x Genomics: 2000207) – 50 ul
- H_2O – 950 ul
- RNAse Inhibitor (Lucigen: 30281-1) – 25 µl
- 0.1M DTT (Invitrogen: y00147) - 10 µl

Harvesting Cells

Note: If using adherent cells, process them as you would if you were passaging them. If using frozen cells, thaw them into a solution with a small amount of FBS or BSA to prevent clumping

1.Count cells in whichever solution they are in
2.take 1-2 million cells total for experiment
3.Centrifuge 300 rcf for 5 mins
4.Resuspend in 475 µl PBS
5.Run through a 40 µm Flowmi cell strainer (Bel-Art: H13680-0040) into a 1.5 ml tube

Fixation and Permeabilization

1.Add 30 µl 16% FA and pipette to mix
2.Leave at RT for 10 mins, swirl/invert occasionally
3.Quench fixation by adding 68.5 µl of 1M Glycine
4.Fill tube with ice cold 1X PBS
5.Centrifuge for 5 mins at 400 rcf at 4C
6.Remove supernatant and add 1 ml cold 1X PBS
7.Centrifuge for 5 mins at 400 rcf at 4C
8.Discard supernatant and resuspend in 100 µl permeabilization buffer
9.Incubate on ice for 5 mins
10.Add 1ml Wash buffer #1, centrifuge for 5 mins at 500 rcf at 4C
11.Resuspend in 100 µl blocking buffer
12.Put on tube rotator at RT for 30 mins

Hashing

Note: This step is optional and the protocol is if you are using four separate hashing antibodies for one sample. For samples that are already separate, leave them separate and hash each one accordingly. Adjust accordingly for more or less hashes used and skip step if not hashing

1.Add 500 µl wash buffer #2, centrifuge for 5 mins at 500 rcf at 4C
2.Remove supernatant, add 300 µl wash buffer #2, split into 4 x 100 µl tubes, add further 400 µl wash buffer to each and spin down 500 rcf at 4C
3.Remove supernatant and resuspend in 100 µl staining buffer with 1 µg hashing antibody per tube
4.Place on the tube rotator for 30 mins at RT
5.In the meantime prepare the SSB-bound antibodies

Binding SSB to antibodies

1.Add an appropriate amount of pooled antibody to a 1.5 ml tube
Note: Usually I add approximately 0.5 µg of antibody mix per antibody – e.g. in a panel of 30 antibodies I would add 15 µg of antibody pool – but this can go as low as 0.1 ug/ab or as high as 1 ug/ab with expected changes in sensitivity.
2.Add H_2O to fill up to 100 µl less 10µl and less the amount of SSB you put in (8ug/ug of antibody) e.g. for 20 µl of antibody pool with 10 ug, you will add roughly 16 µl of SSB, so you would add 100- 20 -10 -16 = 54 µl H_2O
3.Add 10 µl 10X NEB buffer 4
4.Add SSB and pipette mix until well distributed
5.incubate at 37C for 30 mins
6.Add 50 µl of 3X PBS + 9% BSA to make a final concentration of 3% BSA in 1X PBS

Staining and washes

1.After hashing, centrifuge at 600 rcf for 5 mins and resuspend in 500 µl wash buffer #2
2.Remove supernatant and repeat centrifugation
3.Remove supernatant and resuspend in wash buffer #2, count each sample and combine into one tube (1 million cells total)
Note: Counting is unnecessary when starting with small quantities of cells
4.Centrifuge at 600 rcf for 5 mins, remove supernatant
5.Resuspend in the 150 µl of Staining Buffer + ab
6.Put in the tube rotator at RT for 1 hr

7.Centrifuge at 600 rcf for 5 mins, remove super and add 500 µl wash buffer #2. Repeat this step
8.Remove supernatant and resuspend in 500 µl 1x nuclei buffer and run through a 40 µm flowmi filter
9. Centrifuge at 600 rcf for 5 mins to concentrate sample
10.Take off much of the supernatant to have about 30 µl left depending on size of pellet
11.Resuspend cells and count 2-5 µl of cells and adjust concentration accordingly
12.Load 30,000 cells into tagmentation reaction for 20,000 cell recovery overall
13.From here, follow the 10X scMultiome protocol with indicated changes

Adjustments to 10X scMultiome protocol

1. At step 2.1q (barcoding reaction), spike in 0.5 µl of 1 µM of bridge oligo B
Note: This protocol assumes TotalSeqB oligos are being used for the protein antibodies, if Total-seq-A oligos are used, then the bridge oligo is unnecessary as the multiome kit has polyA capture for the RNA modality
2.At step 4.1 (pre-amplifcation PCR) spike in 1 µl of 0.2 µM HTO and ADT additive primers:
HTO (TSA) additive: `CCTTGGCACCCGAGAATT*C*C`
ADT (TSB) additive: `GTGACTGGAGTTCAGACGTGTGC*T*C`
3.At Step 4.3k, after pre-amplification, elute the DNA into 100 µl buffer EB. Use 25% for ATAC (25 µl + 15 µl H_2O for ATAC reaction), 35% for cDNA and the remaining 40% for protein tags
4.If doing both HTOs and ADTs, split the 40 µl fraction in two and input into two separate PCR reactions with the following parameters:

PCR reaction:

50 µl 2X KAPA master mix (KAPA Biosystems: 07958935001)
2.5 µl 10 uM P5 Primer (SI-PCR P5)
2.5 µl 10 uM RPxx (TSA) or D7xx (TSB) primer
20 µl input fragments
25 µl H_2O

RPxx Primer: `CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA`
D7xx Primer: `CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGC`
Generic P5 Primer: `AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC`

X’s denote the i7 barcode in the primers

PCR Program:

1.95 C 3 min
2.95 C 20s Repeat steps 2-4 for 12-16 total cycles
3.60 C 30s
4.72 C 20s
5.72 C 5 min
6.4 C Hold

Note: Depending on the number of cells and antibody input into the reaction, I will remove 2 µl of product after 12 cycles and look for product (188-205 bp) on a 2% e-gel. If no product is visible, I will run 2-4 more cycles.

5. After PCR, clean up the reactions with SPRI beads: Add 160 µl SPRI beads to 100 µl PCR reaction and let bind for 5 mins. Place on magnet and wash 2X with 80% EtOH. Remove all EtOH and resuspend in 20 µl buffer EB. Let bind for 2 mins at RT and place back on magnet. Remove elute which is your ADT or HTO sequencing library.

Sequencing

1. Run all libraries on a Bioanalyzer High Sensitivity DNA chip to determine molarity. For ATAC libraries, look for nucleosome banding pattern. For ADTs and HTOs look for strong, clean products at 188-205 bp.
2.Combine the libraries to aim for 20,000 reads per cell for the ATAC fraction, 20,000 reads for the RNA fraction, 5,000 reads per cell for the ADT fraction and 2,000 reads per cell for the HTO fraction
3.Run on whichever sequencer you have available as long as there is >28 bp Read 1, 8 bp i7 index, >34 bp Read 2 and 16 bp i5 index. A 150 cycle NextSeq high-output kit performs well for 10,000 cells for this with 50/8/16/86 sequencing recipe, however you must use a custom recipe to perform 8 “dark” cycles in the i5 reaction in order to skip over the splint probe. This is unnecessary when using a NovaSeq or equivalent sequencer.