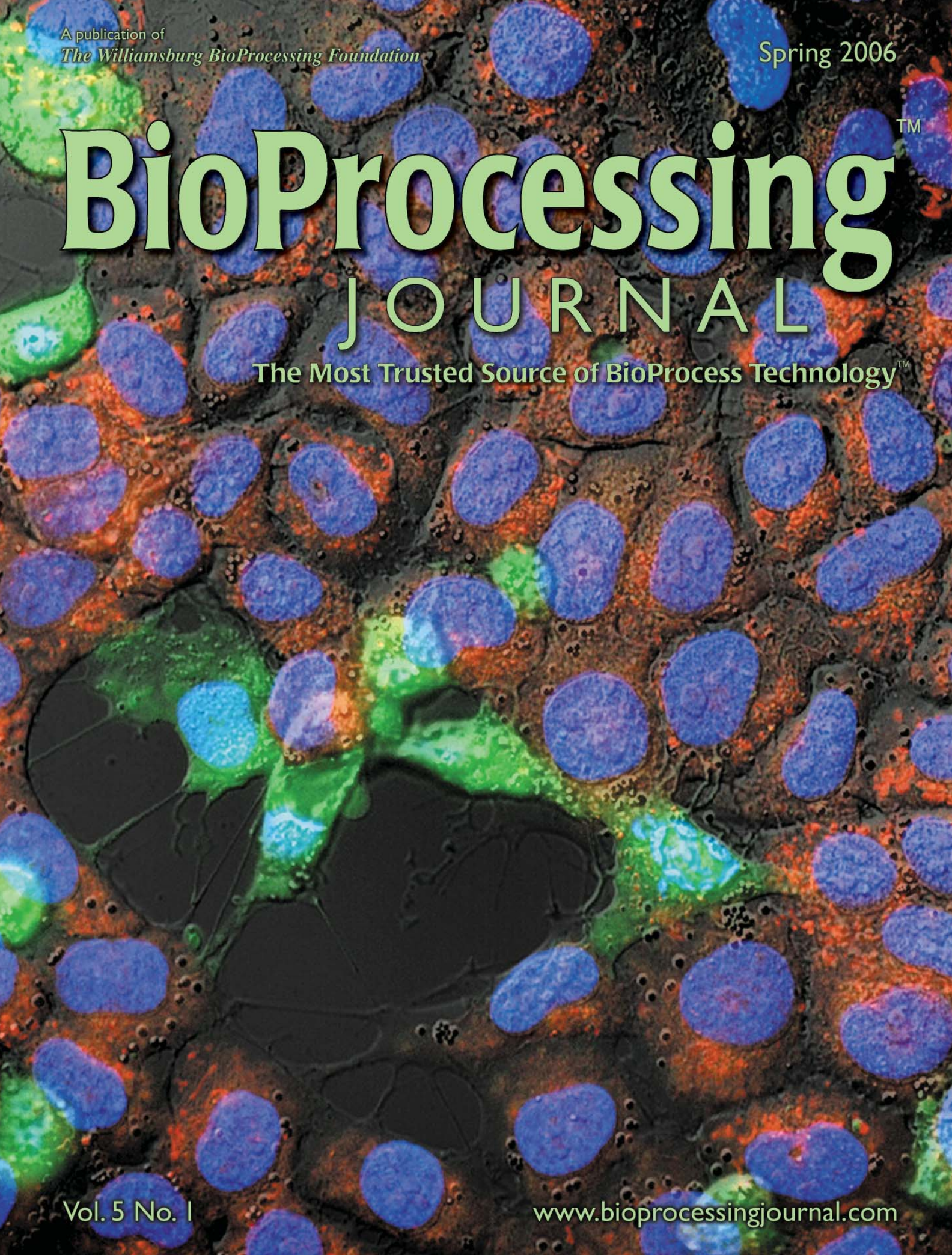
A publication of
The Williamsburg BioProcessing Foundation

Spring 2006

BioProcessing™ JOURNAL

The Most Trusted Source of BioProcess Technology™



Vol. 5 No. 1

www.bioprocessingjournal.com

The Establishment and Stability of the Peripheral Blood Mononuclear Cell Donor Bank for Immunoassay Validation

By JANET LATHEY, SCOTT GREGORY, MIKE EWELL, and SCOTT HICKMAN

The enzyme-linked immunospot (ELISpot) assay is one of the most useful techniques for the immunological monitoring of vaccine trials and has increasing application as a measure of specific T-cell activation. Recently, we developed, optimized, and validated a customized ELISpot kit for the detection of interferon gamma (IFNγ) positive cells. The precision of the ELISpot was good and it varied over the range of the assay values, independent of the stimulus. Here we describe the development of a library of donors with characterized responses to the CEF peptide pool: *cytomegalovirus* (CMV), *Epstein-Barr virus* (EBV), and *influenza* (Flu); pool of 32 peptides which can be used as controls for IFNγ ELISpot and multiple immune monitoring assay validations for use in clinical trials. The ELISpot reactivities of six PBMC donor samples (leukopheresis cell packs from six anonymous, healthy donors) were characterized using standard concentrations of CEF pool (2 µg/ml) with 200,000 cells per well. These samples are representative of a bank of 31 unique PBMC samples from healthy donors (SeraCare Life Sciences, Oceanside, CA).

Table 1. Demographics and Level of CEF Response for Donor Bank

CEF Responder	# of Donors	Mean SFC PHA (range)	Race^ C	Race^ NC	Mean Age (range)	HLA-A type A1/A2	HLA-A type A3/A24
Non (SFC*<10)	2	234 (195-272)	1	1	31 (27-35)	0	1
Low (SFC 11-100)	11	392 (62-855)	3	8	40 (23-48)	7	5
Medium (SFC 101-300)	9	496 (118-963)	6	3	46 (19-66)	5	3
High (SFC>300)	9	774 (311-1126)	6	3	45 (25-51)	8	3

*SFC = Spot Forming Cells calculated per well: 200,000 cells for CEF pool and 100,000 cells for PHA
^C represents Caucasian; NC represents Non-Caucasian

Post-cryopreservation cell recovery and viability were monitored for six months using a Guava PCA. There was little variability in the cell recoveries and viabilities from six donors whose PBMCs were tested over six months. Recovery was 14.3 ± 2.8 million and viability was 91 ± 3%. ELISpot spot forming cell (SFC) values were consistent among fresh, frozen, and frozen for six months, with good precision (average CV = 23%). This study demonstrates that frozen PBMC samples can be used as a convenient control tool for the better standardization of protocols and reagents in obtaining reliable and reproducible cell functionality data among different laboratories. Such standardization of data may support immunogenicity studies, vaccine regulatory submissions, and aid in designing validation studies for immunoassays, including ELISpot assays.

The ELISpot assay is sensitive and reproducible for measuring immune-reactive T-cells in response to prophylactic and immuno-therapeutic vac-

Janet L. Lathey, Ph.D.* *(jlathey@bbii.com), is director of virology/immunology; Scott Gregory is manager of immunology; Mike Ewell is senior scientist in virology; and Scott Hickman is marketing manager; SeraCare BioServices, Gaithersburg, MD.*
**Corresponding author*

cines. ELISpot assays for the detection of cytokine-producing T-cells are becoming more widely adopted for detection of cellular immune responses. The IFNγ ELISpot assay has been used for several years in detecting functional antigen-specific T- and B-cells to measure HIV-1 specific responses in seronegative and seropositive recipients of HIV-1 vaccines and cancer vaccines.[1-8] The ELISpot assay is currently being evaluated as a biomarker for cancer immunotherapeutic and HIV-1 vaccine trials throughout the United States and internationally, resulting in the organization of proficiency testing programs.[9-10] Although the sensitivity and technical ease of the ELISpot assay makes it a useful alternative to traditional analytical methods, there remains a need to monitor reproducibility and precision in detection of the frequency of antigen-specific T-cell responses in all laboratories.

We have developed, optimized, and validated a customized SeraCare ELISpot kit for immunogenicity assessments in NIAID-sponsored vaccine trials. Coating and detection antibodies, Streptavidin-HRP, and substrate were titrated for optimal performance. Precision and linear range were characterized using a standard curve with various concentrations of phytohemagglutinin (PHA) stimulated PBMC and the CEF peptide pool (CMV, EBV and flu peptides).[11] The linear range of the SeraCare ELISpot was 10–1,000 SFC per well. The precision of ELISpot results varied over the range of assay values, independent of the stimulus, demonstrating decreasing variability, correlating with increasing SFC values.[12-14] Several reports have stressed the importance of validating clinical assays, and specifically ELISpot assays, before using the assays to evaluate trial outcomes.[15-17] Validation guidelines for bioassays are based on the International Conference on Harmonization (ICH) document ICH-Q2A.[18] Included in the parameters that need to be validated are precision, specificity, linearity, and range. In order to establish and monitor these validation criteria for clinical assays like the ELISpot assay, it is important to have a consistent source of donor samples whose responses cover the linear range of the assay. We describe here the development of a donor bank that can be used in assay validation, and the ELISpot assay is used as the example assay. The techniques, however, can be adapted for other immune monitoring assays.

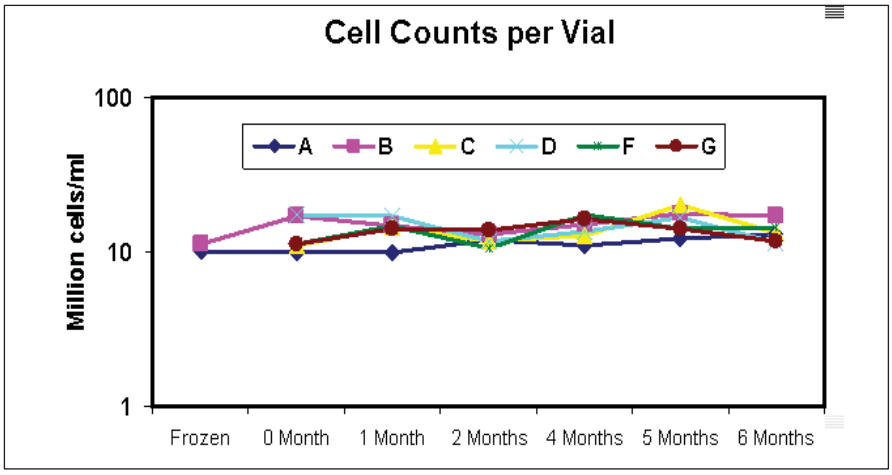


Figure 1. Cell Recovery of Frozen PBMC Over a Six Month Period. One vial of PBMC from each donor was thawed, washed with media, and counted using a Guava PCA at each time point. Counts are shown as million cells per vial.

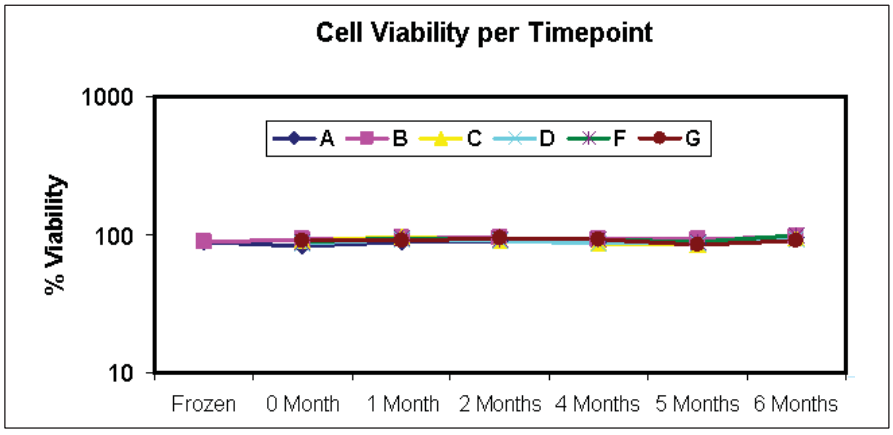


Figure 2. Cell Viability of PBMC Over a Six Month Period. One vial of PBMC from each donor was thawed, washed with media, and counted using a Guava PCA with vital dye at each time point. To determine % viability, the viable cell count was divided by the total count.

Methods

PBMC Sample Preparation

Leukopaks from 31 healthy donors (approximately 450 ml each) were acquired from SeraCare Life Sciences, Oceanside, CA. Acid-citrate-dextrose (ACD) was used as an anticoagulant. Within 24 hours, the PBMCs were isolated and frozen using a programmable rate-controlled freezer. PBMC samples were cryopreserved with 10% dimethyl sulfoxide (DMSO), volume/volume, and stored at –150° C in liquid nitrogen freezers.

ELISpot Assay

The SeraCare ELISpot kit was used. Briefly, sterile Millipore polyvinylidene fluoride (PVDF) plates were pre-coated with IFNγ coating antibody and blocked with 10% FCS-RPMI. The CEF pool of 32 peptides (8–10 mers) from CMV, EBV and flu, was used to measure antigen-specific responses; and phytohemaglutinin (PHA) was used to measure general cell reactivity.[11] The CEF pool was prepared from frozen 10X CEF pool aliquots (200 µg/ml). PHA was prepared from 10X PHA

aliquots (80 µg/ml). CEF pool (20 µg/ml final concentration) and PHA (8 µg/ml final concentration) were added to the appropriate wells on each plate. The CEF pool was tested using 200,000 PBMC, and PHA used 100,000 PBMC. The plates were placed in the 37° C, 5% CO_2 incubator for 18-20 hours. They were washed the next day, six times with wash buffer, and then each plate received IFNγ detection antibody. The plates were incubated for two hours at 37° C, 5% CO_2, followed by six washes with buffer. Next, Streptavidin-HRP was added to each plate. The plates were covered, incubated for one hour at room temperature, and again washed six times with wash buffer. Each plate then received Nova Red solution. The plates were allowed to incubate at room temperature in the dark for five minutes and immediately rinsed with cool tap water for five minutes. Finally, the plates were dried overnight in the dark before analysis with the Cellular Technology, Ltd., (CTL) ImmunoSpot® plate reader.

Evaluations

Assays were performed using samples from the 31 consented donors. Age, gender, and human leukocyte antigen (HLA) type were obtained through SeraCare Life Sciences. Cell recovery and viability were determined by counting cells using a Guava Personal Cell Analyzer (Guava PCA). The CEF and PHA reactivity was determined by ELISpot. The variability in SFC values and cell recovery, over time within an individual lot, were evaluated using PBMC from six donors in multiple assays at different times. Standard statistical analyses were performed in Excel for mean, standard deviation (SD), and coefficient of variability (CV).

Results

Development of the PBMC Donor Bank

PBMCs were isolated from leukopaks of 31 independent donors. Information on demographics and HLA type were provided for each donor. The level of immune response to the CEF peptide pool was determined using an ELISpot assay with a PHA-stimulated positive control to assess general immune function. All PBMC made IFNγ in response to PHA, demonstrating that the cells were functional. Four levels of response were observed for the CEF pool: 2 non-, 11 low-, 9 medium-, and 9 high-responders (Table 1).

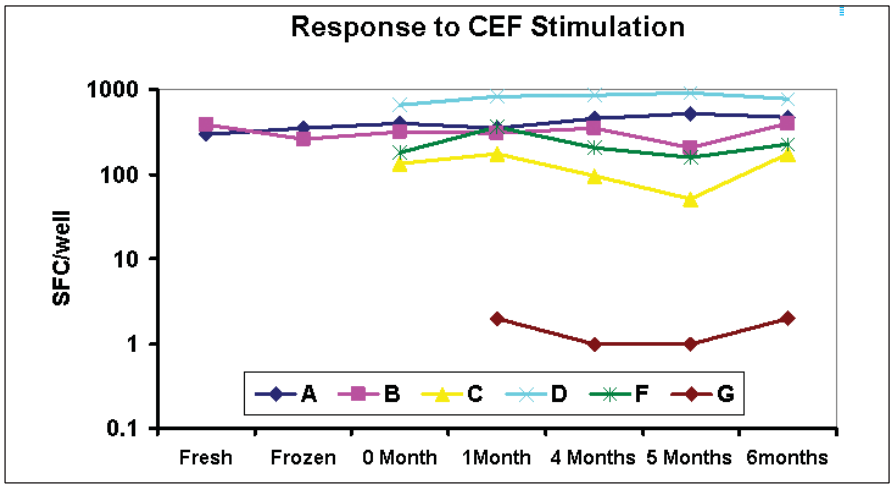


Figure 3. IFNγ ELISpot Response for Fresh and Frozen PBMC. At each time point, one vial of PBMC from each donor was thawed, washed with media, counted using a Guava PCA, and set up in an IFNγ ELISpot assay. Each well received 200,000 PBMC and CEF pool. Data is shown as SFCs per well.

PBMC Stability

In order for a donor bank to be useful as an assay validation tool, the PBMC must retain viability and function with long term storage in liquid nitrogen. Representative samples from the donor bank were thawed and assessed for recovery, viability, and function shortly after freezing, and periodically for six months. As shown in Figures 1 and 2, cell recovery and viability were consistent over the six month period. The responses to CEF for the same PBMC samples are shown in Figure 3. A comparison between levels of response before and after freezing is presented for two samples. The other four were all assayed after freeze-thaw. All donors' PBMC demonstrated consistent responses between fresh and frozen, and when frozen and stored for varying periods up to six months before assay was performed.

Variability

Specific donor's PBMC can be useful as controls for assay precision. Results from the ELISpot assay are very dependent upon the precision of the cell counts. As seen in Table 2, when PBMC from three donors were counted six different times over six months, the variability was approximately 20%. IFNγ ELISpot assays were performed on five of the six sampling timepoints from each of the three donors. The repeatability (intraassay variability) ranged from 5% to 15%, and the intermediate precision (interassay, within laboratory) ranged from 12% to 34% depending on the donor (Table 3).

Summary and Discussion

The donor bank consists of PBMC from donors who generally are over 30 years old, with approximately equal distribution between Caucasians and non-Caucasians, and demonstrate a wide range of HLA-A subtypes. Of the 31 characterized donors, 94% (29) were CEF responders with variable levels of responses. Post-thaw cell viability was ~90% with 4% variability. Cell recovery variability was 20% as measured by cell count. The values obtained for IFNγ SFC were comparable in fresh and frozen samples from the same donor, and variability was ~20% in frozen samples over a six month period. Using the format presented here as an example, the use of cryopreserved PBMC from a donor

bank can be initiated at the first stage of assay development and continue throughout the immune monitoring phase of clinical trials.

Assay Development

An assay being developed from an independent set of reagents needs a consistent set of responding cells (PBMC) in order to properly compare reactivities of varying reagent concentrations. Optimization of the assay with repeatable results is essential to obtain the best sensitivity and specificity.[12-14,17]

Assay Validation

Before an assay can be released for use in a clinical trial, the precision and dynamic range must be determined.[15-17] As demonstrated in this report, cryopreserved PBMC from the donor bank can be used to determine repeatability, (*i.e.*, intraassay precision and intermediate precision) and intralaboratory variability in cell counting and assay performance (Tables 2 and 3). In addition, with a diverse donor bank like that presented here (Table 1), the effective range of the assay can be determined.

Quality Assurance (QA)

Ensuring that assay performance is uniform across an entire trial requires monitoring the performance of a positive control that is identical with each assay. A donor bank of PBMC that perform consistently over time can provide that performance/QA control (Figure 3). Large trials sometimes necessitate the involvement of multiple laboratories at the immune monitoring stage of a trial. A measure of reproducibility across labs may be necessary to accurately compare results. This can be accomplished through proficiency testing. Donor bank samples have been used for ELISpot proficiency testing in clinical trial laboratories participating in both HIV and cancer vaccine clinical trials.[9-10]

The diversity in this donor bank and its resemblance to the overall population, results in a broad range of reactivities, making it a good source of materials for use throughout the stages of assay development, validation, and other clinical trial QA studies.

Table 2. Precision of Cell Counts

	Donor B (n = 6)		Donor D (n = 6)		Donor F (n = 6)	
	Cell Count x million	% Viability	Cell Count	% Viability	Cell Count	% Viability
Mean	15.1	93	13.8	90	13.7	93
SD	2.6	3.5	3.3	3.0	2.5	3.5
CV*	17%	4%	24%	3%	18%	4%

*CV was determined for six independent vials of PBMC thawed on different days.

Table 3. Precision of ELISpot Assay Results

	Donor B (n = 5)		Donor D (n = 5)		Donor F (n = 5)	
	CEF	Media	CEF	Media	CEF	Media
Mean	309*	2*	795	1	237	1
SD	73	3	93	1	83	1
Inter-assay CV^	24%	NA	12%	NA	34%	NA
Intra-assay CV#	7%	NA	5%	NA	15%	NA

*SFC = Spot Forming Cells calculated per well: 200,000 cells for CEF pool and media.
^Interassay CV was determined for five independent assays on different days.
#Intraassay CV was determined for triplicates on the same assay plate.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the invaluable contributions of Wenyi Su, Rodshawn Branch, Hongzhi (Larry) Liang, Lequan Nguyen, Joseph Rogers, and Mary Pearson — all members of the virology/immunology department at SeraCare BioServices.

REFERENCES

1. Evans TG, Kallas EG, Campbell M, Andrews J, Schwartz D, Keefer M, Caugrelier P. Evaluation of canarypox-induced CD8+ responses following immunization by measuring the effector population IFNγ production. *Immunology Letters* 2001:77;7-15.
2. Gahery-Segard H, Pialoux G, Charmeteau B, Sermet S, Poncelet H, Raux M, Tartar A, Levey J, Gras-Masse H, Guillet J. Multiepitopic B- and T-cell responses induced in Humans by a Human Immunodeficiency Virus Type 1 lipopeptide vaccine. *Journal of Virology* 2000:74;1694-1703.
3. Moss RB, Webb E, Giermakowska WK, Jensen FC, Savary JR, Wallace MR, Carlo DJ. HIV-1 specific CD4 helper function in persons with chronic HIV-1 infection on antiviral drug therapy as measured by ELISpot after treatment with an inactivated, gp120-depleted HIV-1 in incomplete Freund's adjuvant. *Journal of Acquired Immune Deficiency Syndromes,* 2000:24;264-269.
4. Arlen P, Tsang K, Marshall J, Chen A, Steinberg SM, Poole D, Hand PH, Scholm J, Hamilton JM. The use of a rapid ELISpot assay to analyze peptide-specific immune responses in carcinoma patients to peptide versus recombinant poxvirus vaccines. *Cancer Immunology, Immunotherapy,* 2000:49;517-529.
5. Asis T, Storkus WJ, Whiteside TL. Evaluation of the modified ELISpot assay for gamma interferon production in cancer patients receiving anti-tumor vaccines. *Clinical and Diagnostic Laboratory Immunology,* 2000:7;145-154.
6. Lewis JJ, Janetzki S, Schaed S, Panageas KS, Wange S, Williams L, Meyers M, Butterworth L, Livingston PO, Chapman PB, Houghton AN. Evaluation of CD8 T-cell frequencies by the ELISpot assay in healthy individuals and in patients with metastatic melanoma immunized with tyrosinase peptide. *International Journal of Cancer,* 2000:87;391-398.
7. Klencke B, Matijevic, Urban RG, Lathey JL, Hedley ML, Berry M, Thatcher J, Weinberg V, Wilson J, Darragh T, Jay N, Costa MD, Palefsky JM. Encapsulated plasmid DNA treatment for human

papillomarvirus 16-associated anal dysplasia: A Phase I study of ZYC101. *Clinical Cancer Research* 2002:8;1028-1037.

8. Scheibenbogen C, Sun Y, Keilholz U, Song M, Stevanovic S, Asemissen AM, Nagorsen D, Theil E, Rammensee HG, Schadendorf D. Identification of known and novel immunogenic T-cell epitopes from tumor antigens recognized by peripheral blood T-cells from patients responding to IL-2 based treatment. *International Journal of Cancer,* 2002:98;409-414.

9. Cox J, Ferrari G, Kalams SA, Lopaczynski W, Oden N, D'Souza MP. ELISpot collaborative study group – Results of an ELISpot proficiency panel conducted in 11 laboratories participating in international human immunodeficiency virus type 1 vaccine trials. *AIDS Research and Human Retroviruses* 2005:21;68-81.

10. Janetzki S, Lathey J, Panageas K. Assay Working Group – ELISpot Panel. Presentation at the *5th Meeting of the Cancer Vaccine Consortium* 2005: Alexandria, VA.

11. Currier JR, Kuta EG, Turk E, Earhart LB, Loomis-Price L, Janetzki S, Ferrari G, Birx DL, Cox JH. A panel of MHC Class I restricted viral peptides for use as a quality control for vaccine trial ELISpot assays. *Journal of Immunological Methods* 2002:260;157-172.

12. Lathey J, Zhang Y, Gregory S, Cox J, Coup R, D'Souza PD, Lopaczynski W. Precision of the ELISpot assay for use in HIV clinical trials. Poster presented at the *11th Conference on Retroviruses and Opportunistic Infections* 2004: San Francisco.

13. Lopaczynski W, Gregory S, Zhang Y, Cox JH, Koup R, D'Souza P, Lathey JL. Optimization of the ELISpot assay for use in vaccine immunotherapy trials. Poster present at *Experimental Biology* 2004: Washington DC.

14. Lathey JL, Gregory S, Zhang Y, Horton H, Koup R, Cox JH, Ferrari G, D'Souza P, Lopaczynski W. Characterization of two ELISpot kits for evaluation of immune responses to vaccines. Poster presented at *12th International Congress of Immunology and 4th Annual Conference of FOCIS*: 2004; Montreal, Canada.

15. Lathey JL. Preliminary steps toward validating a clinical bioassay – A case study of the ELISpot assay. *BioPharm International,* March: 2003;42-50.

16. Lathey LL, Sathiyaseelan J, Matijevic M, Hedley ML. Validation of pretrial ELISpot measurements for predicting assay performance during a clinical trial. *BioProcess International,* 2003: September; 34-41.

17. Janetzki S, Cox JH, Oden N, Ferrari G. 2005 Standardization and validation issues of the ELISpot assay. In: Kalyuzhny LA, editor. *Methods in molecular biology, Vol.3, handbook of ELISpot: methods and procols.* Totowa, NJ: Humana Press 2005; 51-83.

18. FDA Guideline for Industry. Text on Validation of analytical procedures. Available at: www.fda.gov/cder/guidance/ichq2a.pdf; Internet; accessed. August 26, 2004.

ASME
SETTING THE STANDARD

Space is limited! Register Now.

ASME 19th Annual Bioprocess Technology Seminars

October 23–27, 2006 • Montreal Canada

Featuring 11 Concurrent Courses, including...

- Aseptic Fill and Finish for Biopharmaceuticals
- Bioprocess Purification Process Development
- Bio-Downstream Process Design and Economics
- Validation of Biopharmaceutical Facilities and Processes

Plus... tabletop exhibits, plant tour and networking

TO REGISTER
www.asmeconferences.org/bioprocess06
OR CALL
1.800.843-2763

Premier Media Sponsor: BioProcess INTERNATIONAL
Supporting Publications: BioPharm International, BioProcessing Journal
Supporting Organization: Society for Biological Engineering, An AIChE Technological Community