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| Time: 6h30m  I. Sample Digestion with Proteinase K. 1h30m  Thaw 5min  Digestion 30  SpeedVac 30  Adjust Vol 10  II. Bisulfite Conversion of DNA 5h  Conversion 4h  DNA purification 1h | **1. Bisulfite conversion of DNA**  Kit: Zymo Research EZ DNA methylation-Direct Kit D5020  Reagent Preparation(see preparations)  I. Sample Digestion with Proteinase K.  1. Setup digestion (100~10k cells)  Materials:  Cells in PBS  Nuclei in NB (Nuclei extraction Buffer)  After FACS, volume of cells ranges from 20~30µL (10µL before FACS), cells were collected in 1.5 ultra smooth Ependorf tube and were spin down before freeze. To avoid cell loss, no removal of medium is applied in this protocol.  Thaw cells, measure volume of cells using pipette man.  Add equal volume of 2x M-Digestion buffer.  Setup digestion for samples  X µL Sample (2k~100k cells)  X µL M-Digestion Buffer (2x)  1 µL Proteinase K  --------------------------------------  2X+1µL Total  Special case will be  20µL Sample  20µL M-Digestion Buffer (2x)  1µL Proteinase K  --------------------------------------  41µL Total  2. Incubate sample at 50°C for 20min.  2.5. Optional: If in-vitro methylated spike are needed, add spike DNA.  3. Concentrate sample volume to less than 20µL with vacuum dryer. Concentrate samples on SpeedVac for 20min. Check every 10min if the volume reduced to less than 20µL (by comparing it with a tube with 20µL H2O).  4. Add H2O to reach 20µL total volume.  II. Bisulfite Conversion of DNA  1. Add 130µL of CT conversion reagent solution to the sample. Transfer all liquid into 200µL PCR tubes/strips for bisulfite conversion on PCR machine.  2. Setup BS conversion program on PCR machine:  98°C 8 min  64°C 3.5 h  4°C Hold (Use 12°C)  3. To bind DNA. Place a Zymo-spin IC column into a Collection Tube and add 600µL M-Binding Buffer.  4. Load treated sample into Zymo-spin IC column containing the M-binding buffer. Close cap and mix be inverting the column several times.  5. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.  6. To wash. Add 100 μl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Discard the flow-through.  7. To Desulphonate. Add 200 μl of M-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15-20 minutes.  8. After incubation centrifuge at full speed for 30 seconds.  9. To wash. Add 200 μl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 μl of M-Wash Buffer and centrifuge for an additional 30 seconds.  9.5 Optional: Discard the flow-through. Extra 30 seconds centrifugation to remove residual EtOH.  10. To elute. Place the column into a 1.5 ml microcentrifuge tube. Add 10 μl of M-Elution Buffer directly to the column matrix. Let stand for 2 min. Centrifuge for 2min at full speed to elute the DNA.  Store DNA at -20°C, for long term storage, store at or below 70°C. Use 2µL (1-4 µL) of eluted DNA for each PCR. |