

IDS-TCW Freezing Study I

INTRODUCTION

T cell reactivity is thought to play a major role for pathogenesis of type 1 diabetes (T1D). Previous studies have shown that there are multiple different ways to measure islet specific T cell responses, yielding different outcomes with great variability. One important aspect to harmonize in order to compare results from different laboratories is that of preparation of blood cell material from diabetes and non-diabetes subjects.

Previous work has been done to evaluate immune responses to recall antigens and mitogens using fresh and frozen cells, without yielding a clear recommendation. The aim of the current IDC TCW Freezing Study I is to investigate optimal way(s) to prepare and freeze immune cells from T1D and control subjects.

Objective

To define an initial consensus protocol for PBMC freezing and thawing which is able to:

- 1) Yield results that most faithfully match results observed using fresh cells across several T cell assay platforms.
- 2) Provide the best cell recovery and viability.

It is not an objective of this study to evaluate the performance of different T cell assays, e.g., to define their ability to separate type 1 diabetes (T1D) subjects from healthy ones. The novelties of this study as compared to previous efforts are: i) the use of beta-cell antigens (Ags) rather than recall stimuli, as autoimmune responses may be subjected to different rules; ii) multicentric, blinded validation; iii) validation over a wide range of T cell assay platforms rather than a single specific one. This initial, small-size study will subsequently form the basis for further freezing optimization initiatives, to be implemented on a wider scale.

Outline

* Two different freezing protocols will be evaluated:

- 1) “Warm” protocol, where all freezing reagents are used at room temperature
- 2) “Cold” protocol, where all freezing reagents are used at 4°C

* In addition, participating laboratories have an option to add their local freezing protocol of choice, if desired.

* These protocols will be tested in each participating Laboratory using 5 blood draws from T1D patients and 5 blood draws from age- and gender-matched healthy subjects.

* The study will be performed in a blinded fashion. To this end, test Ags will be centrally blinded using randomly generated codes. Different codes will be used for each set of Ag aliquots, where each set is to be used within a single assay session. This means that participating Laboratories will not only be unaware of which Ags they are testing, but also of the correspondence between the coded Ags tested on fresh blood vs. those tested on the frozen fractions. This procedure has been designed to make blinding of the starting fresh blood samples unnecessary. Such blinding would in fact be inappropriate, as it needs to be performed locally rather than centrally, thus resulting open to potential bias.

* Data analysis will also be centrally performed, according to the assay-specific criteria for positive responses defined beforehand by each participating Lab.

* Detailed procedures are outlined in the enclosed Standard Operating Procedures (SOPs).

* Participation to this initial pilot study will be limited to few Laboratories within the T Cell Workshop Steering Committee. These Laboratories have the task to define which one between the “warm” and the “cold” protocol is the best performing (i.e., the one which leads to better cell recovery, viability, and reproducibility as compared to fresh samples).

* The chosen protocol will subsequently be used as a “golden standard”, against which novel freezing procedures will be tested. These further freezing studies will be open to all IDS Laboratories willing to participate.

IDS-TCW Fresh-Frozen SOP 1.3

Materials

Sodium heparin vacutainers (17 I.U. Na Heparin/ml, BD 368480)

Sterile 50 ml polypropylene tubes.

Sterile 15 ml polypropylene tubes

RPMI (e.g. Invitrogen 52400-025) at room temp.

Lymphoprep (Axis Shield)

Freezing solution A (100% human serum) and B (80% human serum + 20% DMSO)

– centrally provided

10ml serological pipettes

Wide bore sterile plastic Pasteur pipettes.

1.8ml cryovials (Nalgene/Nunc 368632 or Fisher12-565-171N)

Nalgene Mr Frosty filled with 2- propanol (Nalgene 5100- 0001 or Fisher#15350-50).

Trypan blue

Neubauer hemacytometer

FACS tubes

Becton Dickinson Cellfix

Monoclonal antibodies:

CD3-FITC

CD8-PE

CD4-PerCP

CD19-PE

CD14-PerCP

CD56-PE

Isolation of peripheral blood mononuclear cells (PBMC) from whole blood

- I1. Obtain blood by venepuncture in a vacutainer and allow the blood to come to room temperature (~30 mins). Invert tube carefully 5 times to ensure complete mixing with heparin. Do NOT shake. Blood samples should be stored at RT (20-25°C) and processed within 16 hr of drawing.

- I2. Transfer blood from vacutainer to sterile 50 ml polypropylene tube(s) and add an equal volume of RPMI (at room temp) mix gently by inversion. In the example below 30 ml of blood is used so 30ml of RPMI is added.

- I3. Aliquot 20ml lymphoprep (stored in the dark) into a 50 ml polypropylene tube, tilt tube and very slowly overlay with 30ml blood/RPMI mix (1:1). For an initial volume of 30ml of blood this is performed in 2 tubes.

- I4. Centrifuge at 400g for 40 minutes at room temperature in a swing-out rotor with brake set to off at room temperature.

- I5. Remove freezing media from the fridge. Place Warm media A and B at room temperature, place Cold media A and B on ice.

- I6. When centrifugation has finished carefully remove tubes. You should see 4 layers – the lymphocytes are in the 2nd layer from the top. The first layer is plasma and the third layer is the lymphoprep with red cells forming the fourth layer. Remove the plasma layer to within 1 cm of the lymphocytes, and transfer to another sterile tube if required for antibody testing otherwise discard. Remove the lymphocytes with a plastic Pasteur into a fresh universal tube. The lymphocyte layer from each universal should be dispensed into a separate 15 ml polypropylene tube (i.e. contents of 1 tube into 1 tube)

- I7. DO NOT TAKE UP THE CLEAR GRADIENT OR RED BLOOD CELLS:

- I8. Fill tubes to the top with 12ml RPMI , balance tubes and centrifuge at 300g, for 10 mins at room temp.
- I9. Remove supernatant carefully with 10ml pipette taking care not to disturb the pellet, (do not pour off). The pellet should be white with no red blood cell contamination.
- I10. Resuspend the pellet gently in fresh RPMI using a 10ml pipette and combine the contents of the two tubes into 1 15 ml polypropylene conical tube, centrifuge again at 200g for 10 mins at room temp.
- I11. Remove supernatant carefully with 10ml pipette taking care not to disturb the pellet, (**do not pour off**). Resuspend cells gently in medium to be used for functional assay. To allow for accurate counting, volume of media added should allow for a final concentration of cells of between $1-4 \times 10^6$ /ml.
- I12. Count PBMC using the protocol shown below.
- I13. Calculate the volume of cells required for the assay using fresh cells. Ensure that the cells are well suspended by gently pipetting up and down 3-4 times and transfer required number of cells for fresh assay to a 15 ml polypropylene conical tube.
- I14. For **FACS analysis** prepare 6 tubes according to the following scheme:
(use 10 μ l of each fluorescence conjugated antibody or titrate)
- Tube 1: CD3-FITC
Tube 2: CD8-PE
Tube 3: CD4-PerCP
- for compensation control.
- Tube 4: CD3-FITC **and** CD8-PE **and** CD4-PerCP
Tube 5: CD3-FITC **and** CD19-PE **and** CD14-PerCP
Tube 6: CD3-FITC **and** CD56-PE **and** CD4-PerCP
- Take 0.6ml of PBMCs (adjusted to 2×10^6 cells /ml) and mix it with 0.6 ml Cellfix (1:10; BD)
- Or:** transfer 1.2×10^6 cells in a FACS staining tube and add BD Cellfix add 1.2ml.

Add 200µl of cell suspension to each tube and vortex well (start with tube 4,5,6)

Incubate for 15 min at room temperature in the dark
Vortex again and incubate another 15 min

Add 1.9ml Cellwash to each tube and centrifuge 5min at 300g, discard supernatant and resuspend the cells in 500µl Sheathfluid

or: add 300 µl Sheathfluid mix tube 1, 2 and 3 in one tube for compensation control

For tubes 4, 5 and 6 acquire 20,000 events in a Lymphocyte gate (R1 = G1) but save without a gate (Acquisition will stop when 20000 events are in R1, but: save all events)

Divide the rest of the PBMC equally between two 15 ml polypropylene conical tubes, one labelled 'cold protocol' and the other 'warm protocol'

- I15. Prepare cyrovials: Freeze cells at $5-10 \times 10^6$ cells/vial, do not freeze less than 5×10^6 cells in a vial. For example, if 17×10^6 cells are to be frozen by the warm protocol and 17×10^6 cells are to be frozen by the cold protocol then prepare 2 cyrovials for Warm and 2 cryovials for Cold and dispense 8.5×10^6 cells in each tube. Freeze the same number of cells per tube and the same numbers per tube for each freezing protocol.
- I16. Label all cyrovials with patient ID, cell number, date, and protocol (Warm, Cold or Lab specific).
- I17. Place cyrovials for Cold protocol on ice.
- I18. Centrifuge PBMC at 300 g, for 10 mins at room temp. Resuspend cells for fresh assay in appropriate volume of media. Proceed with the two freezing protocols prior to setting up assay with fresh cells.
- I19. Establish functional assay with fresh cells as set out in assay SOP.
- I20. Note reference number of coded stimulation aliquots used in assay.
- I21.

Proceed to freezing protocols.

All laboratories will perform the standard cold and warm freezing protocols laid out in the following SOPs. If desired a laboratory may also perform its own freezing protocol

Please alternate the order in which freezing protocols are performed, e.g. on one sample perform the cold freezing protocol first and on the next sample perform the warm freezing protocol first. Similarly, if using a laboratory is using its own specific

freezing protocol then please rotate the order in which each freezing protocol is performed.

Cold Freezing-Thawing Protocol:

Perform all steps on ice ensure cryovials are cold prior to adding cells to them.

- CF1 Resuspend the cell pellet by gentle tapping, add the appropriate amount of ice cold Freezing Medium A to adjust the cell concentration to $20 \times 10^6/\text{ml}$. Mix cells by gently tapping the tube; do not use a pipette. Ensure cells are in a single cell suspension.
- CF2 Slowly, drop by drop, add an equal volume of Freezing Medium B (20%DMSO) to Freezing Medium A containing the PBMCs using a wide bore pastette, mix the cells by a gentle continual swirling motion during the addition to ensure steady mixing of the two freezing solutions. This step should take 2 minutes. GENTLY pipette up and down 3 times using a serological pipette. Avoid bubbles. The final concentration should be $10 \times 10^6/\text{ml}$.
- CF3 Once mixing is complete, aliquot appropriate volume of PBMC suspension per cryovial on ice (note $1\text{ml} = 10^7$ PBMC).
- CF4 Freeze the cryovials using one of the following processes.
- CF5 Place the cryovials into a Nalgene Cryofreezing Container, the box should be at RT and should be filled to prescribed line with fresh RT isopropanol. Put the freezing container into -70°C freezer for a minimum of 12 hours. Then remove cryovials and store in -70°C freezer. During the freezing time avoid opening the freezer in order to avoid shaking the cryovials or raising the freezer's temperature.
- CF6 If Nalgene Cryofreezing Containers are not available, the following "low technology" method works well. Place the cryovials in a Styrofoam tube container (they are supplied with the 15ml conical tubes) to reduce direct contact with cold surfaces and to slow the rate of freezing. Place a second Styrofoam container of the same type over the first one, tape the two containers together, wrap in a plastic bag (leave some air in the plastic bag) and tie the bag shut. Place the bagged container with the cells in -70°C freezer for at least 12 hours. During the freezing time avoid opening the freezer in order to avoid shaking the cryovials or raising the freezer's temperature.
- CF7 Transfer cryovials to liquid nitrogen.

ITN Warm Freezing Protocol:

WF1 After re-suspending the cell pellet by tapping, add the appropriate amount of room temperature Freezing Medium A to adjust the cell concentration to 20×10^6 /ml. Mix cells by gently tapping the tube; do not use a pipette.

WF2 Slowly, drop by drop, add to the side of the tube an equal volume of Freezing Medium B (20%DMSO) using a wide bore pastette to Freezing Medium A containing the PBMCs. To mix the cells, GENTLY pipette up and down 3 times using a serological pipette. Avoid bubbles. The final concentration should be 10×10^6 /ml.

WF3 Once mixing is complete, aliquot appropriate volume of PBMC suspension per cryovial (note $1\text{ml} = 10^7$ PBMC).

WF4 Freeze the cryovials using one of the following processes.

WF5 Place the cryovials into a Nalgene Cryofreezing Container, the box should be at RT and should be filled to prescribed line with fresh RT isopropanol. Put the freezing container into -70°C freezer for a minimum of 12 hours. Then remove cryovials and store in -70°C freezer. During the freezing time avoid opening the freezer in order to avoid shaking the cryovials or raising the freezer's temperature.

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WF7 Transfer cryovials to liquid nitrogen.

Thawing of cells and use in functional assays.

Cells can be thawed for functional assays after a minimum of one month in liquid nitrogen and a maximum of 3 months.

Thaw vials frozen using each method with the appropriate thawing method.

Thawing protocol for Cold Freezing Procedure.

- CT1. Remove the cryotubes from liquid nitrogen and transfer them to the culture room on dry ice
- CT2. Rapidly transfer vials from liquid nitrogen to a 37°C water bath and gently agitate until the contents are almost completely thawed (a small piece of ice should remain at the time of removing cells from the water bath).
- CT3. Add 1ml complete medium/10% human AB serum dropwise to the vial using a wide bore pastette gently mixing by stirring with pastette.
- CT4. Transfer cells to a 15 ml sterile polypropylene tube using a 10 ml serological pipette and add 10 ml complete medium/10% human AB serum dropwise over 3 minutes. During this time, gently mix the cells by swirling to ensure slow and even dilution of DMSO.
- CT5. Centrifuge at 300g for 10 minutes at RT. Remove the supernatant.
- CT6. Resuspend the pellet with 10 ml of complete medium+10% human AB serum.
- CT7. Centrifuge at 300g for 10 minutes at RT. Remove the supernatant.
- CT8. Resuspend the pellet in assay buffer at approximately 4×10^6 /ml.
- CT9. Leave cells at room temperature for 1 hr
- CT10. Count cells using protocol shown below.
- CT11. Perform FACS analysis as described in section I14 above.
- CT12. Resuspend the rest of the cells at required concentration and establish functional assay as set out in assay SOP.
- CT13. Note reference number of coded stimulation aliquots used in assay.

Thawing protocol for Warm Freezing Procedure.

- WT1. Remove the cryotubes from liquid nitrogen and transfer them to the culture room on dry ice
- WT2. Quickly thaw the cryotubes in a 37°C water bath or in a 37°C incubator with gentle agitation.
- WT3. When the tubes start to thaw (there is still a solid frozen core surrounded by liquid), pour the content of the tube in 4 volumes of complete medium with 10% human AB serum that was kept at room temperature. Mix gently by pipetting 3 times.
- WT4. Centrifuge at 300g 10 minutes, remove the supernatant.
- WT5. Resuspend the pellet with 5 ml of complete medium+10% human AB serum.
- WT6. Centrifuge @300g, 10min. Remove the supernatant
- WT7. Resuspend the pellet in assay buffer at approximately 4×10^6 /ml.
- WT8. Leave cells at room temperature for 1 hr
- WT9. Count cells using protocol shown below.
- WT10. Perform FACS analysis as described in section I14 above.
- WT11. Resuspend the rest of the cells at required concentration and establish functional assay as set out in assay SOP.
- WT12. Note reference number of coded stimulation aliquots used in assay.

Counting Cells

CNT1. To get an equal cell distribution mix cell suspension prior to adding the stain and again just before loading the hemacytometer.

CNT2. To prepare the hemacytometer, first clean the hemacytometer with H₂O and then with 70% ethanol. Dry it off with a Kimwipe.

CNT3. Staining cells with Trypan Blue: On a piece of parafilm (parafilm can be replaced by Eppendorf tube or well from 96-well plate) combine 20 μ l of cell suspension that was set aside in 5.2.12 with 20 μ l of 0.4% Trypan Blue (1:1). Mix well with pipette. Note: After mixing cells with Trypan Blue, count cells immediately. Your goal is to achieve an accurate cell distribution with cell clumping kept to a minimum.

CNT4. Center a cover glass over the hemacytometer chambers.

CNT5. Fill one chamber with 10 μ l of the cell dilution using a 20 μ l pipette. The solution will pass under the cover glass by capillary action. Do not over fill. Allow the cell suspension to settle in the hemacytometer for at least 10 seconds before counting. If the solution spreads into the two lateral grooves adjoining the grid table, clean the hemacytometer and repeat the application. If there are any bubbles in the solution covering the grid table clean the hemacytometer and repeat the application. Place the hemacytometer on the stage of a microscope and adjust focus using 10X objective.

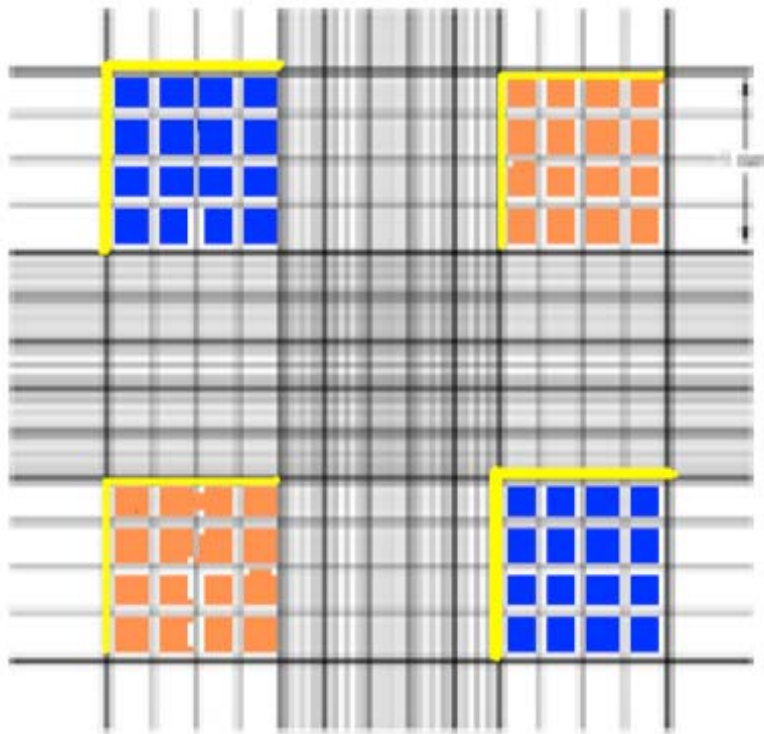
CNT6. magnification, then change to 20X and refocus if necessary.

CNT7. Assess if the cells are evenly distributed among the squares.

CNT8. PBMCs may contain population of erythrocytes. Use caution when counting cells to distinguish lymphocytes from erythrocytes.

CNT9. Count live cells (=not blue) in the four large corner squares. Squares are represented in the image as blue and orange. Include cells that touch either the top line or left vertical perimeter line of any corner square (indicated with yellow lines). Do not count any cells that touch either the bottom line or right vertical perimeter line of any corner square. Determine the number of cells per ml. Blue cells are dead and clear are alive. Count live and dead cells and record the results separately.

View of heamocytometer:



View of one of the blue/orange squares:

