

IDS-TCW Freezing Study I

Analysis of freezing protocols suitable for the detection of islet autoreactive T cells.

Aim: To identify a common protocol for PBMC freezing and thawing which is able to:

- (i) Yield results in functional assays that most faithfully match results obtained using fresh cells.
- (ii) Provide the best recovery and viability.

We will utilize an experimental protocol where we can compare fresh and the 2 main freezing protocols and, if desired a labs own freezing protocol. This will be performed in a multicentric fashion in all Laboratories willing to participate. Each laboratory would use its own T cell assay to examine islet specific T cell responses in locally recruited T1D and healthy subject samples and ask **the two key questions:**

- (i) What is the effect of freezing on assay performance?
- (ii) Which freezing protocol gives results that most faithfully match results observed using fresh cells?

Primary outcome: It is important to stress that this study is **not** designed to identify a ‘good assay’ or ‘good antigens’ for discriminating patients from controls.

Rather we are trying to identify a good freezing protocol that most faithfully mimics the results seen in fresh samples. This will be judged by the concordance of responses to each islet antigen within an individual between fresh and frozen samples.

Proposed protocol:

Each participating lab would prepare PBMC from 5-10 of T1D patients and an equal number of healthy age-matched controls using the attached SOP for Cold and Warm protocols (and a labs own freezing protocol if appropriate).

The *assay of choice* for that laboratory would then be performed on a fresh sample and two cryopreserved samples prepared by each of the standard freezing protocols attached.

The assay of choice must include both a positive and negative control and a number of preparations for detecting islet autoreactive T cells. These may vary slightly for different assays but for all cytokine production assays PMA-ionomycin, the recall antigen Pediacel and the CEF peptide pool will be used as a positive control.

To ensure the data obtained is free from any bias we will require:

(i) Criteria to be defined before starting the experiment on what constitutes a positive response, e.g. a stimulation index of greater than 3 indicates a response to that stimulus. These criteria need to be declared in the individual assay SOP and need not be the same for each assay. It is important that the value reported for a given stimulus gives both a definitive response/no response decision and a measure of the magnitude of response. The assay SOP must be provided to the Central laboratory at the time of antigen blinding (see below).

(ii) Each lab will need to send out the antigens to be assayed and any appropriate negative control (for example if peptides are all dissolved in DMSO then a sample of DMSO should be provided) to the central lab to be blinded and sent back as single coded aliquots for each assay. Results for each stimulus and each individual must then be sent back to the central lab for decode and analysis by the criteria as outlined in (i).

In addition to assay parameters all laboratories would be expected to record yield, viability and composition of fresh and both frozen populations using the standard method provided in the SOP.

Data analysis and interpretation

Results for each of the freeze-thawing protocols will then be compared to the results from fresh samples using 2x2 contingency tables and Chi-squared analysis as illustrated below. This analysis will be performed on an individual lab basis, and also by pooling data from similar assay types (e.g. proliferative assays, cytokine secretion, ELISpots etc).

This analysis will be performed on an individual assay basis, i.e. scoring each individual assay point for each patient or control as positive or negative based on the criteria set out at the start (e.g. an SI of >3 for a single peptide response measured by ELISpot) and summing all responses in the relevant quadrants.

Proposed workflow:

The participating Labs send a (i) copy of their experimental protocol (ii) details of proposed analysis methods, (i.e. how a response will be called as positive or negative and how the magnitude of the response will be assessed) and (iii) a list of the antigens to be tested (in addition to the standard stimuli everyone will use) to the Central lab.

Participating labs send test antigens to Central lab.

Central lab aliquots, codes and returns coded test and standard antigens along with freezing solutions to participating laboratories.

Participating labs perform analysis.

Participating labs return raw data to Central laboratory for decode and analysis.