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| **Reagent List** | |
| **Item Description** | **Reference Number** |
| *Red Top Blood Tube (w/o preservative)* | 366430 - (Bd Vacutainer Labware Medical) |
| *Yellow Top Blood Tube (w/ preservative)* | 364606 - (Bd Vacutainer Labware Medical) |
| *Sterile 50mL Conicals* | 352098 - (Corning Life Sciences Dl) |
| *Sterile Serological Pipettes (5mL/10mL/25mL)* | 356543 / 356551 / 356535 (FALCON) |
| *Ficoll Paque PLUS* | 17-1440-03 - (GE Healthcare - Life Sciences) |
| *Trypan Blue* | T8154 - (SIGMA-ALDRICH) |
| *10X RBC Lysis Buffer* | 420301 - (Biolegend) |
| *1X PBS pH 7.4* | 10010049 - (Gibco) |
| *Hemocytometer* | 3100 - (Hausser Scientific Partnership) |
| *HI FBS* | 10438026 - (Gibco) |
| *DMSO* | D2650-100ML - (Sigma Aldrich Fine Chemicals Biosciences) |
| *Steriflip: 50mL 0.22um Vacuum Filtration System* | SE1M179M6 - (Emd Millipore Corp) |
| *Mr. Frosty Freezing Container* | 5100-0001 - (Thermo Scientific) |
| *Isopropanol* | 190764-4L - (Sigma Aldrich Fine Chemicals Biosciences) |
| *2mL Cryovial* | 5000-0020 - (Nalgene) |
| *0.5 mL Eppendorf Tubes* | 022431005 - (Eppendorf North America Biotools) |

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| **Before Processing** | |
| 1 | Prepare Freezing Media  45mL FBS + 5mL DMSO  Sterile filter the media w/ Steriflip and store @ 4°C  \*\*\*can be stored for up to 2 months once made\*\*\* |
| 2 | Prepare 1X RBC Lysis Buffer  45mL H2O + 5mL 10X RBC lysis buffer  Sterile filter the buffer w/ Steriflip and store @ 4°C |
| 3 | Prepare 1:25 Trypan Blue  2mL Trypan Blue + 48mL PBS |
| 4 | Prepare Mr. Frosty Containers  Fill the internal chamber to the line w/ isopropanol and store in flammables cabinet  \*\*\*refill isopropanol as needed\*\*\* |

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| **Processing (7 yellow + 2 red) for Serum/Plama/PBMCs**  **\*\*\*all processing should take place in a certified BSL2 biosafety cabinet whenever possible\*\*\*** | |
| **This protocol describes the simultaneous processing of iMSMS blood samples for serum/plasma and PBMCs. Note that the protocol switches between these distinct processes to best utilize incubation and centrifugation times.** | |
| 1 | Centrifuge all blood tubes briefly to remove blood from the tops |
| 2 | Take two red top tubes and two yellow top tubes and centrifuge them @2200g for 20 minutes  \*\*\*these will be used for serum (red) and plasma (yellow)\*\*\* |
| 3 | Prepare three 50mL conicals with 15mL of Ficoll at the bottom of each  \*\*\*avoid getting Ficoll on the walls of the conical\*\*\* |
| 4 | Transfer blood from the remaining five yellow top tubes into two empty 50mL conicals evenly  \*\*\*this should be around 50mL total\*\*\* |
| 5 | Dilute the blood 1:2 with PBS and mix by inverting the conicals several times  \*\*\*at this stage you should have 3 Ficoll conicals and 2 blood-PBS conicals\*\*\* |
| 6 | Using a 25mL serological pipette, gently layer the same volume of blood-PBS onto each of the three Ficoll preparations being sure to maintain distinct layers |
| 7 | Centrifuge the Ficoll layers @400g for 40 minutes with NO BRAKE |
| 8 | Retrieve the remaining blood tubes (step 2) from the centrifuge and prepare labelled cryovials for 1mL aliquots of serum and plasma  \*\*\*label: ID#, sample type, date, volume\*\*\* |
| 9 | Using a serological pipette, aspirate as much of the serum or plasma layers as possible without drawing up any cells or touching the blood layer then move the serum/plasma directly into the prepared cryovials  \*\*\*use one 10mL serological pipette to aliquot all plasma from both yellow tops and another 10mL serological pipette to aliquot all serum from both red tops\*\*\* |
| 10 | Place all serum and plasma cryovials into a Mr. Frosty and move into -80°C  \*\*\*these can be moved into 2in. boxes for storage after 24 hours\*\*\* |
| 11 | Once the Ficoll layer spin (step 7) is complete, carefully move the conicals into the hood being sure not to mix the layers |
| 12 | Using a serological pipette, carefully aspirate the cell layers (between the clear Ficoll and yellow plasma-PBS layers) being sure not to take any of the RBC layer and as little Ficoll as possible then transfer evenly into two new 50mL conicals  \*\*\*this is likely to take several passes and may require aspirating most of the Ficoll and  serum-PBS layers\*\*\* |
| 13 | Complete the volume of both sample conicals to 50mL using PBS then centrifuge @400g for 15 minutes |
| 14 | Immediately move the RBC lysis buffer to the hood so it can equilibrate to room temperature |
| 15 | Retrieve the sample conicals (step 13) and pour off the supernatant into waste being sure not to lose the pellet |
| 16 | Re-suspend the cell pellet from one conical in 1mL of RBC lysis buffer by pipette |
| 17 | Transfer 1mL of re-suspended cells into the second conical and re-suspend the pellet by pipette |
| 18 | Using a serological pipette, wash the first sample conical with 4mL of RBC lysis buffer and then transfer the complete volume to wash the second conical |
| 19 | After combining, mix the entire volume by serological pipette  \*\*\*at this stage there is one sample conical with all cells and 5mL of lysis buffer\*\*\* |
| 20 | Incubate @RT for 10 minutes protected from light |
| 21 | Stop the lysis by completing the volume to 50mL with PBS |
| 22 | Centrifuge the sample conical @400g for 10 minutes |
| 23 | Prepare a 0.5mL tube with 90uL of 1:25 Trypan Blue |
| 24 | Retrieve the sample conical (step 22) and pour off the supernatant into waste being sure not to lose the pellet |
| 25 | Re-suspend the pellet in exactly 10mL of PBS |
| 26 | Transfer 10uL of cell suspension into the prepared Trypan Blue and mix thoroughly |
| 27 | Transfer 10uL of the cell-Trypan Blue mixture onto a hemocytometer for cell counting |
| 28 | Count the cells in all four quadrants and then calculate the total number of cells according to the following example |

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| **Example PBMC Counting Calculation** | | | | |
| **Average # of Cells in Quadrants** | **xDilution**  **(Trypan Blue)** |  | **Cells/mL** | **Total PBMC**  **(10mL)** |
| =70 | X 10 | X 104 | 7.0\*106 | 7.0\*107 |

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| 29 | Complete the volume of the sample conical to 50mL with PBS |
| 30 | Centrifuge the sample conical @400g for 10 minutes |
| 31 | Prepare labelled cryovials so that you have one 2mL cryovial for every 1x107-1.5x107 PBMCs  \*\*\*label: ID#, PBMC concentration, date, volume\*\*\* |
| 32 | Retrieve the sample conical (step 30) and pour off the supernatant into waste being sure not to lose the pellet |
| 33 | Re-suspend the pellet in 1mL of Freezing Media by pipette |
| 34 | Using a serological pipette, add an appropriate volume of 4°C freezing media to the sample conical so that the concentration of cells is between 1x107 PBMC/mL and 1.5x107PBMC/mL and is suitable for 1mL aliquots  \*\*\*in the above example you would create seven 1mL aliquots of 1x107 PBMC/mL\*\*\* |
| 35 | Immediately transfer 1mL of the sample into each of the prepared cryovials |
| 36 | Place all PBMC cryovials into a Mr. Frosty and move into -80°C  \*\*\*these can be moved into 2in. boxes for storage after 24 hours\*\*\* |
| Serum and Plasma can be stored @ -80°C long term but PBMCs should be transferred to a -180°C gas phase nitrogen tank within one week. | |