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**CUT&Tag Fixed Nuclei Preparation**

**Materials:**

* Fresh culture(s)
* PBS
* NE1 buffer
* 16% formaldehyde
* 1.25 M glycine
* Wash buffer
* Cryogenic vials
* DMSO
* Isopropanol

**Procedure:**

1. Harvest fresh culture(s) in a conical centrifuge tube (15 ml or 50 ml) at room temperature and count cells. The same protocol can be used for up to ~500,000 mammalian (e.g., human K562 or H1 embryonic stem cells) cells per sample to be sequenced.
2. Centrifuge the tube for 3 minutes at 600 x g in a swinging bucket rotor at room temperature and drain the liquid.
3. Resuspend the cells in 1 volume of PBS at room temperature while pipetting.
4. Centrifuge the tube for 3 minutes at 600 x g in a swinging bucket rotor at room temperature and drain the liquid.
5. Resuspend the cells in 1/2 volume of ice-cold NE1 buffer with gentle vortexing. Let sit on ice for 10 minutes.
6. Centrifuge the tube for 4 minutes at 1300 x g at 4°C in a swinging bucket rotor and drain the liquid by pouring off and inverting onto a paper towel for a few seconds.
7. Resuspend the cells in 1/2 volume of PBS (relative to starting culture).
8. While gently vortexing, add 16% formaldehyde to 0.1% (e.g., 62 µL to 10 ml) and incubate at room temperature for 2 minutes.
9. Stop cross-linking by adding 1.25 M glycine to twice the molar concentration of formaldehyde (e.g., 600 µL to 10 ml).
10. Centrifuge the tube for 4 minutes at 1300 x g at 4°C and drain the liquid by pouring off and inverting onto a paper towel for a few seconds.
11. Resuspend the cells in wash buffer to a concentration of ~1 million cells per ml.
12. Nuclei may be slowly frozen by aliquoting 900 µL into cryogenic vials containing 100 µL DMSO, mixed well, then placed in a Mr. Frosty container filled to the line with isopropanol and placed in a -80°C freezer overnight then stored at -80°C.

**NE1 buffer (for preparing nuclei)** Mix 1 ml 1M HEPES-KOH pH 7.9, 500 μL 1M KCl, 12.5 μL 2 M spermidine, 500 µL 10% Triton X-100, and 10 ml glycerol in 38 ml dH2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free

**Wash buffer** Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 μL 2 M spermidine, bring the final volume to 50 mL with dH2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 week.

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| **Binding buffer**  | \* store RT |  |  |  |  |
|   | volume(uL) | number of library | total volume | volume with attrition | total volume |
| 1M HEPES pH 7.9 | 6.4 | 5 | 32 | 35.2 | 1760 |
| 1M KCl | 3.2 | 16 | 17.6 |
| 1M CaCl2 | 0.32 | 1.6 | 1.76 |
| 1M MnCl2 | 0.32 | 1.6 | 1.76 |
| UltraPure Water  | 309.76 | 1548.8 | 1703.68 |
|  |  |  |  |  |  |
| **Wash buffer** | \* store RT |  |  |  |  |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 1M HEPES pH 7.5 | 70 | 5 | 350 | 385 | 19250 |
| 5M NaCl | 105 | 525 | 577.5 |
| 2M Spermidine | 0.875 | 4.375 | 4.8125 |
| 50X PIC | 70 | 350 | 385 |
| UltraPure Water  | 3254.125 | 16270.63 | 17897.69 |

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| --- | --- | --- | --- | --- | --- |
| **Dig-Wash Buffer** | \* store RT |  |  |  |  |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 1X Wash buffer  | 3267 | 5 | 16335 | 17968.5 | 18150 |
| 5% Digitonin  | 33 | 165 | 181.5 |
|  |  |  |  |  |  |
| **Antibody Buffer** | \* store on ice, prepare just before use |  |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 0.5M EDTA  | 0.2 | 5 | 1 | 1.1 | 277.024 |
| 30% BSA  | 0.168 | 0.84 | 0.924 |
| Dig-Wash Buffer | 50 | 250 | 275 |

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| --- | --- | --- | --- |
| **Tagmentation Buffer**  | \* prepare just before use |  |  |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 1X Dig-300 Buffer  | 300 | 5 | 1500 | 1650 | 1666.5 |
| 1M MgCl2  | 3 | 15 | 16.5 |

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| --- | --- | --- | --- | --- | --- |
| **Dig-300 Buffer** | \* store RT |  |  |  |  |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 1M HEPES pH 7.5 | 70 | 5 | 350 | 385 | 19250 |
| 5M NaCl | 210 | 1050 | 1155 |
| 2M Spermidine | 0.875 | 4.375 | 4.8125 |
| 50X PIC | 70 | 350 | 385 |
| 5% Digitonin | 7 | 35 | 38.5 |
| UltraPure Water  | 3142.125 | 15710.63 | 17281.69 |

**Preparation of ConA beads**

1. Per Library, Add 100uL of 1X binding buffer to a 1.5mL low bind tube or 5mL tube.
2. Resuspend ConA beads by pipetting, transfer and mix 10uL ConA beads to the 100uL Binding Buffer.
3. Place the tube with the mixture on magnetic rack and remove supernatant.
4. Remove the tubes from the rack and add 100uL Binding buffer to the beads, mix by pipetting.
5. Place the tube with the mixture on magnetic rack and remove supernatant.
6. Remove the tubes from the rack and add 10uL Binding buffer to the beads, mix by pipetting.

**Harvest cell of interest**

1. Count cell and measure Viability.
2. Remove medium by centrifugation. Speed(rcf): 500 Time(minutes): 5
3. At room temperature, add 500uL 1X Wash Buffer to resuspend the cell pellet.
4. Remove wash buffer by centrifugation. Speed(rcf): 500 Time(minutes): 5

**Cell-ConA beads mixture**

1. Per Library, add 100uL of Wash Buffer to the washed cell pellet, transfer mixture to a 1.5mL low bind tube.
2. On a bench top vortexer, turn the dial to “5” and turn on the vortex.
3. Open the cap of the tube containing the cell-wash buffer mixture and press on the vortexer.
4. Per Library, add the 10uL of the prepared ConA beads to the cell-wash buffer mixture slowly.
5. Incubate the mixture at room temperature for 10 minutes.
6. Briefly centrifuge the tube to collect the liquid to the bottom of the tube.
7. Place the tube on a magnetic rack and remove supernatant.
8. Remove the tubes from the rack.

**Primary antibody**

1. Per Library, add 50uL of 1X cold Antibody Buffer to resuspend the bead-cells.
2. Resuspend by light vortexing.
3. Place the tube on Ice.
4. Add antibody and note the dilution factor of the antibody.
5. Rotate the tube at 4 degree C for overnight.

**Secondary antibody**

1. Dilute the secondary antibody with 1X Dig-wash buffer. Note the dilution factor.
2. Briefly centrifuge the primary antibody-bead-cell mixture to collect the liquid to the bottom of the tube.
3. Place the tube on a magnetic rack and remove supernatant.
4. Remove the tubes from the rack.
5. Add 100uL of the diluted secondary antibody to the bead-cell mixture.
6. Resuspend by light vortexing.
7. Rotate the tube at room temperature for 60 minutes.
8. Briefly centrifuge the secondary antibody-bead-cell mixture to collect the liquid to the bottom of the tube.
9. Place the tube on a magnetic rack and remove supernatant.
10. Remove the tubes from the rack.
11. Add 1000uL 1X Dig-wash buffer to the beads, invert 10 times o thoroughly mix the beads with buffer.
12. Briefly centrifuge the secondary antibody-bead-cell mixture to collect the liquid to the bottom of the tube.
13. Place the tube on a magnetic rack and remove supernatant.
14. Remove the tubes from the rack.
15. Repeat wash once more time for a total of 3 washes.
16. \* At the last wash, do NOT remove supernatant.

**Addition of pA-Tn5 transposon to the washed beads-cell mixture**

1. Dilute the pA-Tn5 transposon with Dig-300 Buffer

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| --- | --- | --- | --- | --- | --- |
|   | volume | number of library | total volume | volume with attrition | total volume |
| 1X Dig-300 Buffer | 100 | 5 | 500 | 550 | 563.75 |
| pA-Tn5 | 2.5 | 12.5 | 13.75 |

1. Place the tube containing the bead-cell mixture on a magnetic rack and remove supernatant.
2. Add the diluted transposon to the bead-cell mixture.
3. Resuspend by light vortexing.
4. Rotate the tube at room temperature for 1 hour.
5. Briefly centrifuge the transposon-bead-cell mixture to collect the liquid to the bottom of the tube.
6. Place the tube on a magnetic rack and remove supernatant.
7. Add 1000uL 1X Dig-300 buffer to the tube and invert 10times to thoroughly mix the buffer with the beads.
8. Place the tube on a magnetic rack and remove supernatant.
9. Remove the tubes from the rack.
10. Repeat wash once more time for a total of 3 washes.
11. \* At the last wash, do NOT remove supernatant.

**Tagmentation**

1. Place the tube on a magnetic rack and remove supernatant.
2. Add 100uL of 1X Tagmentation Buffer to the tube and mix by pipetting.
3. Incubate at 37C for 1 hour.

**Quenching of tagmentation**

1. Place the tube in room temperature and add 10uL 0.5M EDTA,3uL 10% SDS and 2.5uL 20mg/ml proteinase K
2. Resuspend by full speed vortexing fo 2 seconds.
3. Briefly centrifuge the bead-cell mixture to collect the liquid to the bottom of the tube.
4. Incubate the tube at 55C for 1 hour.
5. Purify the mixture with MinElute Column following Manufacturer's instruction.
6. Elute the DNA with 21uL Qiagen EB

**PCR amplification**

1. Add N70X and N50X primers (2.5uL each) to the 20uL of purified DNA.
2. Add 25uL NEBNext HiFi PCR Mix to the tube.
3. Run the PCR.

|  |  |  |
| --- | --- | --- |
| 72C | 5 min |  |
| 98C | 30sec |  |
| 98C | 10sec | 12-14cycles |
| 63C | 10sec |
| 72C | 1min |  |
| 8C | hold |  |

**Purification of PCR product**

1. Equilibrate beads to room temperature for at least 15 minutes.
2. Add 65uL of the beads to each PCR product. Mix by pipetting. Incubate at RT for 5 minutes.
3. Place the mixture on a magnetic rack.
4. Remove supernatant.
5. Add 200uL 80% ethanol. And incubate for 30 second. Do not remove the tube from the rack.
6. Place the mixture on a magnetic rack.
7. Remove supernatant.
8. Repeat wash for a total of 2 washes.
9. Air dry the beads.
10. Elute the product with 22uL ultrapure Water by incubation at room temperature for 5 minutes.