Establishment of Quality Assurance Program for PBMC Handling to Obtain Reliable Immunological Assessment

Kim Ellefsen, Lucienne Poupon, Emmanuelle Medjitna-Rais, Sara Santoro, Giuseppe Pantaleo and Alexandre Harari

Vaccine and Immunotherapy Center, Service of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

ABSTRACT

Objectives: Appropriate handling of blood samples is a critical issue in clinical trials in order to obtain reliable immunological assessment..
Methods: Within our certified (ISO17025) immune-monitoring platform, the experience acquired over the past decade in the management of multiple multicenter international clinical trials and the knowledge/expertise gained from collaborations with international consortium involved in immune-monitoring allowed us to identify critical steps to control and monitor in order to consistently guaranty reliable immunological assessment.
Results: The following steps and controls need to be implemented, validated and controlled:

- •PBMC isolation, processing and cryopreservation have to be performed according to validated SOP and limited timing. Quality controls on samples obtained from different sites need to be performed on a regular basis.
- •GO/NO-GO steps based on the quality of the samples (based on T-cell viability, recovery and reactivity) need to be implemented for T-cell functional assays.
- •Internal specific controls (e.g. determination of CMV or EBV-specific T-cell responses) have to be implemented in all functional assays monitoring. immunological parameters and these responses must be analyzed longitudinally to evaluate the accuracy and stability of the global process.

Conclusions: Highly reliable and robust data can be obtained by state-of-the-art techniques of immune-monitoring providing implementation of stringent validation steps and recurrent quality controls in the handling of blood samples.
Keywords: Immune-monitoring, validation steps, quality controls.

INTRODUCTION

The Vaccine and Immunotherapy Center (VIC) and the Laboratory of AIDS Immunopathogenesis are renowned for their pioneer activity in the characterization of cellular immunity, in particular in antiviral and vaccine-induced immunity, tolerance in allergy and transplantation, mucosal immunity and T-cell activation. As a fundamental research platform, our laboratory has specifically developed several assays to measure, characterize, quantify and monitor virus-specific or vaccine-induced T-cell responses. The VIC is a laboratory and clinical research platform specialized in vaccinology, clinical research, immune-monitoring and basic immunology. **Our laboratory has implemented a strict methodology to develop, standardize and validate reliable diagnostic tools according to Good Laboratory Practices (GLP) compliance (ISO/IEC 17025).**

Immunological assessments have been found to play a critical role. In the context of vaccine trial, measurement of T-cell responses is really important. The most accessible source of T-cells is peripheral blood. For measuring of T-cell responses, assays such as IFN-γ enzyme-linked immunospot assay (ELISpot), Intracellular cytokine staining assay (ICS) and proliferation assay (CFSE) are currently used. In order to obtain reliable immunological assessments, functional T cells need to be isolated from the blood and stored in a manner that preserves their functional integrity. Thus PBMC isolation, processing and cryopreservation must be performed according to validated SOPs that are the key steps for assessment of immune responses.

Here we summarize appropriate handling of blood to ensure to obtain reliable immunological assessment (Table 1).

Table 1. Summary of Blood handling

Steps	Procedure/technology	Validation Criteria
Blood Collection	Keep at room temperature with agitation	total handling time from collection to initiation of cell freezing should be **less than 8 hours**
PBMC Separation	• Tube selection (EDTA/HeNa) • Leucosep/accuspin • Standard ficoll	• Processing time within **3-4 h** • Expected Cell Yields is a range of **0.8-3.2 x10^6 cells/ml** • Freshly isolated PBMC viability should be **>95 %**
Cryopreservation	• Mr FROSTY Nalgene • Controlled rate freezer	
Counting	• Hemacytometer • Controlled rate freezer	The cell counts between the 4 squares should agree within **+/20%**
Archiving and locating specimen	LDMS, LIMS or others	
Thawing		samples with **<80%** viability are not suitable
Shipping cryopreserved PBMC	• Dry Ice • Dry shipper	PBMC maintained **-80°C** for up to 3 weeks can be shipped in dry ice.

RESULTS

Quality program assurance

a quality program assurance has been implemented to ensure that standards of quality are being met. Each steps of the blood handling are performed according to our accreditation and validated SOPs.

We can separate this program in three parts:

1. Blood collection, laboratory reception, PBMC isolation and cryopreservation are performed following guidelines and validation criteria (See table 1 for summary).
2. Reagents and technical quality controls.
3. Internal specific controls

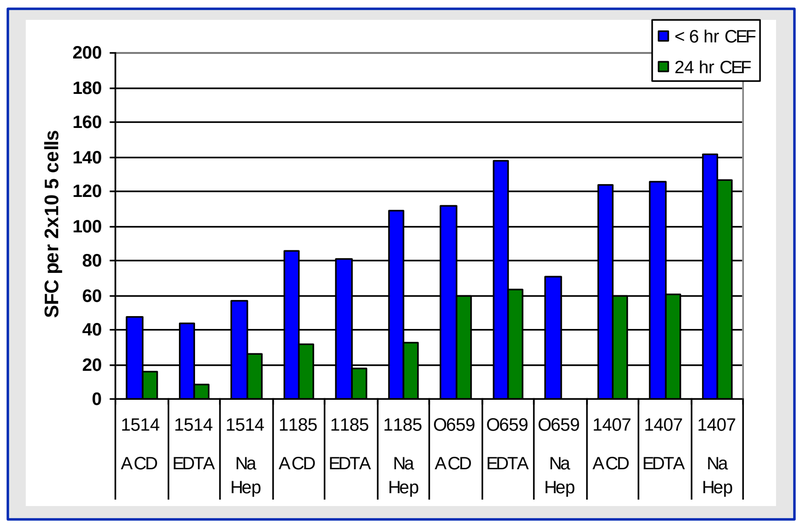
1. Specimen collection/reception

The blood must be collected in EDTA or heparin tubes and specimens are normally transported to the laboratory bags accompanied by a request form within 6 hours after collection. Upon arrival in the laboratory, sample are checked for information regarding patient's ID, that the laboratory number has been correctly assigned, and that the specimen tube and request form bear the same number. The samples are then put on agitation until PBMC processing.

A. PBMC Separation and cryopreservation

To ensure to monitor optimal T-cell responses, appropriate methods specimen handling, PBMC isolation, cryopreservation and storage are critical. It has been shown by Koup and McElrath that the length of time from blood collection to PBMC isolation and cryopreservation strongly affects the frequency and magnitude of T-cell responses that are measured by IFN-γ ELISpot assay. Following two other studies, the total handling time from collection to initiation of controlled-rate freezing should be less than 8 hours.
If this time is delayed, a significant decrease in the magnitude of T-cell responses is induced.

Comparison of different anticoagulants and time delay for the PBMC processing



CEF peptide pool responses detected by IFN-γ ELISpot in 4 donors. *Blood was collected with three different anticoagulants (i.e. ACD, EDTA and NaHep), and PBMC were isolated by Ficoll centrifugation. Cryopreservation of PBMC occurred within 6 or 24 h after blood collection.*

This finding has a major impact in the infrastructure of clinical trial networks. To ensure optimal T-cell measurements, the PBMC processing should be done locally near to the clinic site rather than after shipping overnight to the central processing laboratory.

Internal controls such as cell yield and viability have also been implemented.

A. Expected Cell Yields: In most healthy, adult populations, the average cell yield is approximately 1.5x10^6 cells/ml with a range **of 1-3 x10^6 cells/ml** of usable whole blood volume.

B. Fresh PBMC cell viability is fairly consistent. Long processing time, poor technique and occasionally a specific participant specimen may adversely affect the viability. **Freshly isolated PBMC viability should be >95%.**

B. Specimen shipment

If the samples need to be shipped from local sites to a central laboratory, the optimal method should be used for shipping cryopreserved PBMC. First, once samples have been stored in liquid nitrogen, all transfers or shipments must be maintained in liquid nitrogen (≤ –140 °C). Thus, once samples have been stored in liquid nitrogen, shipping on dry ice is no longer an option.

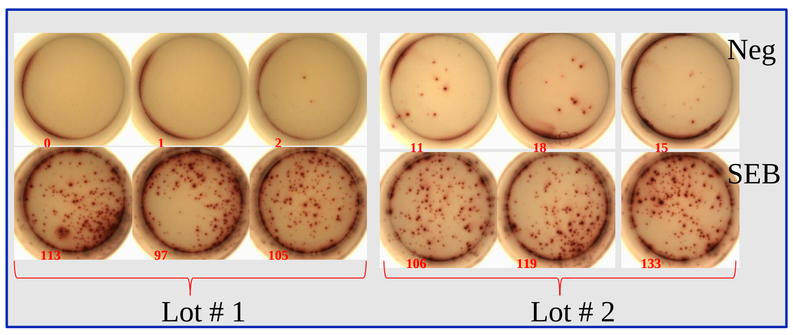
However, if samples are frozen and stored at a T° ranging from –65 °C to –94 °C, they can remain for up to three weeks (21 days) from the date of freezing prior to shipping and relocation in liquid nitrogen without impairment of T-cell function. Within the 21 days following freezing and storage at a T° ranging from –65 °C to –94 °C, shipment using dry ice is an option.

2. Reagents and samples quality control

A. Reagent quality control

Each new lot of reagent should be validated before usage in any clinical trial. For example, Fetal Bovin Sera (FBS) need to be inactivated properly before use. To control the inactivation procedure, an ELISpot assay must be done to evaluate the presence/absence of a background and so permit to use the inactivated FBS in several procedures such as PBMC isolation, cryopreservation, thawing and T-cell functional assays.

Comparaison Between Two Lots of FBS by IFN-γ ELISpot



Comparison of two lots of FBS by IFN-γ ELISpot. The PBMC were stimulated with SEB as positive control and negative control in triplicate. For Lot # 1, the negative control 5 SFU/10^6 cells were detected as compared to Lot #2 73 SFU/10^6 cells. The background must be lower than 3 spots per well to validate the new lot inactivated FBS (i.e. < 15 SFU/10^6 cells.

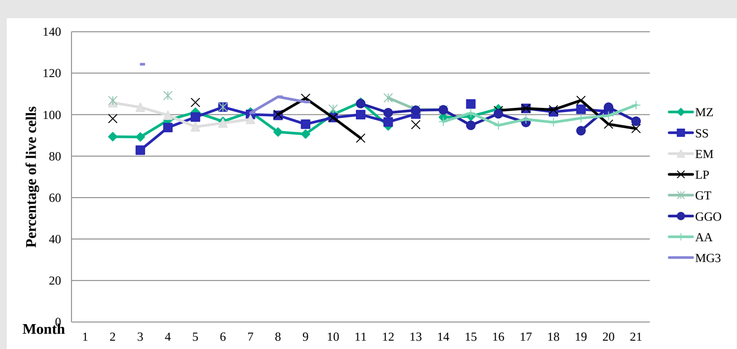
According to our criteria for the selection of a good inactivated FBS, lot # 2 was rejected.

B. Samples quality control:

Each month, blood collection and PBMC isolation/cryopreservation are performed according to the SOP on a blank sample. cells are thawed after 5 to 7 days and PBMC recovery/viability and performance in IFN-γ ELISpot are evaluated.
The quality of the frozen cells is monitored to ensure reliable results in functional IFN-γ ELISpot assay. It has been shown that the relevance of functional assays is strictly dependent on the viability of the cryo-preserved PBMC, such samples with <75% viability are not suitable for cytokine production following stimulation. While cell recovery does not interfere with the results of immunologic functional assays, recovering only a small proportion of cells may preclude running the assays.

We also control at each month counting variability between the technicians. The acceptable variability value is 20% but the average observed variability among several operators is <8 %.

Monthly QC Multi-counting (live cells)

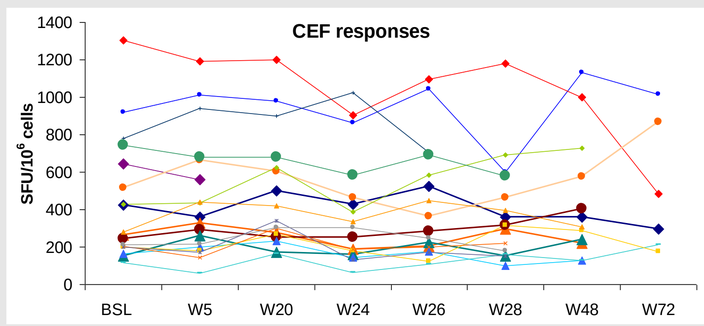


5.6% inter-technician variability

3. Internal quality control

Internal controls (e.g. determination of CMV or EBV-specific T-cell responses have been implemented in all functional assays monitoring immunological parameters. These responses will be analyzed longitudinally to evaluate the accuracy and stability of the global process. In this clinical trial, we have assessed the CMV/FLU/EBV responses at each time point for each donor. This longitudinal control allows us to confirm that each step of this global process is really stable and reliable.

Longitudinal QC ELISpot (EV02)



22.2% inter-time-point variability

CONCLUSION

The elaboration of a quality insurance program handling of blood samples is crucial to generate reliable characterization of vaccine/immunotherapies-induced T-cell responses. **This has been achieved in our certified immune-monitoring platform where we routinely run high-throughput immune-monitoring of clinical trials for both academic and/or pharmaceutical companies.**

EXTERNAL SITES VALIDATION PROCEDURE

Our laboratory is in compliance with GLP guidelines regarding the handling, processing, storage and traceability of clinical trial samples Network. In this regards, procedure to validate external sites is describe below.