Conducting a multi-site ELISPOT proficiency panel.

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We embarked on this project with lots of ideas, three enthusiastic bench scientists, one committed project officer from the Division of AIDS at the National Institutes of Health (NIH) and lots of colleagues who we knew would help us out. While conducting the project we learned many new things and we hope our experiences will help other groups undertake similar endeavors.

The need for an ELISPOT proficiency panel.

A proficiency testing program for the ELISPOT assay serves three purposes: it provides an internal measurement tool for ensuring that the information a laboratory generates and provides is accurate, timely, clinically appropriate and useful; it provides the sponsoring and regulatory agencies with confidence that individual laboratories are generating data with a rigor that will support vaccine licensure in the USA and; it ensures the clinical trial volunteer that the system is working together to provide accurate and reliable information.

Conducting the proficiency panel.

Obtaining peripheral blood mononuclear (PBMC) samples

Our first task was to obtain enough human PBMC samples so that we could perform beta testing in 3 primary laboratories and an independent contract laboratory (BBI Biotech, Gaithersburg, MD), have sufficient PBMC to send out multiple vials to 11 different laboratories for the proficiency panel itself and have enough samples left in storage in case we needed to redo any experiments. To obtain the large volumes of blood required, blood units, buffy coats or leucapheresis samples need to be acquired. Specialized equipment is needed in order to obtain leucapheresis products. Human use protocols are absolutely required for obtaining blood, this is not a trivial undertaking. Blood samples can be obtained from commercial suppliers. While this might seem a bit ghoulish, there are lots of volunteers willing to sell their blood (BRT Laboratories, Baltimore, MD, Research Blood Components, Boston, MA, http://www.bloodbanker.com/plasma/). The cost of a leucapheresis typically runs at about $6-800 and a buffy coat or whole blood unit at $2-300.

Processing, cryopreservation and storage of PBMC is probably the most critical step in this whole endeavor. Why use cryopreserved PBMC and not fresh PBMC? From a logistical point of view a proficiency panel can only be conducted with cryopreserved PBMC. From a general perspective, when conducting a clinical trials, there are clear

advantages to being able to batch assays from multiple time points. For these purposes, it is essential that PBMC are cryopreserved in a manner that maintains their functional capabilities. If the cryopreserved PBMC are not good then the results of the clinical trial and any proficiency panels will be not be interpretable. In our study in spite of centralized PBMC processing, laboratories differed considerably in their ability to recover viable PBMC. The reasons for the discrepancy may lie in different thawing and counting procedures. Key steps to ensure good PBMC are delineated in references 1 to 3. A useful freely available PBMC processing protocol can be obtained from the American AIDS Clinical Trial Group (ACTG) http://aactg.s-3.com/pub/download/vir/freezingprotocol.pdf
For everything about cryopreservation, consult the Nalgene manual; http://www.nalgenelabware.com/techdata/Technical/manual.asp

We obtained 18 PBMC specimens for our initial study, of which 10 were chosen with a range of response to our chosen test antigen. In subsequent proficiency panels the number of PBMC specimens has been reduced to three, however the number of replicate wells plated was increased from 3 to 9 for the test reagents. It was essential to have a laboratory that could ship the PBMC specimens via dry shipper to national and international laboratories. This task requires knowledge and compliance with national and international biohazard shipping regulations.

Standard SOP, ELISPOT kit or laboratory-validated SOP?

We had many discussions about this. Should we have everyone use exactly the same protocol or kit or have everyone use their own protocol? For our proficiency panel, the vote was to use each laboratory's own protocol (reference 4). We chose to assess the performance of the ELISPOT assay based on the premise that each laboratory in this study had optimized or validated their ELISPOT assays and established an SOP. Groups within our proficiency panel used different reagents and equipment. The variables were: plate types, antibodies with up to a ten-fold range in antibody concentration, detection reagents and readers. In addition PBMC handling, incubation and washing procedures differed (reference 4 or contact the author for more details).

Was it the correct decision for laboratories to use their own protocols? Considering that the wide spread use of the ELISPOT assay for conducting vaccine trials is still in its infancy, we think this was the correct decision at this time. The future in which the ELISPOT assay may become a standard clinical assay (e.g. FDA or CLIA approved) that any laboratory anywhere in the world can conduct almost certainly will require standardized SOPs or assay kits. Several organizations are working toward the goal of standardizing cellular immunology assays. A key document "Performance of Single Cell Immune Response Assays; Approved Guideline" has just been released by the Clinical and Laboratory Standards Institute; www.NCCLS.org (formerly the National Committee for Clinical Laboratory Standards). The document covers basic aspects of specimen collection, transport, and preparation, in addition to quality assurance and test

validation approaches. Data acquisition analysis and reporting aspects for assays (ELISPOT, tetramer and intracellular cytokine) are also summarized.

Reagents for the ELISPOT proficiency panel.

The type of reagent used in the proficiency panel should match what will be used for the vaccine trial itself. Examples of the types of reagent that can be used are delineated below and further discussed in reference 2. 1) a pool of 9 mer CMV, EBV and Flu peptides (CEF pool) restricted by common HLA alleles (reference 5) 2) commercially available pools of 15 mer peptides from the CMV pp65 protein (BD Biosciences, San Diego CA). A 15 mer pool would capture both CD4 and CD8 responses 3) live virus preparations such as CMV (Advanced Biosciences Incorporated, Columbia, MD) 4) Tetanus, Candida and tuberculin proteins for induction of CD4 T cell responses 5) Mitogens such as phytohemmaglutinin (PHA), ionophores such as PMA/Ionomycin and staphylococcus enterotoxin B (SEB) can also be used to induce cytokine (s) broadly across T cell subset (s) of interest and could act as positive controls for the proficiency panel. It is critical to test all reagents prior to use in any assay to ensure that there is a low spontaneous background in unstimulated PBMC (nonspecific reactivity) and adequate and reproducible detection of T cell responses in stimulated PBMC (positive control). For the ELISPOT proficiency panel, prior to sending out the testing panel we evaluated the reagents and PBMC in three different laboratories as well as the contract laboratory (BBI Biotech, Gaithersburg, MD).

Follow-up to the ELISPOT proficiency panel.

After we conducted the proficiency panel amongst 11 laboratories, we got very positive feedback. In some cases the results from the ELISPOT proficiency panel was a wake up call. Each laboratory was able to measure itself against its peers and modify protocols and procedures as needed to comply with a new standard. The HIV Vaccine trials network initiated its own 4-laboratory ELISPOT proficiency panel using a single SOP and common reagents. The concordance in spot forming cells (SFC) numbers was much tighter. The same group of original 11 participants is currently repeating the proficiency panel, this time with much better organization and taking advantage of a contract laboratory (BBI Biotech, Gaithersburg, MD) for processing, cryopreserving and shipping all the specimens as well as providing all the common reagents. On this second go round we took advantage of further input from a statistician with regard to the number of replicates as well as a better designed data collection and analysis package (reference 2). Having a good statistician to analyze the huge amounts of data generated during the performance of a proficiency panel is essential. Impartial analysis and determination of what data should be excluded on the basis of pre-defined criteria is also a necessity. We felt it important to define at least three parameters for exclusion of ELISPOT data from our proficiency panel. These were cases where there was a high background response to PBMC only in excess of 100 spot forming cells/million, cases

where the replicates were uneven and cases where low response to a positive control such as PHA was observed. As these proficiency panels develop, pass/fail criteria will be set for participating laboratories.

Timeline for conducting the ELISPOT proficiency panel.

Our first proficiency panel took about 6 months to acquire and test the PBMC and reagents. The participants were asked to perform the proficiency panel with a turnaround time of 2 weeks. We knew this might be unrealistic and assumed we'd have some tardy participants. Initial data analysis took 2 months and refined data analysis further time. So from start to finish it took us one year.

In Conclusion.

The results from our proficiency panel showed that remarkable concordance between laboratories was obtained in defining a qualitative assessment of responder/non-responder status to antigens, but the frequency of responding cells varied significantly among the laboratories. Our study highlighted the need for better standardization of protocols and reagents to obtain reliable and reproducible ELISPOT data.

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