PBMC Thawing

I – Principle

Cryopreserved PBMC/Spleen/Tonsils cells are sensitive to the thawing processes. To ensure optimal cell recovery and viability, thaw protocols are optimised.

II – Safety overview

- All blood samples should be considered an infection risk and all work be performed in Biosafety Cabinet Class II (BSC-II).
- Standard personal protective equipment (PPE) must be worn when performing laboratory work; this includes long-sleeve lab gown, enclosed footwear, safety glasses and gloves.

III – Required reagents and consumables.

MSDS

Chemical Name	CAS. No.
Benzonase Nuclease	9025-65-4
DeNovix Acridient Orange / Propodium Iodide Dye	10127-02-3

Equipment

- Gilson or Finnpipette P10/P20/P200/P1000
- Multichannel pipette
- Eppendorf benchtop centrifuge (Acceleration set at 6, Temp. set at 24 °C)
- Class 2 Biosafety cabinet
- -80 °C Freezer
- MVE 1500 Series -190 °C Liquid N_2 vapour phase
- DeNovix Cell Drop FL

Reagents/Consumables

- Interpath Aerosol Barrier Tips 10 XL For use with Gilson P10 pipette Cat #24300
- Interpath Aerosol Barrier Tips 20 µL For use with F2 Finnpipette P20 Cat #24500
- Interpath Aerosol Barrier tips 200 µL For use with F2 Finnpipette P200 Cat #24700
- Interpath Aerosol Barrier Tips 1000 µL For use with F2 Finnpipette P1000 Cat #24800
- Falcon 50 mL polypropylene tube Cat# 227261
- Copan Interpath sterile 1 mL plastic transfer pipette Cat #201CS01
- Sarstedt 5 mL serological pipette Cat #86.1253.001
- Sarstedt 10 mL serological pipette Cat #86.1253.001
- Costar 25 mL serological pipette Cat #4489
- 1X filtered Phosphate Buffered Saline (PBS), room temp.

- Gibco sterile fetal bovie serum (FBS) Heat-inactivated (HI; 56°C, 45 min) Cat #16000044
- RPMI Cat#22400-089
- Sigma DMSO Hybri-Max Cat #D2650
- CellDrop AO/PI Viability Assay #CD-AO/PI-1.5
- Benzonase Nuclease Cat #15250-061
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IV - SOP

PROCESS	RECOMMENDED TIME/VOLUME/TEMPERATURE/SPEED/ETC
Preparation of media	• Pre-warm water bath to 37 °C. • Warm RPMI and thaw FBS in water bath. • Make up appropriate volume of 10% FBS/RPMI (R10). Approx. 5-10 mL of R10 total is required per PBMC cryovial. • Make up additional volume of R10 containing Benzonase Nuclease (≥250 units/µL). For each PBMC cryovial, need 10 mL with R10 with 2uL of Benzonase. • Warm all media in water bath.
Thaw PBMC/Spleen/Tonsils cells	• Thaw the cells by submerging bottom half of cryovial in 37 °C water bath. Swirl the vials gently, preferably keeping the cap above the water level, until contents of cryovial can move freely. The entire thawing process should be done as quickly as possible. Do not leave the cryovials in the water bath unattended at any time. • Decontaminate cryovial surface by spraying with 80% (v/v) ethanol (EtOH) before placing cryovials in BSCII hood. • Transfer the contents of each cryovial to designated 10 mL tube. • Add 1 mL of warm R10 (containing Benzonase Nuclease) dropwise onto the PBMC/Spleen/Tonsils cells in the 10 mL tube. Rinse the cryovial with an additional 1 mL of the same media and transfer dropwise into 10 mL tube containing PBMC/Spleen/Tonsils cells. • Top up to 9 mL with R10 (containing Benzonase Nuclease) dropwise using a transfer pipette. ○ Centrifuge at 1,500 rpm for 10 min (accel 6, decel 6).
Wash PBMC/Spleen/Tonsils cells	• From this step onwards, Benzonase is NO longer required in any media. Check that media used does not contain Benzonase. • Aspirate supernatant and resuspend the pellet gently with 1 mL of warm RPMI. Top up the tube to 9 mL with warm RPMI.

	• Centrifuge at 1,500 rpm, 5 min (accel 6, decel 6). • After centrifugation, aspirate supernatant and resuspend the pellet in 1-5 mL of RPMI for cell counting. o It is recommended to start with 1 mL RPMI first and proceed with counting. If cell suspension is too saturated, then dilute cell suspension with additional volume of RPMI.

Count PBMC/Spleen/Tonsils cells	• Remove AO/PI staining dye from fridge and equilibrate to room temp.
	• Setup DeNovix Cell Drop FL as described in manufacturer's user guide
	• Open "Primary Cell AO/PI" app and select "BoyleM_PBMC" protocol
	• Label PCR tube and place in PCR tube rack
	• Vortex dye and add 10 µL to empty PCR tube
	• Mix PBMC/Spleen/Tonsils cells in 50 mL tube by gently swirling
	• Add 10 µL cells to PCR tube containing dye and vortex for 3 sec
	• Load 10 µL stained sample into measurement chamber
	  o Check cell solution has evenly loaded ·
	• Allow PBMC/Spleen/Tonsils cells to settle and press "Count 10 µL"
	• Calculate number of live cells
	  o i.e. Live cell conc. x current vol. (mL)

Resuspend PBMC/Spleen/Tonsils cells	• Top up the tube to 9mL of RPMI if volume is <10mL. • Centrifuge at 1,500 rpm, 5 min (accel 6, decel 6). • Aspirate supernatant and resuspend the pellet in the appropriate volume of R10 (WITHOUT Benzonase) to desired cell concentration.