Adapted from the Centre Physiopathologie de Toulouse-Purpan (CPTP), (<https://www.cptp.inserm.fr/wp-content/uploads/2018/01/PBMC-thawing.pdf>)

**Benzonase Treatment for Frozen Cell Samples**

• If PBMCs are not thawed properly, viability and recovery can be compromised. In general, frozen cells should be thawed quickly but diluted slowly to remove DMSO. Cells are then fragile and must be handled gently. Gentle procedures include slow inversions of tubes for mixing (instead of pipetting) and adding the medium on the side of tubes or flasks (instead of on the top of the cells).

• A nuclease treatment step is useful to avoid the cell clumping that results from dying cells releasing DNA. Add pre-warmed complete medium containing 50 units/ml Benzonase per frozen vial followed by a wash step in complete medium (no Benzonase) prior to counting. Cell Technology Limited offers a similar anti-aggregation [product](https://www.fishersci.com/shop/products/ctl-antiagregate-wash-20x-1ml/nc0089914).

 • A 16 to 24 h recovery period after thawing may be beneficial when doing functional studies such as cytokine expression (recovery period is dependent on assay).

 **Reagents**

• RPMI-1640 with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine

• Benzonase nuclease (purity 99%) 250 000 units/ml (Sigma E8263) 🡪 final conc. = 50 units/ml

**Protocol**

1. Warm complete medium to 37°C in a water bath.
2. Remove vials from liquid nitrogen and transport them to the lab on dry ice or liquid nitrogen.
3. Thaw frozen vials in a 37°C water bath. When cells are nearly completely thawed, carry the vials to the hood and wipe with 70% EtOH.
4. Open the vial and slowly add 1 ml of warm thaw media (RPMI + 50 units/ml Benzonase) drop wise (up to 50 million cells per vial).
5. Spin the cells at 300xg for 5 min at room temperature. Remove supernatant carefully with a pipette.
6. Resuspend cells in 1 ml of thaw media and transfer to a 5 mL FACS tube. Wash with a total of 4.5 ml thaw media.
7. Perform an additional wash with complete medium in the absence of Benzonase.
8. Count cells and determine viability.
9. **Optional Recovery Period for Functional Studies.** Incubate PBMCs (1-5 x106 cells/ml) in complete medium without Benzonase at 37°C and 5% CO2 for 16 to 24 hr. After the rest, pellet cells and conduct a second Benzonase wash as described above (steps 6-7).
10. Resuspend as directed in subsequent protocols.
11. Follow with mass cytometry viability stain protocol.