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Analytical Performance of a Standardized Single-Platform MHC Tetramer Assay for the Identification and Enumeration of CMV-Specific CD8+ T Lymphocytes

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Abstract

Major histocompatibility complex (MHC) multimers that identify antigen-specific T cells, coupled with flow cytometry, have made a major impact on immunological research. HLA Class I multimers detect T cells directed against viral, tumor, and transplantation antigens with exquisite sensitivity. This technique has become an important standard for the quantification of a T cell immune response. The utility of this method in multicenter studies, however, is dependant on reproducibility between laboratories. As part of a clinical study using a standardized two-tube three-color single-platform method, we monitored and characterized performance across multiple sites using tetramers against the T cell receptors (TCR) specific for MHC Class I, A*0101—VTEHDTLLY, A*0201—NLVPMVATV and B*0702—TPRVTGGGAM CMV peptides. We studied the analytical performance of this method, focusing on reducing background, maximizing signal intensity, and ensuring that sufficient cells are enumerated to provide meaningful statistics. Inter and intra-assay performance were assessed, which included inherent variability introduced by shipping, type of flow cytometer used, protocol adherence, and analytical interpretation across a range of multiple sample levels and specificities under routine laboratory testing conditions. Using the described protocol, it is possible to obtain intra- and interlab CV's of <20%, with a functional sensitivity for absolute tetramer counts of 1 cell/μL and 0.2% tetramer+ percent for A*0101, A*0201, and B*0702 alleles. The standardized single-platform MHC tetramer assay is simple, rapid, reproducible, and useful for assessing CMV-specific T cells, and will allow for reasonable comparisons of clinical evaluations across multiple centers at clinically relevant thresholds (2.0–10.0 cells/μL). © 2008 International Society for Advancement of Cytometry

Key terms

cytomegalovirus; CD8 T lymphocytes; tetramer assay; flow cytometry

CELLULAR immune responses play an enormous role in a variety of conditions, including infection, autoimmune disease, malignancy, and transplantation. The measurement of an antigen-specific cellular immune response, one of the hallmarks of adaptive immunologic recognition, requires identification, enumeration, and/or characterization of specific lymphocytes (1,2). In contrast to B lymphocytes, which can be tracked by the detection of antigen-specific circulating antibodies, T lymphocytes typically lack the capacity to produce soluble antigen-specific factors. This has historically hampered the direct measurement of T cell responses. T lymphocytes exert effector functions when activated via their T cell receptor (TCR) by specific peptide—major histocompatibility complexes (MHC) on target cells (3). A variety of classic methods for detection and enumeration of antigen-specific T cells has focused on the activation and proliferation of T cells by measuring the incorporation of 3H-thymidine into the DNA of proliferating cells (4) or their ability to kill target cells,

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such as limiting dilution assays and cytotoxic T lymphocyte assays (5). A major limitation of these culture-based techniques is that the magnitude of the immune response may be underestimated (6,7). The introduction of MHC multimers has provided a direct structural basis for detection of T cells specific for, theoretically, any given antigen presented in the context of MHC (8). The first design of this class of reagents is the so-called "tetramers" (9). Tetramers are soluble complexes of four MHC molecules (Class I or Class II) associated with an antigenic peptide and conjugated to a fluorochrome to allow direct visualization by flow cytometry. The antigen-specific T lymphocytes may be enumerated by flow cytometry without the requirement of in vitro stimulation, allowing for rapid and sensitive quantitative measurement of an individual patient's T cell response to a specific antigen (10). Later, other formats for reagents that can bind directly to the TCR of the cognate epitope-specific T cells became available, such as dimers, streptamers, pentamers, and dextramers (8,11–13).

Human cytomegalovirus (CMV) is a herpes virus that infects 60–90% of individuals worldwide. The virus is not eliminated but remains latent in the infected host (14,15). CMV-specific CD8+ cytotoxic T lymphocytes play a critical role in suppressing CMV reactivation (16–19). iTAg™ MHC tetramers (Beckman Coulter, Fullerton, CA) each bound to specific peptides of the structural matrix proteins pp65 and pp50 of human CMV have been used in clinical studies to enumerate CMV-specific CD8+ T cells by flow cytometry (20).

According to this protocol, absolute cell counts are directly assessed with the flow cytometer by a "single-platform" methodology based on the use of counting beads. The iTAg tetramers, which have been constructed from mutated human leukocyte antigen (HLA) molecules to minimize CD8-mediated binding of HLA to cell surface CD8, show reduced binding to the general population of CD8 cells, but retain peptide-specific binding, thus facilitating accurate discrimination of rare, specific T cells (21). Several clinical studies in which CD8+ T cell responses in viral and tumor immunity are monitored have revealed the diagnostic potential of this methodology (22–30). The utility of this method in multi-centre studies, however, still needs to be demonstrated. The objective of this study was to evaluate the use and characterize the performance across multiple sites using iTAg™ MHC tetramers in enumerating CMV-specific CD8+ T lymphocytes. Here, we report inter and intra-assay performance while addressing variability introduced by shipping, cytometer, analytical interpretation, and testing across a range of multiple samples and specificities under routine laboratory testing conditions. In this context, we specifically addressed whether precision levels of less than 20% could be achieved using tetramers representing low, medium, and high-affinity peptides.

Materials and Methods

Study Design

There were three discrete sections to this study. Performance characteristics of the assay were established in Study 1 at the coordinating centre using various methods with multiple alleles, three operators, and a large donor pool.

Sites (labs) were qualified in Study 2 by training, proficiency testing, and a pre multicenter trial (all quality assessment studies) using stabilized samples. Common procedures were provided to each site. Pipetting precision and accuracy was determined. Three blood sample standards were stabilized in either Transfix™ (Cytomark Ltd, Buckingham, UK) or Cytochex™ (Streck Laboratories, Omaha, NE) and tested within 7 days post stabilization. Three rounds of testing were completed for a total of nine samples. The study was performed in five separate laboratories, using seven different instruments and operators. After evaluation of this preliminary data, targets were established for intra-assay and inter-lab precision and a switch to unfixed blood was used for Study 3.

Study 3 was a multi-center trial conducted over a 3 month period between January and March 2005. Samples were selected with an expected range of 0.5 to 10.0 tetramer positive cells/μL. We chose a sample size that would best model the range of biological variability historically observed and test enough donors to mimic laboratory conditions over time (a three month period, a couple samples a week, and variables in shipping conditions). Panels were performed on 27 samples, in duplicate, using five operators, five flow cytometers (i.e. two EPICS XL™ and two FC500™ Beckman Coulter, Fullerton, CA, 1 FACSCalibur™, BD Biosciences, San Jose, CA), at three different laboratories. The coordinating lab retained blood tubes on a rocker for 24–48 h before testing to approximate the condition of shipped samples received by other sites. Seronegative blood samples were included in the study design to validate iTAg specificity and the operator's proficiency in processing received sample. Sites were blinded to the expected CMV iTAg results. Fourteen normal donors were used. Ten donors were CMV seropositive and four CMV seronegative. Some donors were drawn twice or tested for two alleles for a total of 27 blood draws. Five blood samples (no stabilization) were drawn per blood donor and shipped to each site (US and Canada) with testing 24–72 h post draw. Samples were packed in insulated containers to ensure temperature stability. In earlier studies it was shown by the coordinating laboratory that under these conditions cell viability was >90%. Seronegative samples were allele matched for use with the CMV MHC-peptide tetramer. List mode files were analyzed by each individual site.

Specimen and Reagents

Blood was obtained using informed consent and standard IRB protocol review established at the coordinating laboratory (Beckman Coulter Laboratory, San Diego, CA). Peripheral blood samples were collected in K_3EDTA anticoagulant tubes (Becton Dickinson, Oakville, ON) from a pool of apparently healthy donors, previously screened for CMV serostatus using CMV specific IgG and/or IgM ELISA antibody testing (Quest Diagnostics, San Juan Capistrano, CA) and typed for HLA specificity prior to blood draw (UCSD Healthcare Immunogenetics and Transplantation Laboratory, LaJolla, CA). None of

the seropositive donors had a history of CMV-associated disease, and the precise dates of primary infection are unknown.

Reagents were obtained from Beckman Coulter, Inc., Fullerton, CA.: iTAg MHC tetramers, Negative tetramer, VersaLyse (fix-lyse), tetramer fixative reagent, anti-CD3-PC5 clone UCHT1, anti-CD4-PE clone SFCI12T4D11, anti-CD8-FITC clone SFCI21Thy2D3, Flow-count™ fluorospheres, IMMUNO-TROL™ control cells. The following iTAg™ MHC tetramers were used in the study: pp50: A*0101 (VTEHDTLLY)—high affinity; pp65: A*0201 (NLVPMVATV)—medium affinity, B*0702 (TPRVTGGGAM)—low affinity; iTAg™ MHC negative tetramer. All tetramers were conjugated with phycoerythrin (PE).

Enumeration of CMV-Specific CD8+ T Lymphocytes in Whole Blood by Flow Cytometry Using iTAg™ MHC Tetramers

PE-conjugated iTAg MHC tetramers were used to enumerate CMV-specific CD8+ T cells by flow cytometry using a single-platform absolute counting method by combining the results of two distinct panels. Panel 1 determines absolute CD3+CD4+ and CD3+CD8+ lymphocyte subset counts. Panel 2 determines the percentage of CD3+CD8+ T cells that are CMV-specific. The lyse-no-wash procedure for Panel 1 was prepared by adding 10 μL each of CD8-FITC, CD4-PE, and CD3-PC5 to duplicate tubes. Next 100 μL of whole blood was added by reverse pipetting to each tube. The mixture was then vortexed and incubated in the dark for 20–30 min at room temperature. Red blood cells were lysed by adding 1 mL of Versalyse. Suspension was incubated for at least 10 min at room temperature in the dark and subsequently stored at 2–8°C for 1–24 h, until analysis. Just prior to analysis 100 μL of Flow-Count™ fluorospheres were added by reverse pipetting. Tubes were capped and vortexed for 5–10 s. IMMUNO-TROL™ control cells were stained in duplicate using the lyse-no-wash Panel 1 procedure as part of the quality control. Percentage and absolute counts of CD3+, CD3+CD4+, and CD3+CD8+ were compared against results in the package insert. If the results were outside the indicated parameters, the IMMUNO-TROL™ control cells were restained. This was performed on the same days donor samples were processed to validate the process of Panel 1 and the reliability of antibodies and Flow Count beads. Results from Panel 1 provide a direct determination of the CD3+CD4+ and CD3+CD8+ lymphocyte subset absolute counts.

Panel 2 consists of three tubes. Tube 1 was labeled as a negative control and 10 μL of each CD8-FITC, negative tetramer-PE, and CD3-PC5 was added. Results from this tube are used to evaluate the level of background fluorescence present for Class I CMV peptide-specific tetramer.

On the basis of the HLA type of the patient and the corresponding iTAg™ MHC Class I tetramers available, duplicate tubes were labeled. To each tube, 10 μL of corresponding iTAg CMV tetramer-PE and 10 μL each of anti-CD8-FITC and anti-CD3-PC5 were added. Next 200 μL of whole blood was added to each tube. After incubation in the dark for 20–30 min at room temperature, red blood cells were lysed by adding 2 mL of VersaLyse. For specimens with low leukocyte counts ($<3.0 \times 10^3/\mu$L) or low lymphocyte counts ($<0.5 \times 10^3/\mu$L) whole blood volumes up to 400 μL were used. Under these circumstances, up to 4 mL of VersaLyse solution is required. All other reagent volumes remain as described. Samples were capped, inverted, and incubated for at least 10 min at room temperature in the dark followed by a 5 min centrifugation at 150*g*. Supernatant was aspirated and pellet washed with 3 mL of PBS and centrifuged, decanted and then reconstituted with 0.5 mL of 0.1% paraformaldehyde solution (Tetramer fixative reagent diluted 1:80 in PBS). Prepared samples were stored at 2–8°C for 1–24 h until analysis by flow cytometry. Results from Panel 2, Tube 2, are used to determine the percentage of CMV-specific CD3+CD8+ T cells present within a sample.

The absolute count for CD3+CD8+ CMV tetramer+ cells per μL is derived using the absolute CD3+CD8+ cell count determined in Panel 1 and the percent CD3+CD8+ CMV tetramer+ cells determined in Panel 2.

Acquisition and Analysis

Instrument setup, calibration, compensation, and interpretation were performed according to established laboratory procedures.

Panel 1 data was collected using a T gate (CD3 vs. SS) with a stop count on 3,000 singlet Flow-count™ fluorospheres. A listgate was used to exclude the majority of non CD3+ events, to reduce the size of the listmode file. Cells meeting the criteria of CD3+, with low SSC and low to intermediate forward scatter, were enumerated for CD4 or CD8 positive events. Beads were enumerated in the PC5 (FL4 channel).

In Panel 2 the stop count was set at 20,000 $CD3^+CD8^+$ events. However, the minimum number of tetramer+ events was set at 100, which would override the primary stop. All data were acquired using Medium flow rate. Identification of the CD3+CD8+ population is common to both panels. Panel 2 uses a T gate, then CD8 versus PE with cluster analyses to identify CMV peptide-specific T cells. A negative reacting tetramer was used to assess background for Panel 2.

Data analyses were done with CXP or system II software (Beckman Coulter, Fullerton, CA) or Cellquest (BD Biosciences, San Jose, CA). An analysis example of both panels with dot plots and histograms are presented in ascending order in the supplementary information that will be available online.

Statistical Analyses

The following parameters were recorded—CD8 absolute count, CMV tetramer %, and absolute count. Tetramer results were expressed in terms of percentage of the total CD3+CD8+ cells that were CMV-specific, i.e., CD3+CD8+CMV tetramer+ percent (%) and CD3+CD8+CMV tetramer+ absolute counts (cells/μL).

Sensitivity was defined as the predetermined quantitation limit, i.e., the lowest level of detection of percent and absolute tetramers while maintaining CVs less than 20%. These analyses were performed in Studies 1 and 3.

Table 1. Quantitation limit of the iTAg™ MHC tetramer assay

RESULTS BASED UPON CV% OF <20[a]		
	% TETRAMER	CELLS/μL
[b]Allele	$N = 2$	$N = 2$
A*0101	0.16	0.87
A*0201	0.19	0.35
B*0702	0.44	1.51
B*0702	0.16	0.64
Mean	0.22	0.77
1 SD	0.07	0.37

Whole blood from three CMV seronegative donors was collected and spiked with CMV peptide-stimulated T cells and then serially diluted to achieve at least ten to fourteen distinct levels for CMV Tetramer + cells. Samples were tested in replicates of two.

[a] The quantitation limit was defined as the lowest concentration where the estimated CV continues to be less than 20%.

[b] Summary of all alleles tested and reviewed (B*0702 was tested twice).

Table 2. Accuracy of the iTAg™ MHC tetramer assay

ABSOLUTE COUNT (CELLS/μL)				
SPIKED LEVEL	ALLELE	EXPECTED	OBSERVED	% RECOVERY
Low	A*0101	3.14	3.37	107
	A*0201	0.52	0.43	83
	B*B702	2.55	2.49	98
Mid	A*0101	9.23	9.28	100
	A*0201	1.54	1.29	84
	B*B702	7.83	7.7	98
High	A*0101	15.91	15.92	100
	A*0201	2.48	2.13	86
	B*B702	12.14	11.69	96
Mean recovery				95

Whole blood from one CMV seronegative donor per allele was spiked with blood from an allele-matched CMV seropositive donor for A*0101, A*0201, and B*0702 alleles to a low, mid, and high % tetramer positive level. Samples were tested for the Tetramer + cells/μL in replicates of five.

Intra-assay precision was calculated using 10 replicates. Deming linear regression analyses were also used to assess recoveries and linearity. These analyses were performed in Studies 1.

Between-laboratory precision was assessed by determining the CV across sites for sample results above the limit of detection in Studies 1, 2, and 3.

Between-laboratory precision was also calculated using 1 replicate in each panel in Study 3, as if a three tube test was used by the operator by deriving the data from Panel 1, Tube 1 (absolute T cell counts), Panel 2, Tube 1 (irrelevant tetramer staining) and Panel 2, Tube 2 (experimental tetramer staining).

Deming linear regression examining replicate one versus the mean of replicate one and two was analyzed to evaluate intra-assay precision in Study 3. In some cases, total precision (all replicates, all sites) is represented.

As an ongoing assessment of quality assurance, absolute difference of CD8% between Panel 1 and Panel 2 was examined, as was the stop count adherence for number of CD3+CD8+ events and CD3+CD8+Tet+ events collected.

Results

In Study 1, sensitivity (quantitation limit), linearity, and accuracy were evaluated. Whole blood from three CMV seronegative donors was collected and spiked with CMV peptide-stimulated T cells and then serially diluted to achieve at least 10 to 14 distinct levels for CMV tetramer+ cells. Samples were tested in replicates of two. The quantitation limit was defined as the lowest concentration where the estimated CV continues to be less than 20%. Overall, the quantitation limit for the CMV assay was determined to be 1.0 cell/μL for absolute counts and 0.2% tetramer+ percent for A*0101, A*0201, and B*0702 alleles (Table 1). The higher limit of detection that was observed for the B*0702 spiked samples most likely represent undefined assay variability in doing these spike and recovery assays and was therefore repeated. The Deming regression for linearity analysis showed acceptable correlation between the expected and actual values for absolute counts of CMV tetramer+ cells with R^2 values ranging from 0.9980 to 0.9999 with slopes ranging from 0.93 to 1.03% (data not shown).

A spike and recovery assay was performed to evaluate accuracy. Whole blood from one CMV seronegative donor per allele was spiked with blood from an allele-matched CMV seropositive donor for A*0101, A*0201, and B*0702 alleles to a low, mid, and high % tetramer positive level. Separate determinations were made for both Panel 1 (cells/μL) and Panel 2 (% tetramer+) and samples were tested in replicates of five. The overall percent recovery for A*0101, A*0201, and B*0702 CMV tetramers was 95% with a range of 83–107% (Table 2).

Fresh whole blood from three CMV seropositive, donors per allele was tested at the low, mid, and high % tetramer positive level. Samples were tested in replicates of 10 to evaluate intra-assay precision. Imprecision was determined to be less than 10% for the A*0101, A*0201, and B*0702 tetramers for both percentage and absolute count at all test concentrations. An example of the mid affinity tetramer A*0201 is shown in Table 3.

Inter Assay Precision

To assess between-lab precision, Study 1 used three different operators utilizing a distinct combination of technician and flow cytometric instrumentation designated as Sites 1, 2, and 3 to simulate between-laboratory performance in a controlled setting. Separate determinations were made for both Panel 1 and Panel 2. Whole blood from three CMV seropositive donors expressing A*0101, A*0201, and B*0702 alleles were tested at the low, mid, and high % tetramer positive level. Samples were tested in replicates of 10 at three independent sites by three operators for a total of 30 determinations. CD3+CD8+ absolute count CV's were less than 10%, Tetramer percent and absolute count (cells/μL) imprecision for the

Table 3. Intra- and inter-assay precision of the iTAg™ MHC tetramer assay

ALLELE: A*0201		LOW LEVEL		MID RANGE		HIGH LEVEL	
Intra-assay precision[a]				Absolute counts (cells/μL)			
Operator	n	Mean	%CV	Mean	%CV	Mean	%CV
1	10	2.06	6.8	8.62	4.7	14.3	3.1
2	10	1.79	5.6	8.02	4.1	12.36	2.8
3	10	1.83	6.4	7.54	2.9	13.15	3.2
Inter-assay precision[b]							
Allele							
A*0101	30	2.45	13.3	4.64	3.2	29.47	3.7
A*0201	30	1.89	7.7	8.05	6.7	13.27	7.4
B*0702	30	2.5	6.6	4.46	7.5	23.1	5.5

[a] Intra Assay Precision–Whole blood from three CMV seropositive, donors per allele was run in replicates of 10 at the low, mid, and high % Tetramer positive level. Results from mid affinity tetramer A*0201 shown.
[b] Inter Assay Precision–three separate operators ran samples in replicates of 10, for a total of 30 analyses per allele.

A*0101, A*0201, and B*0702 tetramers were determined to be less than 15% at the low test concentrations, and less than 10% for the mid and high test concentrations (Table 3).

Determining Between-Lab Imprecision

In Study 2, seven different operators from five sites utilizing a distinct combination of technician and flow cytometric instrumentation were designated Sites 1–7. Separate determinations were made for both Panel 1 and Panel 2. Preserved whole blood from CMV seropositive donors was tested at a low, mid, and high % tetramer positive level. Alleles tested across the donor pool were (4) B*0702, (1) A*0201, (3) A*0101, and (1) B*3501 for a total of nine results. Results with an absolute CD3+CD8+tet+ value of less than 1 cell/μL. (range, 0.33–0.79/μL) showed expected high, CV's ranging from 25.3% to 27.4%. Of the remaining six samples, with absolute tetramer numbers ranging 0.6–15.9/μL, the CV's ranged from 9.9% to 23.4%, with five of six values less than 16.3%. Inter-lab imprecision matched analytical performance characteristics with a CV $\leq$ 20% for CMV tetramer+ results above the quantitation limit of 1.0 cell/mL, and a CV $\leq$ 15% for CD3+CD8+ absolute counts. These data also show that the laboratories were reporting results as expected which qualified the assay for further study. Figure 1 illustrates the results for absolute CD3+CD8+tet+/μL.

Final Multicenter Study

In Study 3, fresh whole blood was used instead of preserved samples that had been used previously. Intra-assay imprecision was estimated in Study 3 using the duplicates performed by each operator. That is, comparing results from tube 1 from Panel 1 versus the mean of tube 1 and 2 for Panel 1 and then analyzed using a Deming regression analysis. This analysis was repeated for Panel 2. The target was for an R^2 greater than 0.96 for all parameters and a slope from 0.94–1.06. Results were as follows: CD8 absolute counts (CD3+CD8+ cells/μL) R = 0.9953, slope 95% C.I. = 0.9671–1.001. Tetramer % positive (CD3+CD8+CMV-tetramer %) R = 0.9978, slope 95% C.I. = 1.0019–1.0253. Tetramer absolute counts (CD3+CD8+CMV-tetramer cells/μL) R = 0.9970, slope 95% C.I. = 0.9849–1.0118.

After exclusion of CMV seronegative samples and samples not tested due to shipment and/or instrument failure, there were a total of 100 individual results from five sites that could be evaluated for intra-assay precision. Of these 100 results, 73 had a CV $\leq$15%, and 96 had a CV $\leq$20%, see Figure 2. The median intra-assay (within-operator) CVs across all sites were 2.79% (CD8 cells/mL), 5.26% (tetramer % positive), and 6.13% (tetramer cells/μL), respectively.

Quantitation Limit

Intra-assay CVs were plotted against assay values across all seropositive specimens tested and ranked (G-thru-ZZ) to

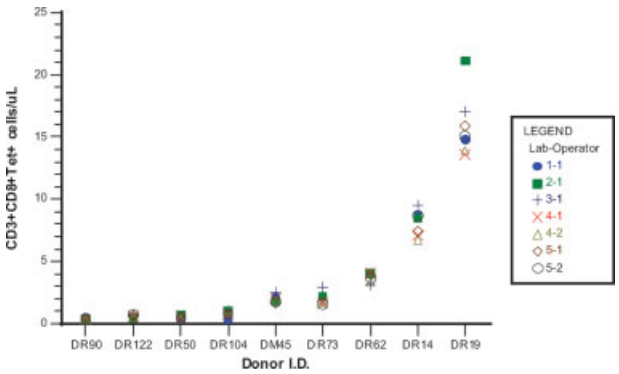

Figure 1. Nine whole blood samples stabilized by fixative and tested by five labs (sites), (seven operators) for the number of CMV tetramer+ T lymphocytes. Mean results of absolute number of CD3+ CD8+ Tetramer+ cells/μL versus donor identification are shown. Samples were rank ordered. Tetramer cells/Operator 1, Lab 1 (closed circle), operator 2, Lab 2 (square), Operator 3, Lab 3 (cross), Operator 4, Lab 4 (x), Operator 5, Lab 4 (triangle), Operator 6, Lab 5 (diamond), Operator 7, Lab 5 (open circle). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

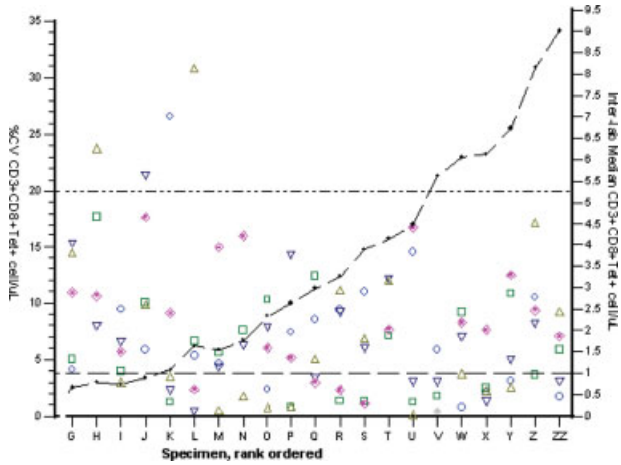

Figure 2. Twenty-one samples from CMV seropositive donors in Study 3 were tested and rank ordered G-thru-ZZ based upon reactivity to Tetramer. Intraassay imprecision (CV) was estimated by duplicate measure and is represented in the symbols identifying each site; Lab 1 (open diamond), Lab 2 (square), Lab 3 (triangle downwards), Lab 4 (filled diamond), Lab 5 (triangle upwards). All data for Tetramer+ cells/μL from labs 1-thru-5 are superimposed as curve. The lower and upper horizontal lines represent the quantitation limit of 1.0 tetramer+ cells/μL and the limit of imprecision of 20%, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

elucidate a functional sensitivity pattern in a true multicenter study setting, (see Fig. 2). An inverse relationship between intra-assay CV and tetramer cells/μL level was expected. This was the general observation, with notable deviations in some cases, especially with Lab 1 and 5. Of the 17 determinations greater than the quantitation limit (1.0 cells/μL), only four (4) had tetramer results with an imprecision >20%. The variability noted was due to a combination of factors including the use of shipped blood, age of blood, and low expression levels of the samples. Furthermore, Lab 1 had notable instrument difficulties throughout the study related to fluidics and quality control.

Interlaboratory Precision

A total of 27 samples $\times$ 5 labs $\times$ 2 duplicate meant there was a total of 270 possible results, (135 with first replicate). Five samples were lost to shipment or could not be tested due to instrument failure, or reported as a missing result. In summary, only 260/270 (130/135) results were obtained (96%). Six samples were tested using A*0101 (producing a total of 19 results CMV+, nine results CMV−), seven samples were tested using B*0702 (30 results CMV+, five results CMV−), and 14 samples were tested with A*0201 (53 results CMV+, 14 results CMV−). From the data, 5.4% (7/130) were excluded from final analyses using a univariate box plot procedure identifying outliers in either Panel 1 or 2 as falling outside of the 1.5$\times$ interquartile range below the 25th percentile rank, or above the 75th percentile rank that were tested within each sample's data set (expected $N = 10$ per set of results). Between-lab imprecision based upon only the first tube is shown in Table 4. In general, precision results were as expected. Between-laboratory imprecision for CD8 absolute counts (CD3+CD8+ cells/μL) was determined to be 6.11% (median CV) (range 1.00 to 18.1%), tetramer % positive (CD3+CD8+CMV-tetramer %) was determined to be 11.1% (median CV) (range, 1.78%–23.7%), and tetramer % positive (CD3+CD8+CMV-tetramer cells/μL) was determined to be 11.4% (median CV) (range, 0.48%–25.2%). Tested values in this experiment ranged from 0.2%1 to 3.14% tetramer positive %, and from 0.68 to 8.95 cells/μL (Fig. 2).

Of note, all labs correctly identified seronegatives (Samples A, B, C, D, E, F) and positives (G thru ZZ), see Figure 3. In general, between-laboratory results were achieved. The CMV tetramer+ absolute count (cells/μL) median was 14.8% including only data points greater than the functional sensitivity of 1.0 cells/μL. The imprecision ranged between 6.8% and 29.6% for absolute count levels between 0.5 and 8.0 tetramer (cells/μL). From the (17) determinations greater than the quantitation limit (1.0 cells/μL), only four (4) had imprecision >20% (Fig. 2).

A quantitation limit analysis was performed with the inter (between-lab) data as well. Total precision CVs were plotted against all assay values across all seropositive specimens tested and ranked (G-thru-ZZ) to elucidate a quantitation limit pattern (see Fig. 3). Only three samples (J, S, T) exceeded a CV of 20% in this analysis. Using total between-lab precision, imprecision for CD8 absolute counts (CD3+CD8+ cells/μL) was determined to be 6.86% (median CV) (range, 2.79%–28.0%), tetramer % positive (CD3+CD8+CMV-tetramer %) was determined to be 10.4% (median CV) (range, 3.89%–20.4%), and tetramer % positive (CD3+CD8+CMV-tetramer cells/μL) was determined to be 15.5% (median CV) (range, 6.59%–24.23%).

Assessment of Quality Assurance

The difference of $CD3^+CD8^+$ percentage between Panel 1 and Panel 2 can be best represented by Study 3 for all labs. The median difference was ~1% between tubes, with most results showing a difference of less than 3%. These results were typical of Study 1–3 (data not shown).

The number of CD3+CD8+ events collected by site was expected to be a minimum of 20,000. Assuming all Panel 2 acquisitions obtained by each operator = 20,000 CD3+CD8+ events, we can calculate yields of expected tetramer positive events per test; using the mean frequency of all sites per sam-

Table 4. Precision of the iTAg™ MHC tetramer assay

% CV[a]	CD3 + CD8 + CMV TETRAMER ABSOLUTE COUNT	CD3 + CD8 + TETRAMER %	CD3 + CD8 + ABSOLUTE COUNT
Median	11.4	11.1	6.11
Min	0.46	1.80	1.00
Max	25.2	23.6	18.1

[a] Precision estimated using only the first tube in the test and across all labs and operators in Study 3, outliers removed. Descriptive statistics of CV results are tabled.

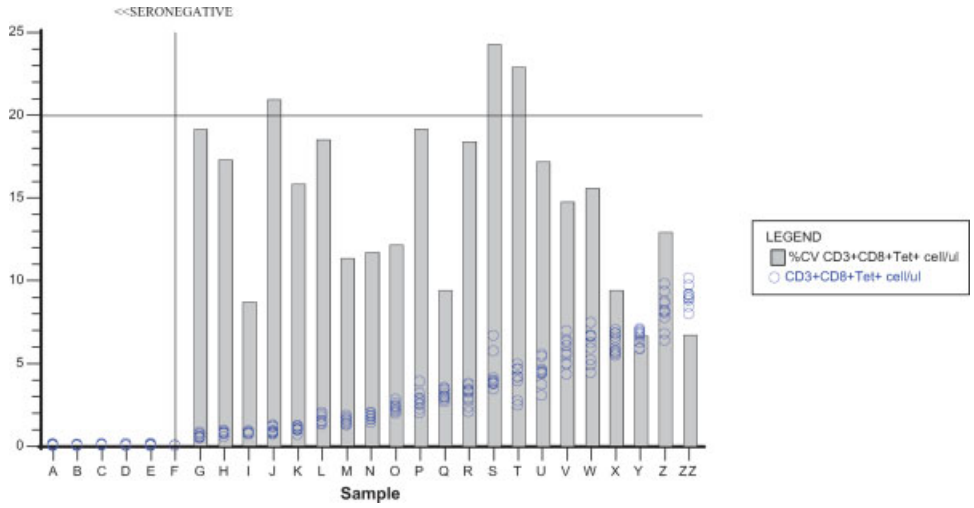

Figure 3. Twenty-seven samples in Study 3 were rank ordered A-thru-ZZ based upon reactivity to Tetramer. Total imprecision (CV) was calculated and is represented in the bar graph above for CMV seropositive samples. All data for Tetramer cells/μL from labs 1-thru-5 are superimposed as (O). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ple ×20,000, we calculate the expected tetramer positive events per result. The actual events "Observed–Expected" were calculated per result and plotted by site. Lab 1 typically did not acquire 20,000 CD3+CD8+ events and under recovered a median of 32 tetramer positive events less than other labs (Fig. 4).

Discussion

The dissection of T cell responses against pathogens, autoantigens, and tumor-derived antigens by MHC multimer staining has become an established technique in preclinical research, and is becoming increasingly important for clinical trial monitoring of responses to vaccines and correlating natural T cell responses with clinical outcomes (8,9,20,30,31). However, a more detailed comparison of the multimer assays used in the numerous reported studies demonstrates that a great variety exists in the flow cytometric methods employed. Moreover the utility of this methodology in multicenter studies still needs to be demonstrated. In addition, there is a need to standardize this method if it is to be implemented as routine diagnostic assay. The benefits of standardizing flow cytometric methods have been illustrated previously by multicenter studies for the enumeration of lymphocyte subsets and of CD34+ hematopoietic stem cells (32–34). Therefore, we studied the analytical performance of a standardized single-platform MHC tetramer assay in a multicenter setting, focusing on reducing background, maximizing signal intensity, and ensuring sufficient cells are enumerated to provide meaningful statistics. As an antigen model, we selected iTAg^{TM} MHC tetramers each bound to specific peptides of the structural matrix proteins pp65 and pp50 of human CMV. The pp65 protein has been identified as a major source of epitopes recognized by CD8+ T cells from CMV-seropositive individuals (35,36), and several studies have shown that MHC pp65 multimers can be successfully used for enumerating CMV-specific T cells in healthy CMV carriers and various clinical settings (16,23,24,37).

Our results showed that the overall quantitation limit, defined as the lowest concentration where the CV continues to be less than 20%, was determined to be 1.0 cells/μL for tetramer+ absolute counts and 0.2% tetramer+ percent. Spiking experiments with blood from CMV seropositive donors revealed a high accuracy of the assay with an overall percent recovery of 95%.

Inter- and intra-assay performance was assessed, which included inherent variability introduced by shipping, type of flow cytometer used, protocol adherence, and analytical interpretation across a range of multiple sample levels and specificities under routine laboratory testing conditions. Absolute count and percent imprecision within laboratory was deter-

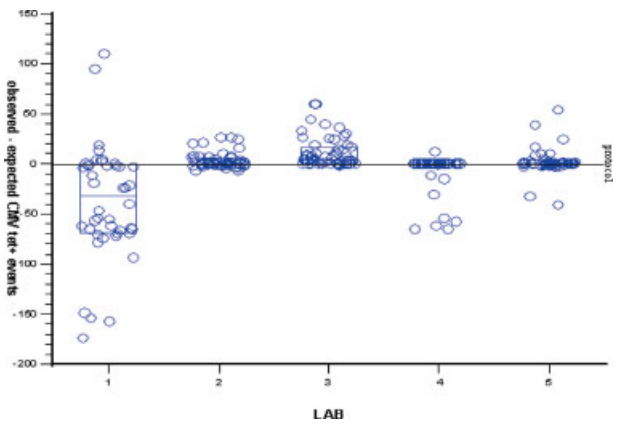

Figure 4. The number of CD3+CD8+ events collected by site was expected to be a minimum of 20,000. Assuming all panel two acquisitions obtained by each operator = 20,000 CD3+CD8+ events, we can calculate yields of expected tetramer positive events per test; using the mean frequency of all sites per sample x 20,000, we calculate the expected tetramer positive events per result. The actual tetramer+ events Observed—Expected were calculated per result and plotted by site. Laboratory site 1 failed to collect the desired number of events that was mentioned in the protocol. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mined to be less than 10% for the used tetramers at all test concentrations. Imprecision between laboratories was determined to be less than 15% at the low test concentrations, and less than 10% for the mid and high test ranges. A comparison of our data with those of other multicenter evaluations showed that the between-site variations of less than 10% for absolute CD8+ T cell counting achieved by us are at least as good as the CVs of 10–15% and 10% reported for the quality assurance schemes in Canada (38) and the Benelux (33), respectively. Our overall between-site and within-site CVs turned out to be better than a recently reported multicenter evaluation by the European Working Group on clinical cell analysis (39). In Study 2, stabilized whole blood was used in an attempt to ensure similar quality of sample for all centers. However, stabilized samples showed reduced PE signals which lead to analytical gating errors and greater imprecision (interpretation problems). Also, a pattern of poor red cell lysing was noted at all sites with stabilized samples and altered FS and SS properties, leading to difficulty in discriminating noise from lymphocytes. For this reason, fresh EDTA samples were used in Study 3, the multicenter trial. Most variability can be attributed to lack of protocol adherence to stop count, an important factor at low levels of detection, with one site in particular failing to achieve target stop counts for either total CD3+CD8+ or tetramer+ events (Fig. 4). Some variability can be attributed to a few samples prone to aging rapidly in transit (scatter changes, increased background). This was seen mostly in samples that were tested after 48 h. Technical issues that were important to standardize included the use of polypropylene tubes to minimize cell aggregates in Panel 2, running samples at medium flow rates to reduce coincidence and ensuring that there was adequate sheath fluid for the lengthy acquisition times required for samples with low cell counts. Panel 2 included a histogram of "time" versus "count" to allow identification of air bubbles or other flow instability during acquisition (see, supplementary information). The importance of inclusion of a negative (irrelevant tetramer) control cannot be overstated, as this allowed monitoring of any increase in background for the specific tetramer tube.

Although our results show that the utilized methodology can be considered as a reproducible assay for routine enumeration of antigen-specific CD8+ T cells, some limitations are evident. First, it is practically impossible to comprehensively cover for a given pathogen all potentially relevant T cell specificities by tetramers due to the combination of the polymorphism of the HLA system and the various antigens being (potential) sources of T cell epitopes. Inherent to the currently available multimers in combination with the selection of HLA alleles, analysis of T cell responses can, therefore, lead to skewed information in the various clinical studies. Second, several studies have shown that CMV-specific CD4+ T cell responses are also important for the protection against CMV disease (40–44). However, the use of Class II tetramers to enumerate antigen-specific CD4+ T cell responses is more problematic due to the difficulty to obtain stable peptide MHC Class II complexes (45). The ELISPOT and intracellular cytokine staining assays (ICS) (46,47) can be used for monitoring functional responses of CD4 as well as CD8 antigen-specific T cells. ICS can provide more detailed information that clearly distinguishes T cell subsets such as CD4 and CD8, as well as a number of other phenotypic markers on the responding cells (48). The difficulty of those dynamic assays to its use in a multicenter setting is related to various variability issues, including instability of reagents, stimulation requirements, duration of time, experiment-to-experiment variability and sample management, whereas tetramer-based assays are static tests that are less technically demanding and therefore more suited for high volume, high throughput sample processing allowing for reasonable comparisons of clinical evaluations across multiple centers. Third, counting antigen-specific T cells by immunophenotyping only does not provide information on their function. For instance functional analyses can also be of importance when immunocompromised patients are studied; for example, many organ and blood SCT recipients are receiving immunosuppressive treatment for graft rejection or GVHD. These therapeutic interventions primarily interfere with function and, to a lesser extent, with T cell absolute counts (49,50). For characterization of immune function it is more appropriate to use the ICS technology, especially when multiparameter (>4-color) flow cytometry is available.

Conclusion

In general, as tetramer values approach the lower detection limit, CVs increase. However, this does not necessarily relate to major changes in the absolute number of tetramers. For example for a tetramer with a "true" value of 1/μL, a range of 0.6–1.4/μL would represent a CV of 20%. Also contributing to CVs >20% were a lack of adherence to protocol and/or testing of samples >48 h old. The assay is simple, rapid, reproducible, and useful for assessing CMV-specific T cells, and will allow for reasonable comparisons of clinical evaluations across multiple centers at clinically relevant thresholds (2.0–10.0 cells/μL) if care is taken to obtain statistically significant events and the sample is tested within 24–48 h. CMV tetramer-based immune monitoring, in conjunction with virologic monitoring, can be an important new tool that permits clinicians to assess the risk of CMV-related complications and to guide preemptive therapeutic choices.

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