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Working

CAN-ASC CONSENSUS PROTOCOL: Isolation, Cryopreservation and Thawing of Peripheral Blood Mononuclear Cells

CAN-ASC Canadian Autoimmunity Standardization Core[1]

[1]BC Children's Hospital Research Institute

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CAN-ASC Canadian Autoimmunity Standardization Core
BC Children's Hospital Research Institute

EXTERNAL LINK

https://www.bcchr.ca/CAN-ASC/protocols

CAN-ASC_PBMC isolation, cryopreservation and thawing_20190321.pdf

GUIDELINES

- Caution: human blood and blood-derived products are a potential source of contagious human pathogens. When working with these products, observe general biosafety level 2 precautions (refer to your local biosafety guidelines for details).
- The blood, all centrifuges and all reagents which come into contact with the blood or blood derivatives should be at room temperature (RT) until peripheral blood mononuclear cells (PBMCs) are frozen. Before starting, ensure all freezing containers are also at RT.
- The cells should not be allowed to sit in a pellet after centrifugation. Always resuspend the cell pellet by flicking before adding re-suspension buffer.
- Age of blood: after about 2-4 h, granulocytes will start to degranulate and sediment with the PBMCs so for the best results, isolate as soon as possible after draw. If blood needs to be shipped to a central site or isolated the next day, downstream assays should be tested on fresh and aged blood to ensure comparable results.
- It should be noted that most of these recommendations are based on experiments with T cells.

MATERIALS TEXT

Note: as we are trying to 'harmonize' and allow flexibility with reagents, we have avoided recommending specific products/manufacturers.

Density Medium: Lymphoprep, Ficoll-Paque
FBS: heat-inactivated for 30 min at 56°C and filtered.
Cryoprotective medium: CryoStor CS-10
Benzonase Nuclease or DNAse I

- Washing buffer: Wash buffer is 2% (heat-inactivated) FBS in PBS; addition of 1-2 mM EDTA can inhibit downstream applications. Serum-free alternatives to FBS still need to be found and tested as 0.5% HSA is not equivalent (see Figure 2).
- Cryopreservation medium: for CAN-ASC protocols we recommend using CyroStor CS-10.
- Thawing medium: 10% FBS in RPMI. Serum-free alternatives still need be tested.

SAFETY WARNINGS

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BEFORE STARTING

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(RT) until peripheral blood mononuclear cells (PBMCs) are frozen. Before starting, ensure all freezing containers are also at RT.

Isolation and Cryopreservation of PBMCs

1 Draw blood using an appropriate anti-coagulant and ensure it is filled to the top and immediately inverted 5 x to prevent clotting.

- *Choice of anti-coagulant will depend on downstream application; heparin is better for processing delays but not for DNA isolation, EDTA may inhibit functional assays.*
- *When possible draw blood at a similar time each day.*
- *If drawing into different collection tube types, draw in the following order: citric acid/serum/heparin/EDTA.*
- *Note time between blood draw and processing.*

2 If collecting plasma, note volume of blood, centrifuge for 5 min at 500 x g. Collect clear plasma into new tubes and chill on ice. Centrifuge at 4°C for 10 min at max speed, collect supernatant and freeze at -80°C.

Store plasma aliquots in screw-top tubes with O-rings to prevent sublimation during long-term storage.

3 Invert blood to mix and transfer from collection tubes into falcon tubes. Note the original blood volume and add the same amount of PBS, first rinsing the collection tubes.
If plasma has been collected, add extra PBS so that the final volume is 2 x the original blood collection volume.

- *Avoid chilling blood before processing; keep at RT. If shipping, use insulated boxes.*
- *Optimally, blood should be processed within 3 h and avoid delays of >24 h.*
- *If starting with buffy coat, the cell suspension should be diluted more to obtain optimal yields. We usually increase the original volume by 3-4 x with PBS.*

4 Add density gradient medium (eg. Ficoll, Lymphoprep) into falcon tubes, 0.43 mL of medium for every 1 mL of blood. (15 mL medium for 35 mL blood).

5 Add the diluted blood to the top of the gradient medium so that it slowly overlays the surface without turbulence and mixing. **A crisp interface is critical for good yields.**
Ensure you move as quickly as possible to step 6 as the gradient medium has toxic effects on cells if they are left sitting on top for too long.

- *Tilt the tube horizontally and let the blood run down the side of the tube and slowly pool over the top of the density medium; as the layer of blood builds up, the blood can be added more quickly.*
- *It is also possible to underlay the blood.*
- *This step can be simplified for less experienced technicians by use of tubes with barriers (eg. SepMate) but these are not foolproof and require proper training.*

6 Centrifuge at 580 x g (1700 rpm) for 30 min at RT with the **acceleration set to 1 and the brake set to 0.** Immediately after centrifugation, carefully remove the tube from the centrifuge without disturbing the layer.

7 Collect the cloudy layer of PBMCs at the interface between Ficoll and diluted plasma (see Figure 1) using a sterile transfer pipette and transfer to a 50 mL Falcon tube containing ~25 mL washing buffer. Try to take over a minimum of density medium while getting all of the PBMCs. Top up to 50 mL with washing buffer (2% fetal bovine serum (FBS) in PBS).

The time in which the cells are in contact with density medium should be kept to a minimum

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Avoid combining more than two PBMCs layers in one wash tube.

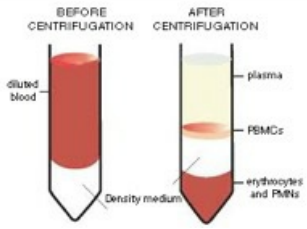

Figure 1: Isolation of PBMCs by density gradient centrifugation. PMNs= polymorphonuclear leukocytes.

8 Centrifuge at 450 x g (1500 rpm) for 10 min at RT and remove supernatant.

Increased density caused by addition of density medium will necessitate longer centrifugation times.

9 Resuspend the pellet in washing buffer. Centrifuge for 10 min at 130 x g (800 rpm) and carefully aspirate supernatant with a large pipette (don't decant!). Discard supernatant.

This step removes platelets which will not pellet at this low speed; however, the PBMC pellet will be loose, so remove supernatant immediately after the centrifugation using a pipette and leave ~ 1 mL of washing buffer in the tube covering the pellet.

10 Resupend the pellet in washing buffer so that the volume is approximately that of the original blood sample. Take a sample for counting.

Red blood cells (RBC) can look very similar to lymphocytes in a hemocytometer post trypan blue stain so it is important to use a counting method that is able to differentiate RBCs from lymphocytes. Either lyse RBCs before counting or include a nuclear stain.

11 Centrifuge for 450 x g (1500 rpm) for 5 min at RT. Prepare cryopreservation tubes.

12 Remove all supernatant. Resuspend the cells in 1 x CryoStor CS-10 at RT so that cell concentration is between 2 x 10^6 and 50 x 10^6 cells/mL.

CAN-ASC protocols should use Cryostor CS-10 when possible.
If preparing alternative 'labmade' cryoprotectant media, make fresh on the day of use. Note that there is a strong exothermic reaction when combining dimethyl sulfoxide (DMSO) with aqueous solutions.

13 Immediately aliquot cells into 2 mL cryopreservation tubes (volume should not be less than 250 mL) and freeze using a CoolCell, a Mr. Frosty (at RT) or a controlled rate freezer. CoolCells or Mr. Frosties should be placed in a -80°C freezer for a minimum of 16h (overnight) before transferring to liquid nitrogen.
Perform transfer to liquid nitrogen tanks on dry ice as cryovial contents can rise from -80°C to over -50°C in less than one minute if exposed to RT air

- *If cryopreserving small volumes (<250 mL), short cryotubes with the same diameter should be used.*

- *Cells should be cooled at a rate of 1°C/min. If using Mr. Frosties, be sure to follow manufacturer's instructions, ensuring to replace isopropanol after every fifth use.*
- *If using a CoolCell ensure the core (black) ring is inserted and all components are at same temperature as cells.*

14 Store in liquid nitrogen (vapour phase is preferable) and if shipping use a liquid nitrogen dewar. Keep temperature as stable as possible.

Thawing of PBMCs

15 Per cryovials thawed, preheat 10 mL of thawing solution to 37°C (avoid chilled medium). Thawing medium: 10% FBS in RMPI.

- *Most thawing solutions contain at least 10% serum, this may be replaced with bovine serum albumin/human serum albumin (HSA) but would need to be tested. A serum free medium could be used but there is not much literature that compares these alternatives.*
- *For blood with high levels of contamination with granulocytes (24 h blood, patients on mobilizing medications) DNAse (10-20 U/mL) or Benzonase (25-50 U/mL) should be included in thawing medium and first wash to prevent clumping.*

16 Rapid-thaw cryovials at 37°C for 5 min by partial immersion in a waterbath, and transfer into the pre-warmed thawing medium. Handle the cells gently and avoid creating bubbles. Gently rinse out cryovial with some of the diluted cell suspension.

- *More than 1 comparison saw no difference between fast and slow dilution of cells with thawing media if the latter was warmed to 37°C*
- *Note: if cryovials were stored in liquid phase of liquid nitrogen tanks then there is a risk of vials exploding. Ensure appropriate personal protective equipment is worn.*

17 Immediately centrifuge cells for 5 min at 500 x g at RT. Remove supernatant.

18 Gently flick cells and resuspend in 10 mL of RT (not chilled) assay buffer. Centrifuge as above. Remove supernatant. If downstream assays are serum-free, repeat the wash to ensure removal of all serum.

There is evidence that cells washed 2 x perform better in downstream assays than cells washed only 1 x due to complete removal of DMSO and dead cells.

19 Resuspend pellet in assay buffer. Take a sample for counting and adjust to assay concentration.

- *Immediate post-thaw viability as measured by trypan blue, fixable viability dye uptake, 7AAD or PI will not accurately reflect the condition of thawed cells; in order to see true health of cells, measure in an apoptosis assay containing a live cell dye and Annexin V (eg. cat.# ab176749 from Abcam).*
- *Or count cells again after incubating in complete media overnight.*

Appendix: Key Points

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- Cold vs. RT cryopreservation medium with conflicting reports. Kreher[1] showed clearly that addition of 25°C was superior while Tree showed that cold was better[2] (although the media was different). Nazarpour[3] showed no significant differences. Several papers show that up to 10% DMSO is not toxic to hematopoietic cells for up to 1 hour even when not chilling.[3-5]
- CS-10 compared to 90% FBS and 10% serum showed no difference in post-thaw viability or recovery[6] (they also did some cell culture of natural killer cells and cytotoxic T lymphocytes so this is based on more than post-thaw viability).

- Post-thaw PBMC can tolerate 10% DMSO up to 30 min even at 37°C.[7]
- There is no difference between partial and complete thawing of cells; leaving them for 5 min at 37°C is fine.[7, 8]
- Thawing medium should be pre-warmed; prewarming medium should contain 10-20% serum if possible.[7, 9] The proprietary anti-aggregate medium sold by Cellular Technology Limited (CTL) is a rare serum-free alternative.
- Honge et al.[8] compared thawing in PBS with 20% and 100% serum but no alternatives; they found better viability but lower recovery in 'harsher' PBS medium reasoning that the dying cells were removed compared to a softer medium.
- A second wash for DMSO removal after thaw improves cell recovery.[7]
- Freeze cells at concentrations from 2 x 10^6/ml [10] up to 50 x 10^6/ml.[10, 11]
- Mr. Frosties and CoolCells are comparable to rate-controlled freezers, if used correctly.[10]
- After thaw, rest PBMCs overnight at 37°C at 2-5 x 10^6/ml before assaying.[12, 13]
- Shipping must be done in liquid nitrogen.[12, 14]
- **Note:** the point was raised that some labs use PBS as a washing buffer without FBS. We compared PBS alone with PBS supplemented with either 2% FBS or 0.5% HSA and found that 2% FBS is superior to both (see Figure 2).

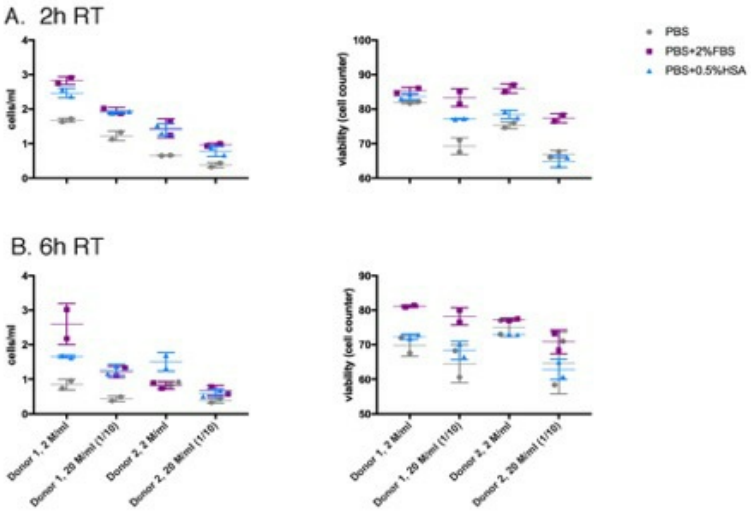

Figure 2: PBS tolerance test.
Thawed PBMCs were plated at 2 x 10^6/ml in either PBS, PBS+2% FBS or PBS+0.5% HSA and left at RT at either 2 x 10^6/ml or 20 x 10^6/ml for either 2 h (A) or 6 h (B). Cells were stained with viability and nuclear dyes (PI and AO) and counted using a Nexcelom Auto 2000 cell counter. Shown are cell counts (left) and viability (right). Data is from 2 independent counts of each sample.

Appendix: References

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