# Demuxlet Cell Preparation Protocol

## List of reagents and materials

1. RPMI (Glu-) (Gibco; Cat. No.: 21870076)
2. Human Serum (HS) (Sigma; Cat. No.: H4522)
3. Fetal Bovine Serum (Sigma; Cat.No: F2442). Try to use the same lot number across all experiments and sample processing sites.
4. Glutamine (Invitrogen/Gibco; Cat.No.: 25030081)
5. Penicillin Streptomycin (Invitrogen/Gibco; Cat. No.: 15140122)
6. Phosphate Buffered Saline (Ca- Mg-) (Gibco; Cat.No.: 14190144)
7. Bovine Serum Albumin (Capricorn Scientific; Cat. No.: BSA-1S)
8. Wide-bore blue tips (Fisher Scientific; Cat. No.: FIS #14-222-703)
9. Trypan Blue 0.4% (Gibco; Cat. No.: 15250061)
10. 30 µm Macs SmartStrainer (Miltenyl Biotec, Cat. No. 130-110-915)
11. EVE Cell Counting slides (NanoEnTek, Cat. No. 10027-446)
12. QIAamp DNA Mini Kit (Qiagen, Cat no. 51306)
13. Illumina Global Screening Array-24 v3.0 BeadChip (Cat. No.: 20030770).
14. An appropriate single cell reagent kit and instrument for your application, e.g., 10x Genomics Chromium Controller

## Preparation of reagents and media

1. Prepare appropriate volume of thawing media (RPMI + 5% HS + 1% Pen/Strep + 1% Glutamine) and keep it at 4 ⁰C.
2. Prepare appropriate volume of wash media (RPMI + 10% FBS + 1% Pen/Strep + 1% Glutamine) and keep it at 4 ⁰C.
3. Prepare appropriate volume of fresh PBS + 0.04% BSA.

## Thawing frozen PBMCs and preparing the suspension mix

1. Warm up thawing media, wash media and PBS + 0.04% BSA in 37 °C water bath.
2. Transfer 9 mL of 37 °C pre-warmed thawing media into each of the 15 mL Falcon tube.
3. Take the cryovial containing PBMCs out of liquid nitrogen storage, place cryovial on dry ice, and transfer immediately to the 37 °Cwater bath. Thaw for 1-2 min until no visible ice crystals remain.
4. After thawing for 1-2 min, open the cryovial in a biosafety cabinet and add 500 µL-1 mL of pre-warmed thawing media into the cryovial using the 1 mL wide-bore blue tips.
5. After adding the thawing media, use the 1 mL wide-bore blue tips to gently transfer the whole suspension from the cryovial into the 15 mL Falcon tube containing 9 mL of pre-warmed thawing media.
6. Mix the suspension extremely gently by inverting the Falcon tube twice.
7. Centrifuge at 300 x g for 5 min at 21 °C and decant the supernatant. Leave around 200 µL of supernatant behind.
8. Using a serological pipette, gently re-suspend the cell pellet (3-5 times) in 5 mL of pre-warmed wash media.
9. Centrifuge at 300 x g for 5 min at 21 °C and decant the supernatant. Leave around 200 µL of supernatant behind.
10. Using a serological pipette, gently re-suspend the cell pellet (7 times) in 3 mL of pre-warmed PBS + 0.04 % BSA. Avoid bubbles.
11. Repeat steps 9-10 for second wash.
12. After the second wash, centrifuge at 300 x g for 5 min at 21 °C. Decant the supernatant until around 200 µL of supernatant is left behind.
13. Gently re-suspend the cells in 800 µL of PBS + 0.04% BSA (pipette ~10 times).
14. Filter the cell suspension through the 30 µm Macs SmartStrainer to remove clumps or debris. After filtering, keep cells on ice.
15. Thoroughly mix 15 µL of cell suspension with 15 µL of trypan blue. Load 10 µL of mixture into each of the two chambers of a cell counting slide.
16. Let the samples sit in the cell counting slide for 1 min before performing cell counting on an automated cell counter.
17. Make aliquots of 1.50 $×$ 106 cells/mL for each sample (100 µL aliquot per sample).
18. Keep the remaining cell suspension from individual samples on ice for DNA extraction using a QIAamp DNA Mini Kit (Qiagen, Cat no. 51306) according to the manufacturer’s protocol. Perform genotyping using Illumina Global Screening Array-24 v3.0 BeadChip (Cat. No.: 20030770).
19. Mix equal volumes (80 µL) of cell suspension from each sample (up to 16 samples) to make a pooled suspension with a final concentration of 1.50 $×$ 106 cells/mL (total volume: 1280 µL). Keep this pooled suspension on ice.
20. Thoroughly mix 15 µL of the pooled suspension with 15 µL of trypan blue. Load 10 µL of the mixture into each of the two chambers of a cell counting slide.
21. Let the samples sit in the cell counting slide for 1 minute before performing cell counting on an automated cell counter.
22. Count cells from the pooled suspension using an automated cell counter and verify that the concentration is in the range of 1.10 x 106 cells/mL to 1.50 x 106 cells/mL.
23. Proceed with single cell capturing: Load 40,000 cells per 10x well using an appropriate 10x reagent kit for your application and run the 10x Genomics Chromium Controller according to the manufacturer’s protocol.