World Health Organization

WHO/BS/2017.2322
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 20 October 2017

Report of the WHO Collaborative Study to establish the First International Standard for Detection of IgG antibodies to Cytomegalovirus (anti-CMV IgG)

Nina Wissel[1], Kay-Martin Hanschmann[2], Heinrich Scheiblauer[1]
and the Collaborative Study Group*

[1] *Testing Laboratory for in-vitro Diagnostic Medical Devices and* [2]*Division of Biostatistics, Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany*
**See Appendix 1*

NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **18 September 2017** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr C M Nübling** at email: nueblingc@who.int

Background/Objectives

The aim is to develop a CMV IgG antibody (anti-CMV IgG) standard for diagnostic purposes, to improve comparability of the divergent result outputs of current anti-CMV IgG assays.

Material & Methods

A WHO collaborative study was conducted with 16 participants from 9 countries using 16 anti-CMV tests of different formats. A candidate standard A1, the anti-CMV IgG reference preparation (A2) of the Paul-Ehrlich-Institut (PEI), and 8 additional study samples with different levels of anti-CMV IgG/IgM and IgG avidity were used. The endpoint titers were determined by linear interpolation at the assay cutoff and by parallel-line-assay. The results were evaluated for potency ratios vs A1, correlation of analytic sensitivity relative to A1 by Spearman's rank correlation coefficient, and spread of the results.

Results

The candidate material A1 resulted in a mean end point titer of 46.4, which was used as overall potency. The titer range was 26-102 for the anti-CMV IgG assays. The additional study samples led to closely related titers and potency ratios within twofold range vs A1 in the majority of the tests. Correlation of the analytical sensitivity between A1 and the study samples ranged from low to moderate and high. However, there was also a group of tests with higher titer variation and lower correlation vs A1 in some study samples. This was associated with low anti-CMV IgG avidity resulting in reduced anti-CMV IgG titers in 4 test kits and poor reproducibility in 2 test kits. Other properties for a candidate standard such as homogeneity for high intra-assay precision (9% mean geometric coefficient of variation) and long-term stability under the recommended storage temperature (-20° C) were available.

Complementary Study

The analytical sensitivity ratio of 4 anti-CMV IgG test kits with candidate material A1 in the collaborative study was consistent with the diagnostic sensitivity score in 5 CMV seroconversion panels. Conversion of the test kit specific signals of the undiluted serial panel samples into A1 units led to comparable values.

Summary & Conclusion

A candidate material A1 was developed for CMV IgG antibody detection with an overall titer of 46.4. A1 showed commutability with the study samples in the majority of the assays by close potency ratios and positive correlation as well as consistency of the analytical and seroconversion sensitivity. For tests with low commutability, test kit related sources of variation were identified. Calibration of the tests using candidate material A1 was effective in harmonizing the results. Preparation A1 may be suitable for test calibration, comparisons of the analytical sensitivity and quality control. Candidate material A1 is proposed as the 1st international standard for anti-CMV IgG with an assigned unitage of 46.4 International Units per vial.

Table of Index

1 Introduction

Cytomegalovirus (CMV) is the most spread human herpesvirus with a seroprevalence from 40% to 100%. Transmission occurs by contact to body fluids and vertically in utero or during delivery. CMV can also be transmitted by blood transfusion, transplantation of organs and stem cells. Once established, CMV results in a lifelong latent infection that can reactivate later. In addition, reinfection may occur by a new viral strain [1]. CMV infection is a major cause of disease and death in immunocompromised people and patients under immunosuppressive therapy, including organ transplant recipients [2], as well as the leading viral cause of birth defects in the world [3]. As a result, control of CMV infection and reduction of CMV transmission by blood and tissue preparations are in the interest of public health, including the question of the best diagnostic methods and optimal prevention [3, 4].
Serological diagnosis of CMV is based on testing for CMV immunoglobulin G antibodies (anti-CMV IgG) along with anti-CMV IgM and IgG avidity. Anti-CMV IgG is used for screening, to assess serological status, to determine immunity and to evaluate the risk of CMV disease. Seroconversion to anti-CMV IgG is evidence for recent primary infection. Anti-CMV IgG in combination with IgM indicates primary or recurrent infection, and without IgM indicates past infection. CMV IgG avidity can distinguish primary from nonprimary CMV infection. A titer increase in sequential samples may indicate active infection [1, 5]. Provision of CMV seronegative blood or selection of long-term seropositive donations can be effective to reduce transfusion-mediated CMV [2, 6]. A further strategy is leukoreduction of the cell-associated CMV with the remaining infection risk being discussed [6]. The highest transmission risk is associated with new seropositive donors [6]. Therefore, CMV detection by highly sensitive serological assays is required [2, 6]. Enzyme immunoassays (EIA) are currently the most common anti-CMV assays, as well as passive hemagglutination (PHA) and immunofluorescence (IFA), and most tests are quantitative [1]. However, there is neither definition of International Units (IU) nor of a protective antibody level. As a result, anti-CMV tests differ by a variety of arbitrary units and cutoff definitions, resulting in test outcomes varying by orders of magnitude. Serological diagnostics therefore depend strongly on the assay used, and output values of different tests are not comparable. On the other hand, there is demand for anti-CMV standardization. A diagnostic anti-CMV IgG reference preparation from the Paul-Ehrlich-Institut (PEI) was regularly ordered by the manufacturers (7-10 vials/year, until recently 04-2017) and is used for batch control of anti-CMV tests at PEI.

The aim of this study is to develop an international anti-CMV IgG standard for diagnostic anti-CMV IgG assays. At the 2nd WHO Collaboration Centre's meeting in 2009, anti-CMV reference materials for IgG and IgM for diagnostic purposes were deemed required [7]. The study was therefore designed to study anti-CMV IgG as well as IgM. In 2012 samples were sourced by PEI and tested for suitability for a possible candidate material and for accompanying study samples. In April 2013 the project was presented to the WHO Collaborating Centre's meeting and the proposal provided to the ECBS was adopted in October 2013. The Collaborative Study was carried out between 2014 and 2016.

2 Material and Methods

2.1 Samples used in the Collaborative Study

The characteristics of the samples used in the collaborative study are shown in Table 1. All samples were pre-screened at the Paul-Ehrlich-Institut (PEI) using 3 anti-CMV IgG and IgM assays (Abbott Architect CMV IgG/IgM, Abbott Axsym IgG/IgM, Siemens Enzygnost Anti-

CMV IgG/IgM), CMV IgG avidity (Abbott, Architect CMV IgG Avidity), and the antibody profile in immunoblot (Mikrogen GmbH, recomBlot CMV IgG [Avidity] IgM). In addition, the material was tested for CMV DNA (RealStar CMV PCR Kit 1.0, altona Diagnostics, LoD 91.38 IU/mL), for anti-EBV (Enzygnost Anti-EBV IgG) and anti-HHV-6 IgG (Panbio Pty Ltd, HHV-6 ELISA). The samples were negative for HBV-DNA, HCV-RNA, HIV-1-RNA, HIV-2-RNA, anti-HIV 1/2, anti-HCV, HBsAg, Syphilis, and contain no additives.

2.1.1 Candidate for the anti-CMV IgG standard (Sample A1)

The material should be used for diagnostic and screening purposes [7] and should correspond in composition and antibody concentration to naturally occurring specimens. Candidate material A1 is a pool from 3 human plasmapheresis units (citrate plasma) collected in Germany (each 730 ml) in 2007 and 2009 (purchased from Biomex GmbH, Heidelberg, Germany). Pre-testing of A1 showed the following profile: highly positive for anti-CMV IgG (endpoint titers 37.5 to 118), high IgG avidity (81%), high reactivity for all CMV proteins in immunoblot (IE1, p150, CM2, p65, gB1, gB2), negative for anti-CMV IgM, negative for CMV DNA, and positive for anti-EBV-IgG and anti-HHV-6 IgG. The pool of 2100 ml was filled in 1 ml aliquots in 3 ml vials (neutral amber glass, 15.5 mm polypropylene screw cap, 14 mm freeze dry rubber stopper). After pooling, A1 was tested with the same test kits as the individual donations. A total of 1907 vials were freeze-dried in 2013 at Greiner Diagnostics AG (4900 Langenthal, Switzerland), and stored at -20°C. The wet fills had a coefficient of variation of 0.9% and the lyophilisate has residual moisture of 0.6% (Karl Fischer titration). Tests before and after freeze-drying showed no difference in the anti-CMV IgG reactivity.

2.1.2 Study samples used in conjunction with A1

2.1.2.1 Study sample A2

Sample A2 is the diagnostic anti-CMV IgG PEI reference preparation from a serum donation prepared by PEI in 1991, lyophilized and stored at -20°C in glass ampoules (0.5 mL, 300 arbitrary units (AU) per mL). A2 is anti-CMV IgG positive and the antibody pattern (p150, gB1, gB2) indicates long past infection. This material is weakly positive, can usually be diluted up to 1:4 on average, and has run out.

2.1.2.2 Study samples B1 – B8

Samples B1-B8 were additionally included to test commutability of A1. They represent clinical samples with different profiles for anti-CMV IgG/IgM and IgG avidity. B1-B7 are positive for both anti-CMV IgG and IgM in varying amounts, B8 is anti-CMV IgG only positive. Avidity ranges from low (B1-B3, B5), intermediate (B4, B7) to high (B6, B8). The various anti-CMV IgG and IgM band patterns were examined by immunoblot. The following CMV infection stages are overall represented: B1 and B5 recent infection, B2 and B7 primary infection, B6 could be from reactivation or reinfection (high IgG, reactivity to p150 and all other proteins, positive IgM), B3 is likely late primary infection, B4 possibly longer-term infection (>14 weeks). Samples B1-B7 were also positive for anti-EBV IgG and anti-HHV-6 IgG. Samples B1, B2, B3 and B4 were obtained from Biomex GmbH (Heidelberg, Germany) and B5, B6, B7 from Trina Bioreactives AG (Zürich, Switzerland). Study sample B8 is a plasma donor from 2001 obtained from Aachen/Germany and was selected because it had the highest IgG titer of 30 pre-tested anti-CMV positive samples.

2.2 Panel (C1 – C53)

The panel consists of 53 plasma samples which were collected in 2013 and were kindly provided by the German Red Cross (Frankfurt/M, Germany). The samples are negative for anti-CMV IgG/IgM and CMV DNA and positive for either Epstein-Barr virus (EBV) IgG and/or human Herpesvirus-6 (HHV-6). The purpose is to identify potential cross-reactivity between CMV and the other herpesviruses that could affect study results.

2.3 Design of the collaborative study

Participants were recruited based on a questionnaire and the study was carried out according to an agreed study plan. The study samples were delivered on dry ice with specific instructions for storage and reconstitution. Dilution ranges to obtain endpoint titers for the various samples were recommended based on pretests at PEI, and the dilutions should be performed with the matrix commonly used by the laboratory. If no suitable dilution matrix was available, PEI provided anti-CMV negative normal human serum. The results should be evaluated in accordance with the test-specific interpretation criteria of the manufacturer's instructions for use, and the data be returned in prepared data sheets. The study plan in detail:

- Three vials of lyophilized A1 and A2 were sent, and one vial in liquid per samples B1-B8 and C1-C53. Lyophilisates should be reconstituted in distilled water (A1 1 mL, A2 0.5 mL).
- Two-fold serial dilutions for each sample should be prepared: A1 1:8 to 1:4096; A2 1:8 to 1:1024; B1-B8 1:8 to 1: 1024. Further dilutions should be performed if the end point titers were not reached. The dilution matrix used should be tested in parallel in each individual run in triplicate as a control.
- Samples should be centrifuged 10-15 minutes at 3000 g prior to testing.
- Samples A1 and A2 should be tested in triplicate and in 3 independent runs on 3 different days, for each day a fresh vial.
- Samples B1-B8 should be tested in triplicates only once.
- Samples C1-C53 should be tested single and initially reactive results repeated in duplicate.

All samples were also tested for anti-CMV IgM with the test of the respective IgG assay manufacturer in separate dilution series (not shown).

2.4 Participants

The participants were selected to cover a wide variety of different anti-CMV IgG test kits and test formats, for global representation, and to represent different scopes, i.e. public-health, users of diagnostics and manufacturers. Twenty-four laboratories were invited, 16 laboratories from 9 different countries participated in the collaborative study (Appendix 1). A random number was assigned to each participant.

2.5 Test kits

Sixteen different anti-CMV test kits were used and assigned test kit numbers (Table 2) which are used throughout the report. Twelve test kits were anti-CMV IgG only assays, and 4 were anti-CMV total kits that detected IgG as well as other Ig classes (kits # 4, 9, 12, 13). The following test formats were included: 14 enzyme immunoassays (EIA) in the variants ELISA, ECLIA, ChLMIA, ELFA, 12 of the EIAs had an indirect test format, 2 EIA a sandwich format; 2 non-EIA test kits, passive hemagglutination (PHA) and immuno-fluorescence assay (IFA), which are read visually with non-numerical values. Test kit #4 was classified as anti-

CMV total assay due to its Ig class independent sandwich format, as stated by the manufacturer itself [8], as well as by lower anti-CMV IgG detection in the presence of anti-CMV IgM (see below section 3.9 and complementary study). The assays were mostly based on viral lysate or antigens derived from CMV strain AD169, while test kits #4 and #11 used recombinant CMV antigens (not specified). The test result interpretation was quantitative in 12 test kits, and 4 kits were qualitative. For all tests it was characteristic that they had very different cutoff or unit definitions, resulting in signal outputs differing several orders of magnitude; this diversity of result reporting was one of the starting points for the project.

2.6 Statistical methods

Endpoint titers with the various samples were calculated by linear interpolation at the intercept of the dilutions series with the assay's cutoff. Mean titers for each test kit and lab were calculated as the geometric mean value (GMV) over all replicates and repeat assays. Relative potency was also calculated by parallel line assay method (PLA) (for ln-transformed response data where necessary) [9], but PLA was valid only in 68% of the data sets (due to significant non-parallelism, non-linearity, or too few evaluable dilutions). Since most anti-CMV tests are quantitative, relative potencies were compared to linear interpolated titers, but not further analyzed. Spearman's rank correlation coefficient was used to assess correlation of analytical sensitivity between candidate material A1 vs the study samples. Correlation strength was interpreted as weak (0.20-0.49), moderate (0.50-0.69), strong (0.70-0.89), and very strong (>0.90) [10]. The potency ratio of each study sample relative to A1 was calculated to determine the consistency of the effect of A1 with the various tests in each sample. An agreement of the ratios between the test kits for the same sample within a twofold range was considered appropriate according to the relevant requirements for serological tests [11] and is regarded permissible batch testing. Repeatability (same test within lab) and reproducibility (same test between labs) of A1 and A2 were described as geometric coefficient of variation (GCV) [12]. Variability was evaluated by means of a mixed linear model (an Analysis of Variance, ANOVA, using fixed and random factors). The inter-assay / inter-lab-precision (intermediate precision) is described by the standard deviation and the coefficient of variation, derived from the total variation using all results. For the intra-assay-precision (repeatability) the residual variance is used. The measurement uncertainty then describes the estimated total variance from the ANOVA, also denoted as coefficient of variation. Analyses were performed using SAS version 9.4 [13], R version 2.6.1 [14] and CombiStats version 5.0 [15].

3 Results and discussion

3.1 Data received

Twenty-three data sets from the 16 laboratories and the 16 anti-CMV test kits were evaluated.

3.2 General description of the evaluation of the study

The presentation of the results is first outlined below, then the detailed results and performance of A1 are presented and discussed in detail in the following sections for the individual study samples and topics.

1. The geometric mean values (GMV) of the anti-CMV IgG endpoint titers with the candidate material A1 and the study samples are shown in Table 3. The distribution of the GMV titers with the various tests in the samples is also displayed in histograms (Figure 1).
2. The performance of the candidate material A1 versus the additional study samples is described, (i) by determination of the potency ratios relative to A1, and (ii) by correlation of the analytical sensitivity between A1 and the study samples. The potency ratios vs A1 are shown in Table 4 and graphically in Forest plots as median of the potency ratios with all tests for each study sample with a twofold range (Figure 2). Correlation of the analytical sensitivity of A1 with the study samples according to Spearman's rank correlation coefficient is shown in Table 5, and in scatter plots of the value pairs between A1 and the study samples (Figure 3).
3. Measurement error estimation in the results with the candidate material A1 and sample A2 is shown for repeatability and reproducibility of the results, expressed as GCV% (Table 6). The variability of the results in the study samples B1-B8 was analyzed as GCV% of the potency ratios relative to A1. The inter-laboratory variability (same test, different laboratories) in study samples B1-B8 was analyzed as coefficient of variation (CV) of the titers of the respective test between the laboratories. In addition, the influence of anti-CMV IgG avidity on the variability of the test results is shown (Figure 4 and Figure 5).
4. Finally, the antibody profile of the study samples (Table 1) was examined to see whether certain anti-CMV IgG patterns could explain differences between tests.

3.3 Candidate material A1

The endpoint titers for the candidate material A1 with all the assays are shown in Table 3. The distribution of the titers is also visualized in a histogram (Figure 1). The mean endpoint titer with all assays was 46.4 in a range of 26 (kit #6/lab 9) to 102 (kit #5/lab 7) in the anti-CMV IgG only assays and 26-233 including the total anti-CMV (IgG/IgM) assays. The two outer values were (i) from the IFA test (#29), whose visual reading allowed only a lower titer gradation or where the endpoint titers could not be unambiguously determined (section 3.9) and (ii) from a total anti-CMV Test kit (#9) with a sandwich test format showing a higher titer (233) than all other assays. The anti-CMV total test kits (# 12, 13) with indirect test format detected anti-CMV IgG within the range of pure anti-CMV IgG test kits. Anti-CMV test kit #4 was excluded from the potency evaluation as obvious outlier and unclear design as discussed below (section 3.9). Intra-assay (mean GCV 9%) and inter-laboratory variability (mean GCV 6-32.3%) with A1 was acceptable (Table 6). Since there is no pre-existing international standard for anti-CMV IgG to compare with and no acknowledged anti-CMV reference method, the mean titer of 46.4 with all assays was used as the overall potency for A1.

3.4 Study sample A2

Study sample A2 represents the current PEI anti-CMV IgG reference material which is low positive for anti-CMV IgG. The mean endpoint titer of A2 (Table 3 and Figure 1) was 7.8, in a range of 4 (kit #10/lab 8) to 14.7 (kit #3/lab 4) in the anti-CMV IgG only test kits and a range of 4 to 26 including anti-CMV total test kit #9. Test kit #13 (PHA) with discontinuous values is regarded as outlier because it was positive only in the undiluted sample. The mean potency ratio relative to A1 was 5.6 within a narrow range of GCV 28% (Table 4 and Figure 2). Correlation of A2 with A1 by Spearman's rank coefficient (Table 5-1, Figure 3-1) was strong (r 0.80, p 0.0002). Intra-assay and inter-lab variability was low with all test kits similar to A1 (Table 6). Overall, sample A2 behaved like A1 consistently across the different assays. The antibody profile of A2 positive only for the structural proteins (p150, gB1 and gB2) was sufficient for a similar relative potency vs. A1 with complete antibody profile across all assays.

3.5 Study samples B1-B8

Study samples B1-B8 comprise a variety of different combinations of anti-CMV IgG, IgM and anti-CMV IgG avidity reflecting the diagnostic range for anti-CMV serology. Since the total anti-CMV test kits inherently react with anti-CMV IgM, the IgG/IgM mixed samples B1-B7 were evaluated only with the pure anti-CMV IgG assays.

The GMV titers of B1-B8 and their distribution are shown in Table 3 and Figure 1. The anti-CMV IgG only sample B8 showed similar titers as A1 with a narrow spread. The titers in study samples B1-B7 with mixed IgG/IgM antibodies and different avidity profiles were more heterogeneous. The spread of the values was analyzed using the potency ratios relative to A1 (Table 4 and Figure 2). In the majority of the tests the potency ratios were within a twofold range around the median. A twofold difference was considered appropriate according to relevant requirements for serological tests [11]. In terms of variability by GCV, the majority of the tests were in a range of 28% and 83% (Table 4). However, a greater spread in a range of GCV 28-156% was observed for test kits # 1, 2, 10, 11.

The relationship of the analytical sensitivity between candidate material A1 and the study samples was analyzed by correlation according to Spearman's rank coefficient as shown in Table 5-1 and Figure 3-1. Overall, candidate material A1 showed positive correlation with the study samples in varying degrees: high correlation for B5 (r_s 0.91), moderate correlation in samples B1-B3, B7 (r_s 0.56-0.61), and low correlation in samples B4 (r_s 0.46), B6 (r_s 0.25) and B8 (r_s 0.19).

Two causes were identified for the above described value dispersion and less pronounced correlation vs A1: (i) Test kits # 1, 2, 10, 11 showed lower anti-CMV IgG titers with decreasing IgG avidity of the samples (Figure 4). (ii) Test kits # 5 and 7 showed poor inter-lab reproducibility also associated with low anti-CMV IgG avidity (Figure 5). Both causes are considered test-related. It is known that the antibody signal in immunoassays is dependent on the antibody concentration and the avidity [11, 16, 17]. In addition, dilution of low-avidity antibodies may cause non-linearity and reduced test response. Adjustment of the data by removal of these two variability sources resulted in significantly higher correlations of samples B4, B6, B8 vs A1 as shown in Table 5-2 and Figure 3-2.

In summary, the candidate material A1 and the samples B1-B8 showed similar behavior in the majority of the anti-CMV IgG tests, indicating commutability of A1. There were, however, test kits that showed less commutability to A1. The source for this was identified and was essentially due to the response of the respective test kits to low avidity IgG antibodies.
Finally, the antibody profiles of the study samples (Table 1) in immunoblot did not explain the differences between the tests. As indicated above for sample A2, p150 appears to be the main protein for CMV antibody formation which occurs in all CMV infections [18, 19]. Obviously, only a few CMV proteins (p150 primarily and to lesser extent p65 and p52 [CM2 in the blot used]) are necessary for detection of antibodies and changes in the antibody titer, as reported [18]. Despite the complexity of herpesvirus proteins, the antibody response against CMV appears to be uniform.

3.6 Repeatability and reproducibility

The variability of the GMV titers with samples A1 and A2 was analyzed for repeatability (same test within laboratory) and reproducibility (same test between laboratories) using the geometric coefficient of variation (GCV) as shown in Table 6. Repeatability with candidate material A1 was generally high at mean 9% GCV, range GCV 0.7% (test #7/lab 12) to 16.1% (test #2/lab 3). This represents low variability within or below normal imprecision of serological assays (CV 10-15%). The assay accuracy in the study with candidate material A1 was thus not biased and homogeneity of A1 can be assumed. The reproducibility for candidate material A1 could be calculated for test kits #2-5 and 7 which were used in 2-3 laboratories. The GCV values were mostly in a range of 6-15%, as expected greater than the intra-laboratory variability but in an acceptable range for serological assays (20-30%). However, a high inter-laboratory variability was obtained with test kit #5 in one laboratory (#2) of GCV 42.5% compared with the 2 laboratories (GCV 13.8%) and with kit #7 between the 2 laboratories of GCV 32.3%. Laboratory #2 with test kit #5 overall showed high variability both intra- and inter-laboratory, presumably due to a matrix effect (see section 3.9). The repeatability with sample A2 was on average the same as for candidate material A1, and also the reproducibility with sample A2 was comparable to that of candidate material A1. Slightly lower inter-lab GCV values for A2 are probably due to the lower dilution error compared to A1.

The variability of the results with the additional study samples B1-B8 was analyzed by the potency ratios relative to A1 and described as GCV% (Table 4). The distribution of potency ratios is shown graphically in Figure 2. The overall GCV ranged from 27.5 to 155.6%. Variability in study samples B1-B3 and B6-B7 was mainly traceable to test kits # 1, 2, 10, 11 which yielded lower anti-CMV IgG titers at low-avidity as described above. Exclusion of these test kits resulted in GCV ranges of 27-83% for the majority of the tests. The effect of low avidity on the variability of the results was investigated closer. While the average titers of all test kits showed no dependence on avidity in the study samples (Figure 4, A), the anti-CMV IgG titers for test kits # 1, 2 10, 11 were decreased with decreasing avidity (Figure 4, B). In comparison, the titers for the pure IgG samples with high avidity were the same in all test kit groups (Figure 4, C). It should be noted that there were also individual results from other test kits that deviated from the average: Kit #3 in sample B4, kit #5 in sample B6, and kit #7 in sample B7.

Inter-laboratory variability in study samples B1-B8 was tested for test kits # 2, 3, 5, 7 as coefficient of variation (CV %) between laboratories. As shown in Figure 5, test kits #5 and #7 showed greater variability than test kits #2 and #3. The variation was up to CV 43% high in test kit #5 and CV 57% for test kit #7 and increased with decreasing avidity of the samples.

In contrast, test kits #2 and #3 showed a consistent low inter-lab variation with CV maxima of 27% and 13%, and constant across the avidity range.

3.7 Neutralization assay

Determination of neutralization antibodies to CMV can help distinguishing primary from secondary CMV infection and can improve diagnosis of recent primary infection because neutralization antibodies appear only after 13-15 weeks post-infection [20]. In addition, neutralization antibodies may be important for assessment of CMV protection [21]. A microscale neutralization test (NT) was used in the study based on reverse transcription quantitative PCR (RT-qPCR) in ARPE-19 epithelial cells with CMV strain AD169^{wt131} [21]. The results are shown in Table 7. Candidate material A1 had the highest neutralization mean titer (347) of all study samples. Sample A2 had an NT titer of 67 with potency ratio vs A1 of 5.2 which was substantially consistent with the potency GMV ratio of 5.6 in the other methods (Table 4). Samples B1-B8 showed neutralization capacities corresponding to their serostatus and/or reactivity against the viral glycoproteins (gB) in immunoblot, contributing to the initial classification of the serostatus of the samples (Table 1). It is finally noted that different reactivity in the samples for neutralization-inducing gB proteins did not explain differences between the tests in the study. Overall, there was correlation with candidate material A1 between the CMV IgG antibody concentration in immunoassays and the CMV neutralization titer. The candidate standard can thus help to define an immune protection level against CMV or to investigate the efficacy of CMV vaccines.

3.8 Panel C1-C53

This panel should assess possible cross-reactivity related to human Herpesvirus-6 (HHV-6) and Epstein-Barr virus (EBV) since the candidate material A1 is positive for both viruses. EBV and HHV-6 are the second most common herpesviruses, HHV-6 is closely related to CMV (beta-herpesvirus), and false positive anti-CMV results due to EBV were reported [22, 23]. The results are shown in Table 8. Ten anti-CMV tests showed specificity of 100%, and 6 test kits showed specificities of 98.1-74.6%. Samples C11, C19, and C36 were false positive in 2 or 3 different test kits, indicating a common cause. The IFA test (#29) was remarkably often false positive with 13 samples. This may be related to the known nonspecific binding to Fc receptors of CMV-infected cells. Test kit #11 was false positive in 3 samples after initial testing and in 2 after repeat testing. Overall, there was no detectable cross-reactivity against HHV-6 and EBV with candidate A1 or the other study samples that would have biased the study results. However, the IFA test (#29) showed high non-specificity which affected evaluation of this test.

3.9 Special findings

In the following, results are described which were conspicuous or inconsistent and which led to identification of outliers.

Test kit #4 was not included in the overall evaluation, because the anti-CMV IgG titer was excessively high in the anti-CMV IgG-only samples while it was disproportionately low in the presence of anti-CMV IgM, and due to its sandwich test formats it reacted like an anti-CMV total assay as also stated by the manufacturer itself [8]. Test kit #4 also showed low anti-CMV IgG detection in the presence of IgM in some seroconversion panels of the

complementary study. The other anti-CMV total sandwich assay (#9) in the study, which also showed higher titers in the pure anti-CMV IgG samples (A1, A2, B8) compared to the other anti-CMV tests. Whether this is specific for the sandwich test format or for the individual tests cannot be decided from the study.

The data set for test kit #5/lab 2 was excluded. The data points towards a possible matrix effect due to lower gradation in endpoint titration and no endpoint titers in samples B3 and B5, as well as poorer repeatability and reproducibility compared to the other 2 laboratories with the same kit (intra-lab GCV 32% vs 8.4-9% and inter-lab GCV 42.5% vs 13.8%).

Test Kit #11 showed a high outlier titer in sample B7 and comparatively low titers with samples B6 and B8 which did not match the data of this test for the other samples and the results of the other tests. Test #11 uses recombinant antigens in contrast to most other tests. The antibody pattern of the respective test samples in the immunoblot could not explain the different reactivity of the test kit # 11.

The IFA test (#29) tests showed comparatively higher titers or did not lead to a titration endpoint probably related to a high non-specificity (see section 3.6). In PHA test (#13) all study samples containing anti-CMV IgM were positive only undiluted without titration graduation (test kit #13 is meanwhile no longer available). The difficulties with these two tests, which are read visually and which give discontinuous values, led to the exclusion of their evaluation for samples B1-B8.

Moreover, there were the following deviations from the study plan, which may have affected the statement about the accuracy of the mean values and/or about the intra-assay precision: Laboratory 6 /test kit #25 and laboratory 9 /test kit #6 tested A1 and A2 only in one day, Laboratory 4 /test kit #3 tested only 1 of 3 replicates of the A1 and A2 dilution series in day 2.

3.10 Stability of the freeze-dried candidate material A1

Real-time stability (Table 9) is examined after storage at the recommended temperature of -20°C at the following time intervals: 1, 3, 6, 12, 24 months and then annually. At the given times the vials are tested by default with test kit #3 (Architect CMV IgG). Up to the currently last measured time point (12 months) the recovery compared to time point 0 was 99.3% and the stability kinetics shows no out-of-trend results. Accelerated stability was examined at 4°C, room temperature (24°C) and 37°C for 7, 14 and 21 days (Table 9). After 21 days there was no indication of reduced reactivity of A1 at any elevated temperature. The recovery compared to storage at -20°C was 95.2% at +4°C, 95.8% at RT, and 98.3% at 37°C. Additional vials were kept for 6 months at +4°C and for 1 year at room temperature: at 4°C there was no stability reduction, at room temperature after 12 months there was a reduction in reactivity of 24%. Overall, the data obtained so far show sufficient stability of A1 for long-term storage at the recommended temperature of -20°C.

4 Complementary Study

4.1 Objective

Aim of the complementary study was to compare the analytical sensitivity of the candidate material A1 obtained in the collaborative study with the diagnostic (seroconversion)

sensitivity in native clinical samples. It was also examined whether test calibration using the candidate material A1 was effective in harmonizing the results.

4.2 Materials and methods

Four anti-CMV IgG test kits were selected to cover the analytical sensitivity range of the collaborative study from low to high (Table 3). Five CMV seroconversion panels represent neat clinical samples with a total of 75 serial samples (Table 10). The analytical sensitivity of the 4 anti-CMV IgG assays was grouped in descending order of the endpoint titers with candidate material A1 (kits # 5, 3, 2, 6) and compared with the total number of positive samples of the respective assays in the 5 seroconversion panels. The panels were selected to provide a graded sensitivity assessment through narrow bleeding intervals and with a negative onset. The panels were also characterized for anti-CMV IgM, antibody profile in immunoblot and anti-CMV IgG avidity. The avidity of the serial samples was in the range of <0% to 80%. Conversion of test-specific kit signals to A1 units was performed by linear interpolation of the serial panel samples on the calibration curve with the candidate material A1 or by linear regression if the values were outside the calibration curve.

4.3 Results

Comparison of the scores of analytical sensitivity and seroconversion sensitivity of the test kits is shown in Table 11. The individual results of the 4 tests with the 5 seroconversion panels are shown in Table 10. Overall, there was agreement between analytical sensitivity ranking in the collaborative study with the positive score ranking obtained with the seroconversion panels. The differences in seroconversion sensitivity between the anti-CMV assays however were rather low. Test kit #3 was one sample more positive than test kit #5, deviating from the analytical sensitivity rank, with both test kits being adjacent in the ranking, and the sample in question was in both test kits near the cutoff. The diagnostic sensitivity of the IgG tests was therefore relatively similar. Also pairwise comparison of the values with the 75 serial samples between the 4 anti-CMV tests showed strong correlation coefficients of r_s 0.80-0.96 (Figure 6). The correlation covers the entire avidity range of the serial samples from low to high. It is noteworthy, that test kit #2, which was affected by reduced titers at low avidity in the diluted samples of the collaborative study, did not show these reduced signals in the undiluted serial panel samples. This suggests that with neat samples the anti-CMV IgG tests are less affected by low avidity than with diluted samples for analytical methods. Despite comparable sensitivity, however, the value output of the tests is very variable, which calls into question the ability of the tests to classify the magnitude of the test output and the quantitative test interpretation. By normalizing the test-specific signals into A1 units, a substantial harmonization of the initially different values could be achieved, as shown in Figure 7.

Test kit #4 was also tested with the 5 CMV seroconversion panels because it had been excluded in the collaborative study. It was confirmed that this test kit did not behave like an anti-CMV IgG-only test but rather as an anti-CMV total test. In panel SCP-CMV-005 and SCP-CMV-006 the signals followed the anti-CMV IgM course and there were gaps in detection of anti-CMV IgG in panels SCP-CMV-003 and SCP-CMV-007 (data not shown; also included in the data sheets of the panel vendor). In addition, anti-CMV IgG detection of kit #4 in the panels (56 positives) was lower than the other 4 tests as opposed to its analytical sensitivity.

4.4 Summary

The analytical results of the Collaborative Study were reflected in the seroconversion panels of the Complementary Study. Candidate material A1 was commutable with native clinical samples for 4 methods covering the analytical sensitivity range of the collaborative study. Calibration by use of candidate material A1 may contribute to harmonization of anti-CMV IgG results for native clinical samples.

5 Overall summary and conclusion

A candidate standard for detection of antibodies to CMV IgG (anti-CMV IgG) has been developed. Suitability of candidate standard A1 was demonstrated for the intended purpose, i.e. calibration of anti-CMV IgG detection in diagnostic tests. The titers for A1 were uniform in the majority of the anti-CMV tests. Commutability of A1 for the additional study samples was shown by relatively uniform titers, narrow potency ratios relative to A1 within a twofold range, and by correlation according to Spearman's rank coefficient in the majority of the anti-CMV IgG tests. On the other side, there were tests that were less commutable due to higher titer variation and lower correlation compared to A1. The sources for this variation of the results could be identified and were mainly test-related, i.e. test-specific lower anti-CMV IgG detection at low IgG avidity and poor inter-laboratory reproducibility. Adjustment of this test-induced variability improved homogeneity of the data and correlation vs A1 for all study samples to higher levels. In addition, the anti-CMV IgG titration with A1 correlated with the CMV neutralization titer, which can contribute to the interpretation of a protective immunity level. In a complementary study, the analytical sensitivity from the collaborative study with the candidate A1 material correlated with the diagnostic sensitivity in CMV seroconversion panels. Calibration of the tests by using candidate material A1 and converting the different test signals of the undiluted panel samples to A1 units resulted in harmonization of the test results.

In conclusion, candidate standard A1 can be useful for the assessment of the analytical sensitivity of anti-CMV IgG detection as well as for the quality control testing and quantitative measurement of anti-CMV. In view of the variety of current anti-CMV tests and their non-comparative performance, calibration of the anti-CMV IgG test kits with the candidate standard A1 should therefore significantly facilitate comparability between the tests and make interpretation of results more reliable. Therefore it is proposed, that the candidate material A1 is established as the 1st WHO International Standard for anti-CMV IgG for serological assays with an assigned unitage of 46.4 IU per mL or per vial.

6 Comments from participants

All participants were asked to comment. Eleven out of 16 participants answered, one participant could not be reached. Eight participants agreed to the report. The other answers were neutral or included only minor corrections. There was no negative opinion. Here the individual comments:

1 Dr David Padley (NIBSC): “This is a really good study & will hopefully have a positive impact on CMV IgG screening. This study also points out how important avidity of

antibodies is. This should be taken into account for all future IgG standards, where possible. Finally was the CMV IgG standard screened for HTLV1&2?"

2 Dr Evi Struble (FDA): "Congratulations on the completion of this report and thank you for sending it. I too think this is an important activity that will help with screening clinical samples but also in other areas of clinical relevance. As has been our experience while searching for an appropriate kit to use in our research, this study also shows that CMV kits in the marketplace differ quite a bit in terms of sensitivity and accuracy. Having an appropriate standard will help alleviate that. Best of luck with the ECBS meeting."

3 Dr Haru Murata (OVRR/CBER/FDA): "Thank you very much for sending this interesting report. I have minor revisions on P. 12, P. 24, and P. 38 (identified by the "track changes" function in Word). Please let me know if you have any questions or if you need additional information. We very much appreciate the opportunity to participate in this study."

4 Eva Wald (Virion-Serion GmbH): Minor typing error corrected.

5 Stefanie Schneider (medac GmbH): Minor typing error corrected.

6 Dr Luca Pallavicini (Diasorin S.p.A.): No comments.

7 Dr Anna P Obriadina (RPC Diagnostic Systems): "We review the report and do not have any comments or additions. Thank you and the entire team for a very necessary and useful study."

8 Dr Sheila Dollard (CDC/OID/NCIRD): "The manuscript looks fine and I approve without any specific comments. It is impressive and looks like it was a tremendous amount of work. I have a question that was not easily gleaned from the discussion, charts and tables; did your analysis show considerable discordance among the various CMV IgG tests, and specifically how concordant was the VIDAS test that my lab uses? When discussing CMV serology testing I have always said commercial IgG tests are generally reliable and it is mainly the IgM tests that are discordant and difficult to use. Maybe this is not exactly true."

9 Dr Marcia Otani (Hemocentro de São Paulo): "Congratulations for the excellent work."

10 Dr Kay Hourfar (German Red Cross, Frankfurt): Minor comments, i.e. to the CMV-DNA method and that test kit #13 is no longer available.

11 Dr Emilio Perreira (Biokit S.A.): "Regarding the report of the 1st IS CMV IgG from WHO, the results obtained from our kits are the expected, there are nothing strange in the results. Also we can see that our kits have quite similar performance in comparison with the others."

7 References

1. Revello MG and Gerna G. State of the Art and Trends in Cytomegalovirus Diagnostics. Chapter II. 18, in: Cytomegaloviruses: From Molecular Pathogenesis to Intervention, Matthias Johannes Reddehase, Niels Lemmermann (eds.), Caister Academic Press, Norfolk UK, 2013, pp 380-399.
2. Kotton CN, Kumar D, Caliendo AM, Asberg A, Chou S, et al. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation. 2013 Aug 27;96(4):333-60.
3. Rawlinson WD, Boppana SB, Fowler KB, Kimberlin DW, Lazzarotto T, et al. Congenital cytomegalovirus infection in pregnancy and the neonate: consensus recommendations for prevention, diagnosis, and therapy. Lancet Infect Dis 2017, Jun;17(6):e177-e188. doi: 10.1016/S1473-3099(17)30143-3. Epub 2017 Mar 11.

4. Stratton KR, Durch JS, Lawrence RS (Eds). Vaccines for the 21st Century: A Tool for Decisionmaking. The National Academies Press. 2000;165-72.
5. Dollard SC, Staras SA, Amin MM, Schmid DS, Cannon MJ. National prevalence estimates for cytomegalovirus IgM and IgG avidity and association between high IgM antibody titer and low IgG avidity. Clin Vaccine Immunol. 2011;18(11):1895–1899.
6. Ziemann M and Hennig H. Prevention of Transfusion-Transmitted Cytomegalovirus Infections: Which is the Optimal Strategy? Transfus Med Hemother 2014, 41:40-44.
7. Report of the 2nd meeting with the WHO Collaborating Centres for Biological Standards and Standardization, 17-18 February 2009. http://www.who.int/medicines/publications/WHO_CCs2nd-IVD_MeetingReport.pdf. Status 2017/05/04.
8. Roche Diagnostics Ltd. Elecsys CMV Panel fact sheet - cobas. Roche (2011). http://www.cobas.com/content/dam/cobas_com/pdf/product/Elecsys-CMV-Panel/Elecsys%20CMV%20Panel%20Fact%20Sheet.pdf. Status 2017/05/04.
9. Finney D J. Statistical methods in biological assay (3rd Edition), 1978. London: Charles Griffin.
10. Zöfel Achim Bühl/Peter: SPSS für Windows 12.0, 9. Auflage, 2005.
11. Porstmann T, Kiessig ST. Enzyme immunoassay techniques. An overview. J Immunol Methods. 1992 Jun 24;150(1-2):5-21.
12. Kirkwood TEL. Geometric means and measures of dispersion, Biometrics 1979. 35, 908-909.
13. SAS 20132 - 2008. SAS Institute Inc., Cary KC.
14. R: The R foundation for statistical computing.
15. CombiStats v5.0 EDQM - Council of Europe, www.edqm.eu/en/combistats.
16. Reverberi R and Reverberi L. Factors affecting the antigen-antibody reaction Blood Transfus 2007; 5(4):227–240.
17. Lagrou K, Bodeus M, Van Ranst M, Goubau P. Evaluation of the New Architect Cytomegalovirus Immunoglobulin M (IgM), IgG, and IgG Avidity Assays. J Clin Microbiol, 2009 Jun;47(6):1695–1699.
18. Lazzarotto T, Paya CV, Smith TF, Wiesner RH, Krom R, Landini MP. Antibody response to cytomegalovirus (CMV) polypeptides in liver transplant recipients with CMV hepatitis. Microbiologica. 1992 Jan;15(1):15-22.
19. Kaden J, Ludwig U, Preyer R. Serum IgG, IgM, and IgA Antibody Response against Cytomegalovirus-Specific Proteins in Renal Transplant Recipients during primary and secondary/recurrent Infection as determined by Immunoblotting Technique. Transplantationsmedizin 2005, 17. Jahrg., S. 61-74.
20. Eggers M, Radsak K, Enders G, Reschke M. Use of recombinant glycoprotein antigens gB and gH for diagnosis of primary human cytomegalovirus infection during pregnancy. J Med Virol 2001; 63:135–142.
21. Wang X, Peden K, Murata H. RT-qPCR-based microneutralization assay for human cytomegalovirus using fibroblasts and epithelial cells. Vaccine 2015, 33:7254–7261.
22. Robertson P, Beynon S, Whybin R, Brennan C, Vollmer-Conna U, Hickie I, Lloyd A. Measurement of EBV-IgG anti-VCA avidity aids the early and reliable diagnosis of primary EBV infection. J Med Virol. 2003; 70:617–623.
23. Weber B, Berger A, Rabenau H. J Virol Methods. Human cytomegalovirus infection: diagnostic potential of recombinant antigens for cytomegalovirus antibody detection. 2001 Aug;96(2):157-170.

8 Acknowledgements

We gratefully acknowledge the important contributions of the collaborative study participants.

We would like to thank very much the DRK Frankfurt Germany, Dr. Kai Hourfar, for providing the donations used for the negative panel C1-C53.

Sincere thanks go to our dedicated colleagues of the Paul-Ehrlich-Institut, Section 2/4 (Molecular Virology) for testing the samples on CMV DNA, and Section 3/1 (Product Test of Immunological Biomedicine) for Karl Fischer titration of the lyophilized fill of the candidate material A1.

We are very grateful to Dr. Sigrid Nick and Dr. Micha Nübling for critical review of the report.

9 Tables and Figures

Table 1: Samples used in the Collaborative Study.

Study	Ab Titer [1]		Avi [2]	Antibody profile[3] IgG						Antibody profile[3] IgM						CMV	EBV/	CMV	Specimen source
sample	IgG	IgM	%	IE1	p150	CM2	p65	gB1	gB2	IE1	p150	CM2	p65	gB1	gB2	inf. stage[4]	HHV-6[5]	DNA[6]	and type
A1	60.8	neg	81.4	3+	3+	2+	2+	3+	3+	–	+	+/–	–	–	–	past	pos	neg	Pool of 3 plasma units, Germany, 2007/2009
A2	14.1	neg	79.3	–	3+	–	–	+	+	–	–	–	–	–	–	long past	n.d.	neg	PEI reference preparation, 1991
B1	79.6	38.0	17.0	+/–	+	2+	3+	+/–	–	+/–	2+	2+	2+	–	–	early primary	pos	neg	Citrate plasma, Germany, 2008
B2	93.5	49.5	24.5	–	+	+	3+	2+	2+	–	2+	2+	–	–	–	primary	pos	neg	
B3	61.0	36.9	32.7	+/–	2+	+	2+	2+	3+	–	2+	–	–	–	–	late primary	pos	neg	
B4	67.8	23.1	48.8	+	3+	+	+/–	+/–	–	+	2+	–	–	–	–	longer-term	pos	neg	
B5	14.6	115.7	16.6	+	2+	+	+	+	–	–	3+	3+	+	–	–	acute primary	pos	pos[7]	ACD-A plasma, 2012, M, age 37
B6	50.1	15.7	68.3	2+	+	2+	2+	+	+	–	2+	3+	2+	–	–	recurrent (?)	pos	neg	CPD plasma, 2012, F, age 18
B7	8.1	188.6	53.6	3+	2+	2+	2+	–	–	–	2+	3+	2+	–	–	primary	pos	neg	CPD plasma, 2012, F, age 37
B8	111.1	neg	75.4	3+	3+	3+	3+	3+	3+	–	+	–	–	–	–	past	n.d.	neg	Plasma donation, Germany, 2001
C1–C53	Negative for anti-CMV IgG and IgM															neg	pos[8]	neg	Human plasma donations, 2013

Abbreviations: Ab=antibody; Avi=avidity, Inf.=infection; pos=positive; neg=negative; ACD-A=Anticoagulant Citrate Dextrose A, CPD=citrate phosphate dextrose; F=female; M=male.

Legend:

1) Mean value pre-screening at PEI with: Abbott Architect CMV IgG/IgM, Siemens Enzygnost CMV IgG/IgM, Abbott Axsym CMV IgG/IgM.

2) Abbott Architect CMV IgG Avidity: <50.0 % =low avidity; 50.0–59.9%=grey zone; ≥60.0 %= high avidity.

3) Evaluation of band intensity and test interpretation according to the instructions for use for Mikrogen CMV IgG [Avidity]/ IgM recomBlot.

4) Possible infection stage according to, the antibody titers for anti-CMV IgG and IgM, avidity, immunoblot pattern, and neutralization assay.

5) Siemens Enzygnost EBV IgG, Panbio HHV-6 ELISA.

6) Altona Real Star CMV PCR Kit 1.0.

7) CMV DNA result, 153 IU/ml.

8) 40 out of 53 EBV IgG positive; 41 out of 53 HHV-6 IgG positive; all 53 C samples positive for either EBV IgG and/or HHV-6.

Table 2: Test kits used in the Collaborative Study.

	Kit #	Product name	Cat. no.	Manufacturer	Test principle	Unit / interpretation	Interpretation of results	Antigen coated on the solid phase
Anti-CMV IgG only EIA test kits	**1**	Bio-Flash CMV IgG	3000-8563	Biokit	ChLMIA, indirect anti-IgG detection	AU/ml, qual/ quantitative	Non-reactive <8.0, ind ≥8.0-<10.0, reactive ≥10.0	CMV antigen
	2	Bioelisa CMV IgG	3000-1216	Biokit	ELISA, indirect anti-IgG detection	s/co or IE/ml, qual/ quantitative	Non-reactive <0.9; ind. ≥0.9-<1.0, reactive ≥1.0	Inactivated CMV antigen
	3	Architect CMV IgG	6C15/B6C150	Abbott	ChLMIA, indirect anti-IgG detection	AU/ml, qual./ semiquantitative	Non-reactive ≥6.0, reactive ≥6.0	Viral lysate (AD169)
	5	Enzygnost Anti-CMV/IgG	OWBA1510446580	Siemens	ELISA, indirect anti-IgG detection	ΔA, qual/ quant	Non-reactive <0.100, eq. 0.100-≤0.200, reactive >0.200	Inactivated CMV antigen CMV of infected human fibroblast cells
	6	Liaison CMV IgG II	310745	Diasorin	ChLMIA, indirect anti-IgG detection	U/ml, quantitative	Non-reactive <12.0, ind. 12.0-<14.0, reactive ≥14.0	CMV Antigen (AD169)
	7	CMV-IgG-ELISA PKS medac	115-Q-PKS	medac	ELISA, indirect anti-IgG detection	AU/ml, quantitative	Non-reactive <0.45, greyzone 0.45-0.65, reactive ≥0.65	Viral lysate of infected human fibroblasts (AD169)
	8	Serion ELISA classic/ Cytomegalovirus IgG	ESR109G	Virion\Serion	ELISA, indirect anti-IgG detection	PEI-U/ml, qualitative/ quantitative	Non-reactive <25 U/ml, BR 25-40 U/ml, reactive >40 PEI-U/ml	CMV antigen
	10	Cytomegalovirus IgG ELISA II	425200CE	Wampole	ELISA, indirect anti-IgG detection	Index (OD ratio), qualitative	Non-reactive <0.90, eq 0.91-<1.09, ≥1.10 reactive	Inactivated CMV antigen (AD169)
	11	DS-EIA-Anti-CMV-G	CM151	RPC Diagnostic Systems	ELISA, indirect anti-IgG detection	OD ratio or U/ml qual/ quantitative	Non-reactive <cutoff, reactive ≥cutoff ($\bar{x}$OD value of Calibrator)	Mix of recombinant proteins as analogs of CMV antigens
	23	Immulite CMV IgG	LKCV1	Siemens	EIA, indirect anti-IgG detection	s/co ratio, qualitative	Non-reactive <0.9, ind 0.9-<1.1, reactive ≥1.1	Inactivated, partially purified CMV antigen (AD169)
	25	VIDAS CMV IgG	30204	Biomerieux	ELFA, indirect anti-IgG detection	AU/ml quantitative	Non-reactive <4,ind. ≥4-<6, reactive ≥6	CMV antigen (AD169)
Anti-CMV EIA total EIA test kits	**4**	CMV IgG	4784596	Roche	ECLIA, one step sandwich	U/ml, quantitative	Non-reactive <0.5, ind. 0.5-<1.0, reactive ≥1.0	Recombinant CMV antigens (pp150, pp28, p52, p38)
	9	CMV TA EIA	60109	Trinity Biotech	ELISA, two-step sandwich	s/co, qualitative	Non-reactive <0.9, eq 0.9-<1.1, reactive ≥1.1	Antigens derived from virus cultured in human fibroblast cells
	12	Bioelisa CMV Colour 2.0	3000-1249	Biokit	ELISA, indirect anti-IgG and anti-IgM detection	s/co, qualitative	Non-reactive <0.9, ind. ≥0.9-<1.0, reactive ≥1.0	Inactivated CMV antigen
Visually readable	**29**	Cytomegalovirus IgG	CMG-120	MBL Bion	Indirect anti-IgG fluorescent detection	Signal intensity, qual/ semiquantitative	Fluorescent intensity of ≥1+ at ≥1:10 dilution	Viral lysate (AD169)
	13	Lab21 CMV HA	60136	Lab21 Healthcare	Passive hemagglutination (IgG, IgM, IgA)	Agglutination, qualitative	Threshold parameters defined in the PK7200 / PK7300 manual	Avian erythrocytes coated with CMV antigen

Abbreviations:

EIA=enzyme immunoassay; ChlMIA=chemiluminescent microparticle immunoassay; ELISA=enzyme linked immunosorbent assay; ELFA=Enzyme linked fluorescent assay; CLEIA=chemiluminescent enzyme immunoassay; ECLIA=electrochemiluminescence immunoassay; qual=qualitative; s/co= sample to cutoff ratio; ind=indeterminate; eq=equivocal; AU=arbitrary units; ΔA=Delta absorption; OD=optical density; BR = borderline range; PEI=Paul-Ehrlich-Institut.

Legend:

Characteristics according to the assay's instruction for use.

Table 3: Mean endpoint titers of candidate material A1 and additional study samples.

				A1	**A2**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
		IgG avidity[2)]		81.4	79.3	17.0	24.5	32.7	48.8	16.6	68.3	53.6	75.4
Assay		IgM Titer[3)]		n.a.	n.a.	36.2	15.9	12.5	8.1	78.2	13.9	194.1	n.a.
category		**Kit[1)]**	**Lab**	**Anti-CMV IgG GMV endpoint titers by linear interpolation**									
Anti-CMV IgG only	EIA	6	9	26.1	6.5	58.5	40.5	35.9	16.6	1.6	30.2	3.7	30.3
		10	8	27.3	4.0	6.8	4.0	3.5	3.8	2.7	9.8	5.4	39.1
		1	11	27.8	5.0	6.4	5.9	6.4	5.6	2.3	12.0	3.1	103.8
		8	10	30.3	7.3	49.5	35.1	26.9	20.1	6.6	31.0	4.9	36.4
		23	15	30.6	6.5	60.1	34.5	27.6	17.6	4.0	44.4	3.7	61.5
		2	3	37.8	6.0	10.3	9.7	10.2	9.0	6.8	22.3	12.8	61.2
		25	6	42.1	8.3	64.0	64.0	50.8	22.6	5.2	26.5	5.3	57.7
		2	11	44.7	6.9	13.0	7.8	10.6	10.5	6.0	29.0	15.3	106.9
		3	15	51.5	12.8	82.7	58.0	46.0	52.9	8.8	52.2	5.2	53.3
		7	12	51.6	8.2	56.7	24.9	24.3	17.1	7.9	54.6	29.4	51.8
		3	5	55.0	14.0	78.1	50.2	46.2	46.5	9.1	51.0	5.8	52.9
		11	14	56.0	6.8	20.2	10.4	10.3	8.3	13.6	9.2	335.1[4)]	25.2
		3	4	59.2	14.7	77.8	59.0	58.8	55.1	9.9	66.7	4.4	51.5
		7	1	79.9	9.8	123.0	83.2	61.4	27.5	29.2	119.8	54.4	119.4
		5	15	85.1	11.8	72.3	93.0	62.5	24.7	22.4	28.1	7.5	59.0
		5	7	102.3	14.1	64.0	43.8	30.3	13.5	8.9	20.0	12.2	58.0
	IFA	29	6	16.0	8.0	64.0	128.0	>64[5)]	32.0	16.0	>64 [5)]	>32[5)]	32.0
Total anti-CMV	EIA	9	13	232.7	26.0	9.7	9.9	15.5	7.1	15.6	114.4	61.5	1101.6
		12	11	43.8	6.8	7.1	6.3	8.3	6.7	4.5	14.6	7.8	92.5
	PHA	13	13	29.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	128.0
Not included.		4	4	1274.0	249.6	5.1	5.1	7.6	13.8	8.0	2.9	7.4	1230.5
		4	15	1013.0	206.3	6.0	6.1	8.0	19.3	6.1	3.4	7.4	1439.0
		5	2	173.6	23.7	138.8	73.1	>64[5)]	38.1	>32[5)]	51.1	28.9	163.3
Mean all anti-CMV tests [6)]				**46.4**	**7.8**	**38.3**	**27.1**	**24.0**	**17.0**	**6.9**	**30.3**	**7.7**	**67.0**
Mean anti-CMV IgG only [7)]				46.2	8.3	38.3	27.1	24.0	17.0	6.9	30.3	7.8	53.7

Abbreviations:

GMV=geometric mean value; EIA=enzyme immunoassay; IFA=indirect immunofluorescence; PHA=passive hemagglutination; n.a.=not applicable.

Legend:

1) Grouped for the EIAs according to increasing A1 titers.
2) Architect CMV IgG Avidity
3) Overall anti-CMV IgM titer of test kits used in the collaborative study.
4) Outlier value (see section 3.9)
5) No endpoint received.
6) GMV without kit #4 and kit #5/lab 2; for sample B7 without kit #11; for samples B1-B7 without anti-CMV total assays (kits # 9, 12, 13) and kit #29.
7) Anti-CMV IgG only EIAs.

Table 4: Potency ratios relative to A1 of the test kit endpoint titers in each study sample.

Kit No. [1]	Lab No.	Potency ratio A1 vs study sample [2]								
		A2	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
1	**11**	5.5	4.3	4.7	4.3	5.0	11.9	2.3	9.0	0.3
2	**3**	6.3	3.7	3.9	3.7	4.2	5.6	1.7	3.0	0.6
2	**11**	6.5	3.4	5.7	4.2	4.3	7.5	1.5	2.9	0.4
3	**4**	4.0	0.8	1.0	1.0	1.1	6.0	0.9	13.5	1.1
3	**5**	3.9	0.7	1.1	1.2	1.2	6.1	1.1	9.4	1.0
3	**15**	4.0	0.6	0.9	1.1	1.0	5.8	1.0	9.9	1.0
5	**7**	7.2	1.6	2.3	3.4	7.6	11.5	5.1	8.4	1.8
5	**15**	7.2	1.2	0.9	1.4	3.4	3.8	3.0	11.4	1.4
6	**9**	4.0	0.4	0.6	0.7	1.6	16.9	0.9	7.0	0.9
7	**1**	8.2	0.6	1.0	1.3	2.9	2.7	0.7	1.5	0.7
7	**12**	6.3	0.9	2.1	2.1	3.0	6.5	0.9	1.8	1.0
8	**10**	4.1	0.6	0.9	1.1	1.5	4.6	1.0	6.2	0.8
10	**8**	6.8	4.0	6.9	7.8	7.2	10.0	2.8	5.1	0.7
11	**14**	8.2	2.8	5.4	5.5	6.8	4.1	6.1	0.2	2.2
23	**15**	4.7	0.5	0.9	1.1	1.7	7.7	0.7	8.3	0.5
25	**6**	5.1	0.7	0.7	0.8	1.9	8.1	1.6	8.0	0.7
Median		**5.9**	**0.85**	**1.05**	**1.35**	**2.95**	**6.3**	**1.3**	**7.5**	**0.85**
Mean (GMV)		5.6	1.2	1.7	1.9	2.7	6.7	1.5	5.9	0.8
GCV%		27.5	98.3	103.8	87.5	78.9	49.5	77.6	155.6	57.2
GCV% [3]		28.2	37.9	42.7	44.8	67.1	53.3	70.5	83.3	36.4

Abbreviation:

GMV=geometric mean value; GCV% = geometric coefficient of variation.

Legend:

[1] Anti-CMV IgG only test kits.

[2] The titer of each test kit for A1 divided by the titer in the respective study sample.

[3] GCV without kits # 1, 2, 10, and 11.

Table 5-1: Spearman's rank correlation coefficients A1 vs study samples.

Study sample	r (Spearman)	N	p value
A2	0.800	18	0.0002
B1	0.609	16	0.0123
B2	0.594	16	0.0152
B3	0.571	16	0.0210
B4	0.459	16	0.0738
B5	0.912	16	<0.0001
B6	0.253	16	0.3446
B7	0.564	15[1)]	0.0284
B8	0.185	16	0.4921

Table 5-2: Spearman's rank correlation coefficients revised for samples B4, B6, B8.

Study sample	r (Spearman)	N	p value
B4	0.727	14[2)]	0.0032
B6	0.596	14[2)]	0.0246
B8	0.424	14[3)]	0.1306

Legend:

Spearman's correlation coefficient reported as r_s, n values, significance $p<0.05$.

1) Without kit 11.

2) Without kit 11 and data set 5/7 (kit/lab).

3) Without kits 1 and 11.

Table 6: Repeatability (intra-lab) and reproducibility (inter-lab) with samples A1 and A2.

Kit code	**Lab code**		**GCV (%)**	
			A1	**A2**
1	11	intra-lab	4.3	3.1
2	3	intra-lab	16.1	8.2
2	11	intra-lab	7.1	4.0
2		***inter-lab***	***10.8***	***8.5***
3	4	intra-lab	10.0	10.4
3	5	intra-lab	1.5	1.1
3	15	intra-lab	7.0	4.6
3		***inter-lab***	***6.0***	***6.5***
4	4	intra-lab	7.1	0.4
4	15	intra-lab	1.1	11.1
4		***inter-lab***	***15.2***	***13.3***
5	2	intra-lab	32.0	30.3
5	7	intra-lab	9.0	20.3
5	15	intra-lab	8.4	10.3
5		***inter-lab***	***42.5 (13.8[1)])***	***41.1 (8.7[1)])***
6	9	intra-lab	1.7	0.4
7	1	intra-lab	8.8	5.5
7	12	intra-lab	0.7	1.7
7		***inter-lab***	***32.3***	***11.5***
8	10	intra-lab	0.9	8.8
9	13	intra-lab	5.7	4.1
10	8	intra-lab	10.0	17.0
11	14	intra-lab	4.2	2.5
12	11	intra-lab	9.8	6.2
13	13	intra-lab	13.4	<0.1
23	15	intra-lab	3.2	1.6
25	6	intra-lab	4.1	5.5
29	6	intra-lab	n.e.	n.e.
		Overall intra-lab GCV%	9.0	9.0
Overall inter-assay/inter-lab GCV%			**72.7**	**80.8**
Overall uncertainty of measurement%			**73.6**	**81.6**

Abbreviations:

GCV = geometric coefficient of variation; n.e. = not estimable.

Legend:

Bold/*italic* represent intra-lab-variability, inter- assay/inter-lab-variability, and overall measurement of uncertainty (combined evaluation for all labs and assays, without kit 4 and kit 5 / lab 2).

[1)] Without kit 5 / lab 2.

Table 7: CMV Neutralization titers of candidate material A1 and study samples A2, B1-B8.

		NT90 (90% inhibition of RT-qPCR signal relative to virus control)			
		Rep 1	Rep 2	Rep 3	GMT
A1	Vial 1	421	384	339	380
	Vial 2	314	422	333	353
	Vial 3	341	287	311	312
					347
A2	Vial 1	67	67	72	69
	Vial 2	78	66	58	67
	Vial 3	62	64	70	65
					67

	NT90 (90% inhibition of RT-qPCR signal relative to virus control)			
	Rep 1	Rep 2	Rep 3	GMT
B1	108	114	124	115
B2	80	56	53	62
B3	119	122	144	128
B4	242	318	293	283
B5	174	152	154	160
B6	< 8	8	8	8*
B7	11	13	15	13
B8	176	140	154	156

Abbreviations:

NT90 = 90% neutralization titer; GMT = geometric mean titer; Rep. = replicate

Legend:

Neutralization titers were expressed as the highest sample dilution causing 90% reduction in RT-qPCR signal quantifying CMV IE-1 mRNA compared with infected control wells in the absence of antibodies.

* Calculated from Replicate 2 and Replicate 3.

Table 8: Results of the test kits used with Panel C1-C53.

Kit #	Lab #	Specificity %	N false pos/ total N samples	Panel number of false positive results
1	11	100.0	0/53	–
2	3	100.0	0/53	–
2	11	100.0	0/53	–
3	4	98.1	1/53	C11
3	5	98.1	1/53	C11
3	15	98.1	1/53	C11
4	4	98.1	1/53	C19
4	15	100.0	0/53	–
5	15	100.0	0/53	–
5	2	100.0	0/51[1)]	–
5	7	100.0	0/53	–
6	9	98.1	1/53	C11
7	1	100.0	0/53	–
7	12	100.0	0/53	–
8	10	100.0	0/53	–
10	8	100.0	0/53	–
11	14	96.2	2/53	C29, 36, 33[3)]
23	15	100.0	0/53	–
25		100.0	0/51[1)]	–
29	6	74.6[2)]	13/51[1)]	C1, 4, 6, 8, 9, 15, 19, 27, 36, 37, 38, 44, 48
12	11	100.0	0/53	–
13	13	98.1	1/53	C36
9	13	100.0	0/53	–

Legend:

1) Only 51 samples (without C52, 53) were sent to the participants.

2) No repeat testing done, data represent the initial reactive rate.

3) Initially positive, negative after repeat.

Table 9: Stability of candidate material A1.

Real time stability

		Endpoint titers		
Month	**Replicate**	**-20°C**	**4°C**	**RT**
0	1	47.3	n.a.	n.a.
	2	44.8	n.a.	n.a.
	GMV (GCV%)	46.0 (3.8)	n.a.	n.a.
1	1	44.7	48.0	42.1
	2	42.7	49.3	39.3
	GMV (GCV%)	43.7 (3.2)	48.7 (1.9)	40.8 (4.8)
	Recovery %	**94.8**	**111.4**	**93.3**
3	1	45.5	50.6	42.8
	2	47.6	50.7	40.9
	GMV (GCV%)	46.6 (3.2)	50.7 (0.2)	41.8 (2.9)
	Recovery %	**101.1**	**108.8**	**89.7**
6	1	44,97	51.1	31.5
	2	46,30	51.5	32.9
	GMV (GCV%)	45,67 (2.1)	51.3 (0.6)	31.82 (3.1)
	Recovery %	**99.1**	**112.3**	**69.7**
12	1	44.6	n.t.	32.0
	2	46.8	n.t.	37.3
	GMV (GCV%)	45.8 (3.4)	n.t.	34.8 (10.9)
	Recovery %	**99.3**	n.t.	**76.0**

Accelerated stability

			Endpoint titers		
Day	**Replicate**	**Baseline -20°C**	**4°C**	**RT (20-24°C)**	**37°C**
0	1	47.3	n.a.	n.a.	n.a.
	2	44.8	n.a.	n.a.	n.a.
7	1	n.a.	41.9	41.5	43.9
	2	n.a.	43.5	42.1	48.0
14	1	n.a.	42.7	41.8	45.9
	2	n.a.	45.0	47.6	44.0
21	1	n.a.	43.9	44.3	45.6
	2	n.a.	44.4	45.9	44.8
GMV		46.0	43.8	44.1	45.3
GCV%		3.8	3.5	3.1	1.2
Recovery %		n.a.	95.2	95.8	98.3

Abbreviations:

RT=room temperature (20-24°C); GMV=geometric mean value; GCV=geometric coefficient of variation; n.a.=not applicable.

Table 10: Reactivity of 4 anti-CMV IgG test kits in 5 CMV seroconversion panels.

			Assays [2]								Additional tests		
			5		**3**		**2**		**6**				
			A	B	A	B	A	B	A	B	**Blot**[5]	**IgM**[6]	**Avi**[7]
	Bleed	Day	0.10[3]	0.58[4]	6.0[3]	0.99[4]	1.0[3]	1.10[4]	14.0[3]	1.61[4]	6	1.0	60.0
Panel #	day[1]	interval	ΔA	A1-U	AU/ml	A1-U	s/co	A1-U	U/ml	A1-U	Points	Index	%
SCP-CMV-001-01	0	0	0.06	0.29	9.3	1.40	0.28	0.16	9.44	1.25	0	0.93	9.9
-02	4	4	0.13	0.80	23.3	3.26	0.58	0.48	22.10	2.98	0	2.79	7.5
-03	8	4	0.30	2.20	54.6	8.02	1.88	2.78	49.20	7.89	7	3.52	-4.0
-04	51	43	0.79	10.34	89.6	13.15	4.26	11.41	66.90	10.64	14	1.03	20.4
-05	55	4	0.83	10.86	94.6	13.89	4.32	11.55	69.70	11.07	14	0.97	22.5
-06	59	4	0.95	12.37	92.9	13.64	4.54	12.07	68.30	10.86	14	0.88	25.5
-07	65	6	1.00	12.97	89.8	13.18	4.45	11.85	62.60	9.97	14	0.90	30.3
-08	67	2	0.76	10.09	91.0	13.36	4.28	11.45	61.50	9.80	14	0.86	30.3
-09	72	5	0.75	9.96	82.5	12.11	3.95	10.67	58.20	9.29	14	0.85	33.8
-10	74	2	0.72	9.54	82.3	12.08	3.91	10.58	57.50	9.18	14	0.84	33.2
-11	79	5	0.70	9.30	84.1	12.35	3.90	10.56	57.10	9.12	14	0.79	34.9
-12	84	5	0.72	9.58	89.8	13.18	4.12	11.08	61.10	9.74	14	0.81	35.5
-13	88	4	0.76	10.03	95.9	14.08	3.84	10.41	64.50	10.27	14	0.81	34.2
-14	95	7	0.82	10.74	91.3	13.40	3.60	9.85	53.10	8.50	14	0.75	44.1
-15	99	4	0.72	9.61	88.7	13.02	3.89	10.53	55.60	8.89	14	0.74	43.8
SCP-CMV-002-01	0	0	0.03	0.04	0.80	0.12	0.21	0.11	5.0	1.04	0	0.18	n.a.
-02	5	5	0.04	0.11	0.60	0.09	0.17	0.08	5.0	1.04	0	0.16	n.a.
-03	8	3	0.03	0.05	0.70	0.10	0.19	0.06	5.0	1.04	0	0.17	n.a.
-04	12	4	0.03	0.00	0.80	0.12	0.12	0.05	5.0	1.04	0	0.19	n.a.
-05	15	3	0.03	0.07	0.60	0.09	0.20	0.09	5.0	1.04	0	0.19	n.a.
-06	21	6	0.04	0.15	0.90	0.13	0.30	0.17	5.0	1.04	0	0.19	n.a.
-07	26	5	0.05	0.18	1.50	0.22	0.48	0.36	5.0	1.04	0	0.30	n.a.
-08	29	3	0.05	0.18	3.00	0.50	0.50	0.38	5.6	1.14	0	0.68	n.a.
-09	33	4	0.11	0.65	12.70	1.84	0.69	0.62	7.7	1.46	5	3.33	2.2
-10	36	3	0.19	1.22	20.50	2.82	1.22	1.46	10.0	1.33	6	4.70	8.8
-11	43	7	0.52	5.56	99.80	14.65	2.83	5.74	49.3	7.91	12	5.71	10.4
-12	50	7	0.54	7.42	111.20	16.32	2.94	8.03	60.7	9.68	14	4.97	7.8
-13	57	7	0.76	9.99	114.60	16.82	3.71	10.10	73.8	11.71	14	4.10	12.8
-14	68	11	0.79	10.38	128.00	18.79	4.37	11.66	84.3	13.34	14	3.25	18.1
-15	75	7	0.94	12.22	122.20	17.94	4.72	12.49	89.8	14.19	14	2.70	20.4
-16	82	7	1.02	13.21	113.00	16.59	4.79	12.66	88.8	14.03	14	2.46	21.2
-17	86	4	0.99	12.85	115.50	16.95	4.48	11.92	87.9	13.89	14	2.31	21.8
-18	89	3	0.87	11.39	102.50	15.05	4.67	12.37	83.7	13.24	14	2.25	22.4
-19	96	7	0.91	11.82	114.10	16.75	4.79	12.66	84.9	13.43	14	2.13	23.6
-20	104	8	0.94	12.25	109.00	16.00	4.51	12.00	79.0	12.52	14	2.03	21.5
-21	109	5	0.90	11.74	98.20	14.41	4.56	12.11	75.8	12.02	14	1.85	27.6
-22	113	4	0.95	12.28	113.90	16.72	4.32	11.54	73.5	11.66	14	1.85	27.6
-23	116	3	0.91	11.83	113.40	16.65	4.60	12.20	82.8	13.10	14	1.86	27.1
-24	121	5	0.90	11.69	104.10	15.28	4.14	11.13	66.7	10.61	14	1.79	32.2
-25	124	3	1.06	13.69	104.30	15.31	4.41	11.76	78.8	12.48	14	1.39	32.3
SCP-CMV-003-01	0	0	0.03	0,07	0 .8	0,12	0.15	0.06	<5.0	0.60	0	0.08	n.a.
-02	8	8	0.03	0,05	0 .7	0,10	0.14	0.05	<5.0	0.60	0	0.07	n.a.
-03	18	10	0.07	0,36	7.4	1.14	0.28	0.15	<5.0	0.60	1	0.27	80.2
-04	25	7	0.25	1,73	73.8	10.83	1.57	2.16	37.8	6.13	1	3.18	78.6
-05	33	8	0.35	5.11	147.9	21.71	3.02	8.49	76.7	12.16	12	6.14	49.1
SCP-CMV-005-01	0	0	0.02	0.01	0.8	0.10	0.04	0.03	<5.0	0.60	0	0.44	n.a.

	-02	6	6	0.55	7.47	27.8	3.99	0.23	0.12	49.9	8.00	1	4.69	-11.6
	-03	10	4	0.78	10.28	38.8	5.70	1.01	1.12	63.7	10.14	6	5.27	-14.1
	-04	24	14	0.95	12.26	50.5	7.41	1.84	5.69	76.1	12.07	6	3.17	-2.0
	-05	29	5	0.94	12.16	54.1	7.94	1.88	5.79	76.7	12.16	6	2.48	4.3
	-06	35	6	0.89	11.65	55.5	8.15	1.65	5.24	80.8	12.79	6	2.28	22.2
SCP-CMV-006-01		0	0	0.03	0.04	0.2	0.03	0.20	0.09	<5.0	0.60	0	0.26	n.a.
	-02	6	6	0.10	0.57	3.9	0.65	1.13	1.31	9.6	1.27	0	0.93	n.a.
	-03	9	3	0.18	1.07	8.3	1.27	1.60	2.22	17.8	2.38	0	1.37	18.8
	-04	13	4	0.30	2.23	13.0	1.91	2.30	3.92	28.0	3.98	1	1.64	40.9
	-05	17	4	0.40	3.39	18.8	2.76	2.12	3.41	38.1	6.17	1	1.63	38.1
	-06	23	6	0.56	6.26	26.9	3.95	2.46	4.38	48.6	7.80	6	1.86	40.4
	-07	43	20	1.27	16.11	74.9	11.00	4.12	11.08	84.0	13.29	7	1.50	61.6
	-08	45	2	0.98	12.69	77.1	11.32	4.32	11.55	84.6	13.38	7	1.47	62.1
	-09	48	3	1.13	14.46	79.1	11.61	3.84	10.41	91.6	14.47	7	1.52	60.9
	-10	50	2	1.20	15.29	87.5	12.84	4.45	11.85	92.6	14.62	7	1.44	61.0
	-11	52	2	1.67	20.95	88.8	13.04	4.20	11.26	94.6	14.93	7	1.57	66.0
	-12	64	12	1.49	18.81	140.8	20.67	5.07	13.32	118.0	18.56	7	1.25	55.5
	-13	66	2	1.46	18.45	117.8	17.29	4.85	12.80	106.0	16.70	7	1.52	64.6
	-14	69	3	1.58	19.94	126.4	18.55	4.84	12.78	115.0	18.10	7	1.52	60.5
	-15	72	3	1.74	21.77	134.6	19.76	5.04	13.25	119.0	18.72	7	1.42	59.8
	-16	76	4	1.49	18.86	126.9	18.63	4.91	12.94	116.0	18.25	7	1.72	61.0
	-17	78	2	1.44	18.17	129.4	18.99	4.94	13.01	116.0	18.25	7	1.70	63.1
	-18	80	2	1.39	17.55	125.5	18.42	4.99	13.13	112.0	17.63	7	1.80	65.0
	-19	84	4	1.59	20.03	130.5	19.16	4.86	12.82	117.0	18.41	7	1.80	63.7
	-20	86	2	1.41	17.88	122.2	17.94	5.07	13.32	114.0	17.94	7	1.82	64.9
	-21	92	1	1.59	19.99	142.8	20.96	4.94	13.01	121.0	19.03	14	1.71	62.7
	-22	94	2	1.50	18.88	135.6	19.90	5.00	13.15	117.0	18.41	14	1.83	66.2
	-23	127	33	1.57	19.80	137.1	20.12	5.14	13.48	119.0	18.72	15	1.09	61.5
	-24	130	3	1.68	21.04	137.7	20.21	5.16	13.53	118.0	18.56	15	1.14	61.3

Abbreviations: ΔA=absorbance; A1-U=A1 Units; AU=arbitrary units.

Legend:

Grey shading=positive; plain text=negative.

Lane A: Test-specific value or unit respectively.

Lane B: Converted value on A1 units of the respective test by linear interpolation against the calibration curve with A1.

1) From the data sheets of the panel supplier (Biomex GmbH, Heidelberg, Germany).

2) Test kit numbering according to Table 2 and in decreasing order (left to the right) of their analytical sensitivity in the Collaborative Study.

3) Cutoff value of the test kit as specified in the respective instructions for use.

4) Cutoff corresponding to A1 units at the end point titer of the calibration curve with A1.

5) RecomBlot CMV (Mikrogen GmbH; Neuried, Germany). The sum of the point values of positive rated reactivities are shown (≥6 positive).

6) Architect CMV IgM (Abbott Diagnostic Division, Sligo, Ireland).

7) Architect CMV IgG Avidity (Abbott Diagnostic Division, Sligo, Ireland).

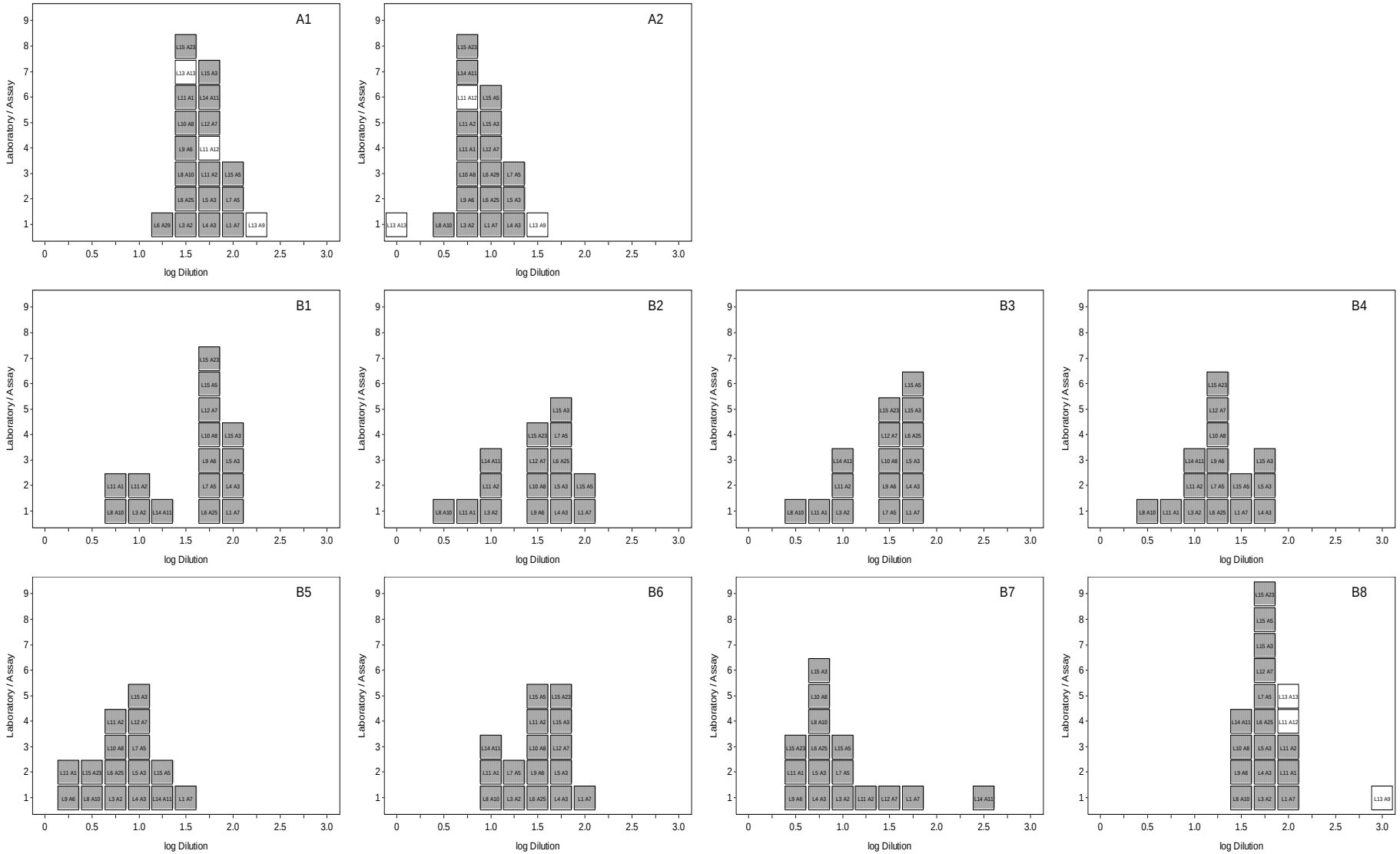
Table 11: Comparison of the analytical sensitivity in the Collaborative Study with the sensitivity in CMV seroconversion panels.

Test kit no. [1)]	Mean endpoint titer A1 [2)]	Analytical sensitivity A1 [3)]	N positives in all panels [4)]
5	93.7	0.53	61
3	55.1	0.89	62
2	41.1	1.17	58
6	26.1	1.61	58

Legend:

1) In decreasing order of the analytical sensitivity in the collaborative study.
2) Mean endpoint titer of test kits # 2, 3 and 5 represent the average of the tests in the different laboratories of the collaborative study.
3) Conversion of A1 titer into "A1-units" by linear interpolation.
4) Aggregated positive number in all serial samples of the 5 CMV seroconversion panels.

Figure 1: Distribution of the mean endpoint titers of A1 and additional study samples A2, B1-B8.



Legend:

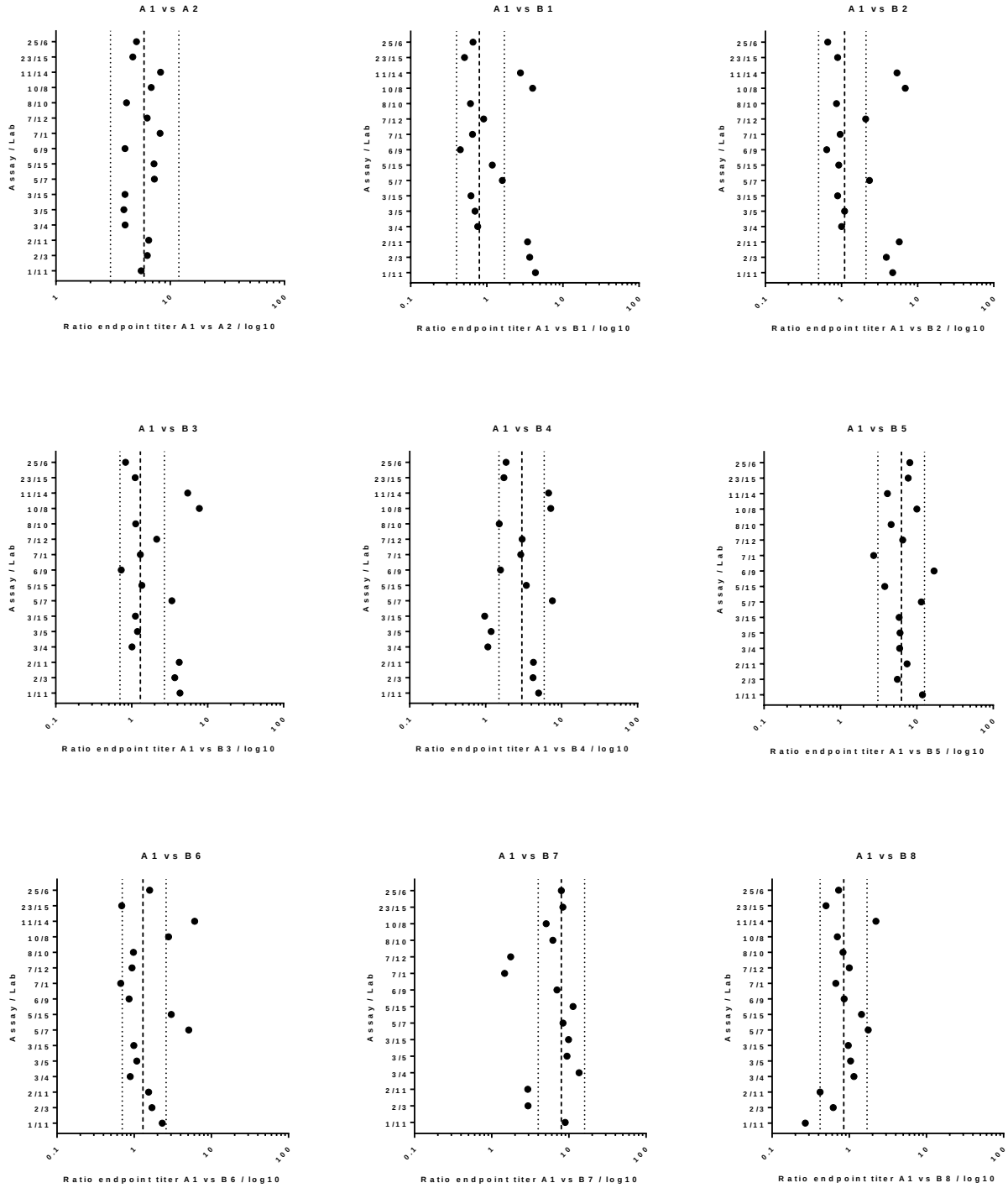
Each box represents the geometric mean endpoint titer of one test kit labeled with the assay and laboratory code number, values from Table 3.

Grey shaded boxes represent anti-CMV IgG test kits; white boxes represent anti-CMV total test kits.

The x-axis represents the geometric mean endpoint titer of an individual test kit (displayed in $\log_{10}$ scale), the y-axis the number of test kits.

The pure anti-CMV IgG samples A1, A2 and B8 are shown above together. Samples B1-B7 are without anti-CMV total test kits and without kit #29 (IFA).

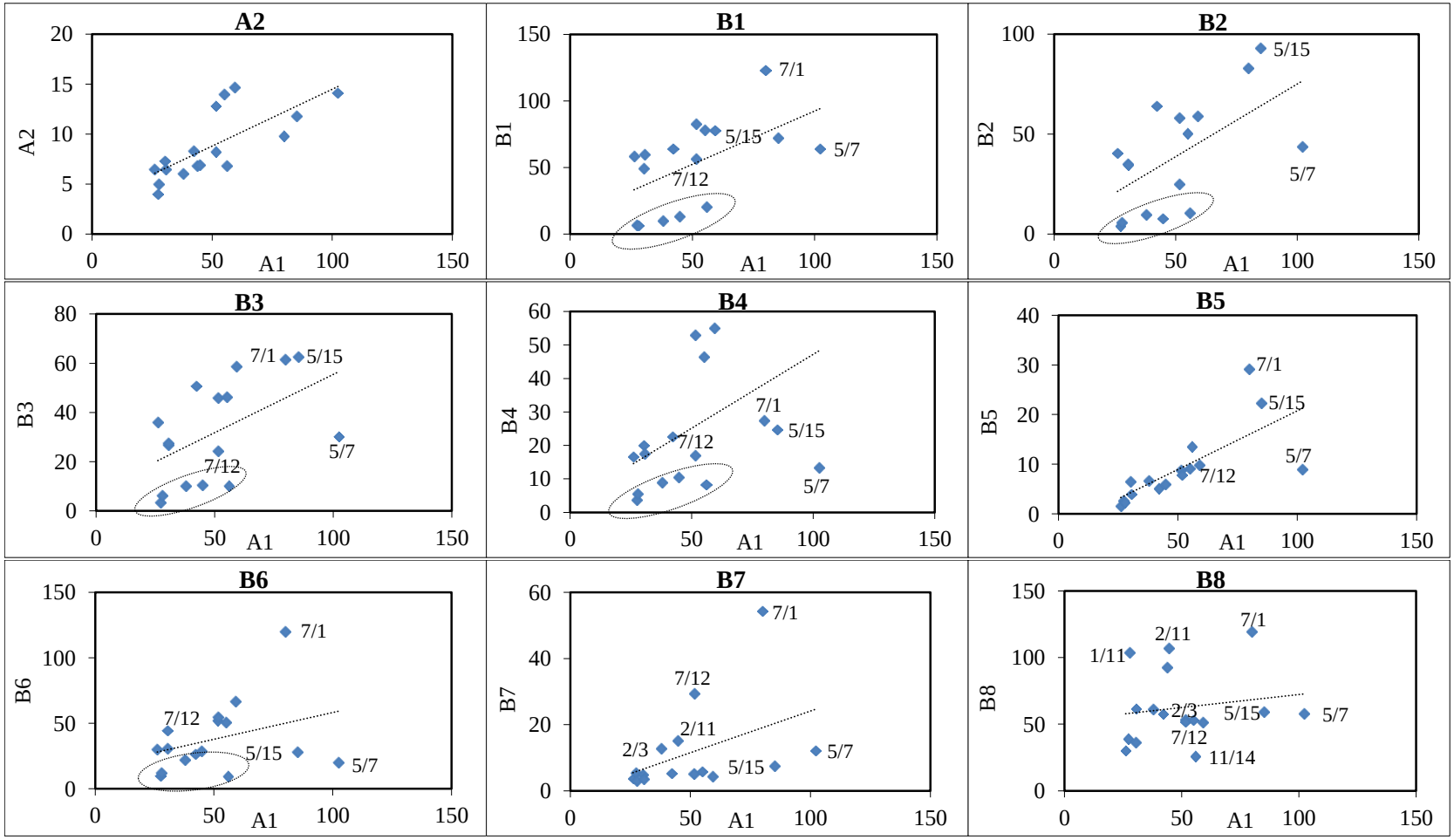
Figure 2: Distribution of the potency ratios of A1 relative to study samples A2, B1-B8.



Legend:

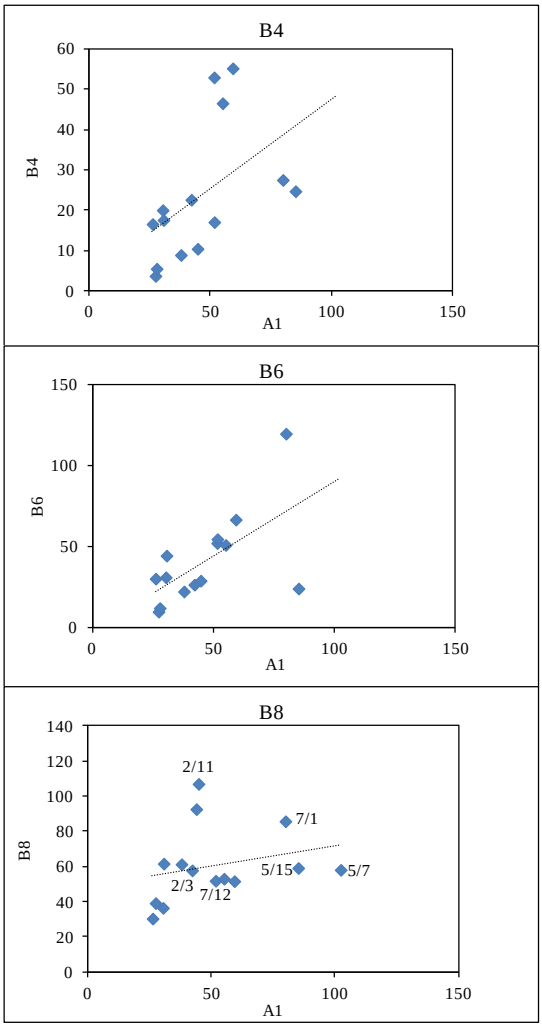
1. X-axis logarithmic plot of the ratios of the titers relative to A1 (values from Table 4).
2. Y-axis values per test kit and laboratory.
3. Solid line median of all test kits, dotted line 2-fold area.
4. Samples B1-B7 shown without anti-CMV total test kits; B7 additionally without test kit #11.

Figure 3-1: Scatter plots, A1 plotted against the study samples A2, B1-B8.



Legend:
X-axis: titer values (from Table 3) for A1 grouped according to decreasing titer.
Y-axis: titers values (from Table 3) of the additional study samples.
The dotted line represents the regression line. The correlation coefficients according to Spearman are given in Table 5-1.
Data points marked by assay/lab value indicate inter-laboratory variability (same test different labs).
Data points enclosed in circles represent test kits # 1, 2, 10, 11 (results and discussion presented in section 3.5).

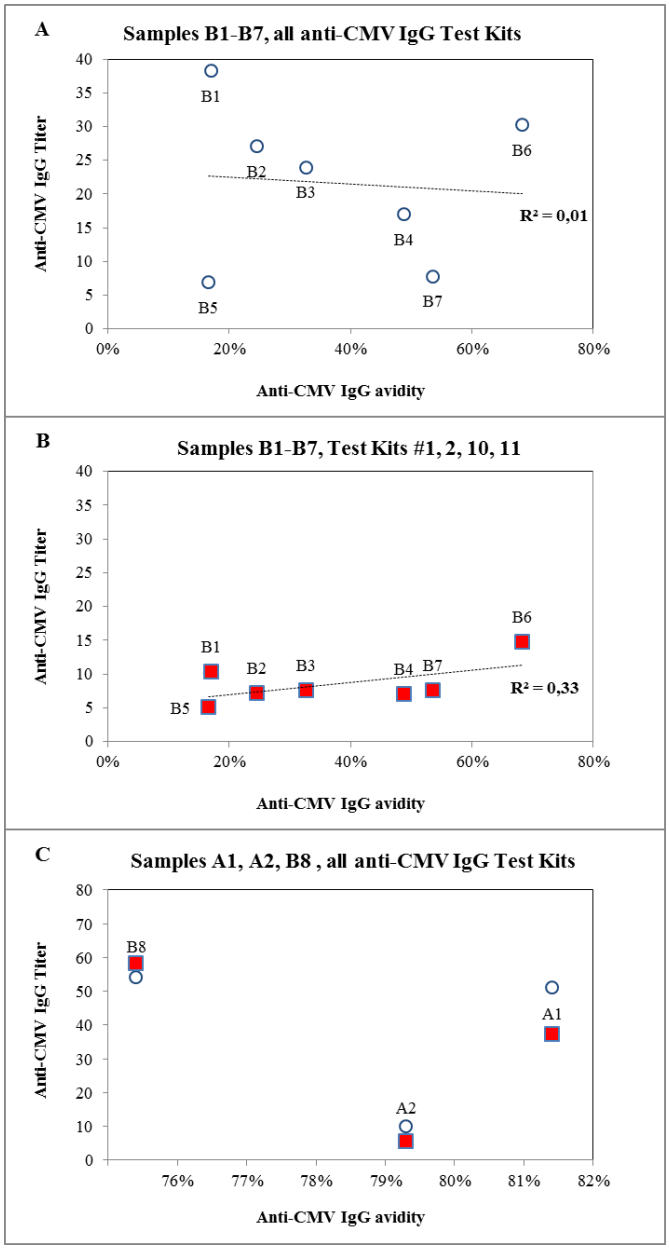
Figure 3-2: Scatter plots of study samples B4, B6 and B8 after data adjustment.



Legend:

1. Same illustration as Figure 3-1 above after revision of outliers in study samples.
2. Results and explanations in Table 5-2.
3. Data marking in graph B8: Test kits that show inter-laboratory variability.

Figure 4: Variation of anti-CMV IgG titers of the test kits by low avidity of the study samples.



Legend:

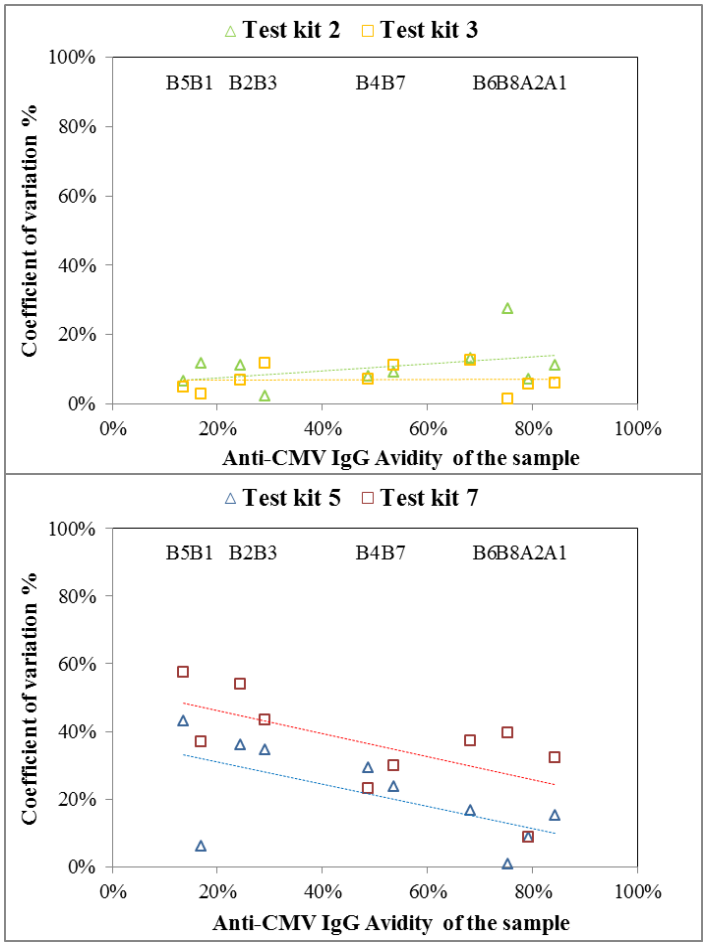
Anti-CMV IgG titers of test kits (y-axis) plotted against IgG avidity of study samples (x-axis).

Graph A Average titer of all test kits with study samples B1-B7 (empty circles): no correlation of titers with avidity (dotted regression line, r^2 0.01).

Graph B Titers of test kits # 1, 2, 10, 11 with study samples B1-B7 (filled squares): Correlation of titers with low avidity (dotted regression lines, r2 0.33).

Graph C Titers of all test kits with the pure anti-CMV IgG study samples A1, A2, B8 of high avidity: no difference of titers between test kits # 1, 2, 10, 11 (filled squares) and the other tests (empty circles).

Figure 5: Variation of results by inter-laboratory variability of test kits # 5 and 7.



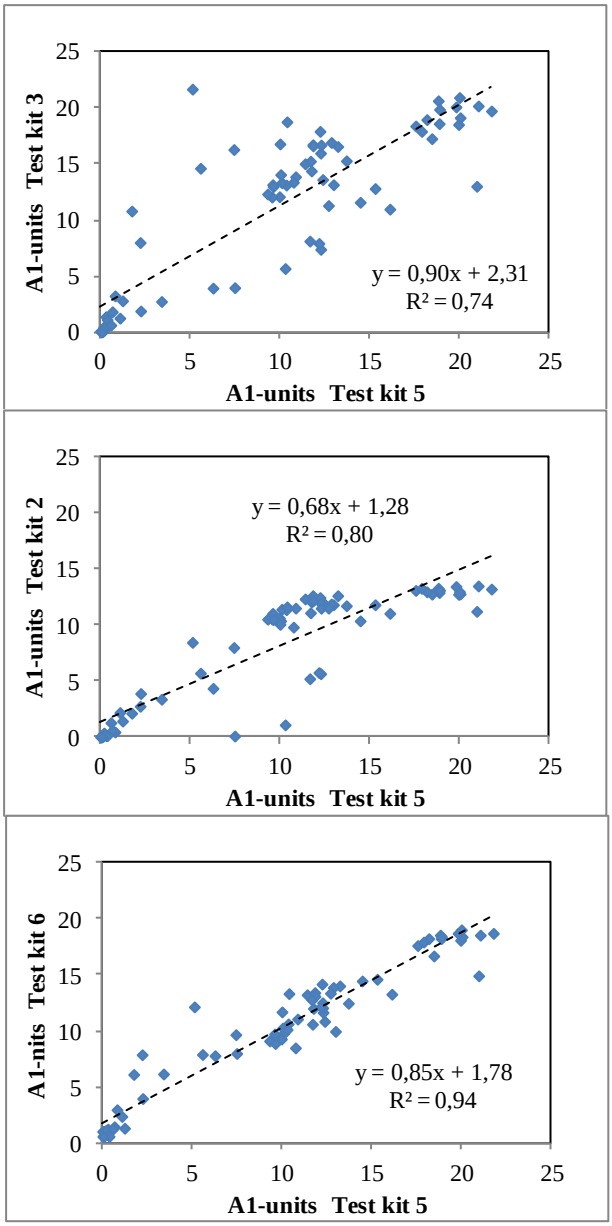
Legend:

Coefficient of variation of inter-lab variability (y-axis) plotted against decreasing IgG avidity of the study samples (x-axis). Number of the study sample to each data point at the top of the diagram.

Graph A Test kits # 2, 3: Low and constant variation over the avidity range of the study samples.

Graph B Test kits # 5, 7: Increasing variation with decreasing avidity of the study samples.

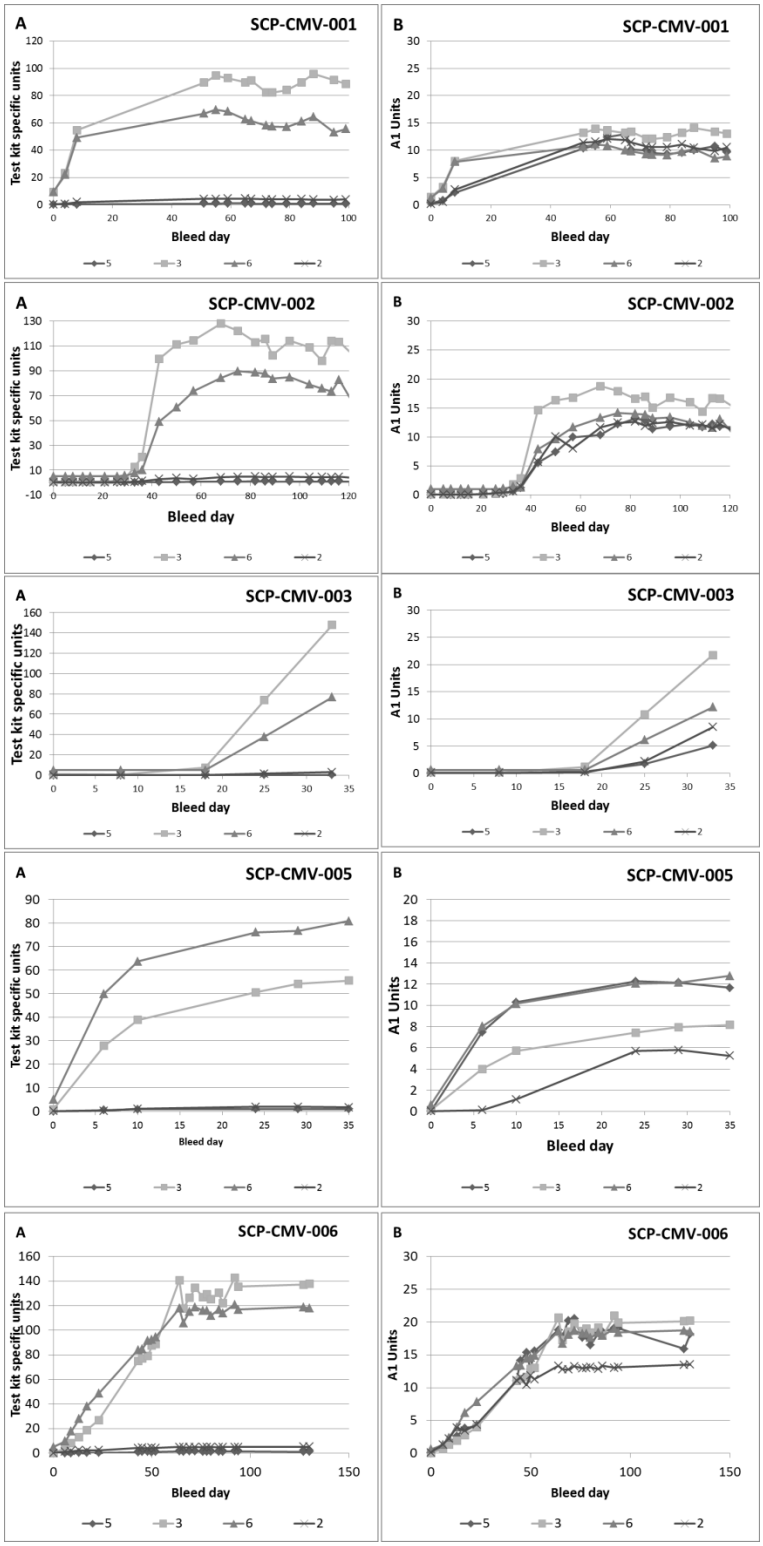
Figure 6: Correlation between the anti-CMV IgG test kits with the serial samples of the seroconversion panels.



Legend:

Test kit #5 was selected as a reference kit (arbitrary) and sorted pairwise each with the other test kits # 3, 2, 6.
X axis: Data from test kit #5 as reference.
Y-axis: Data of the respective other test kits.
Dashed line is the regression line.
R2 corresponds to determination coefficient for linear regression.
Unit of measurement are A1 units for the X and Y axes (after conversion).

Figure 7: Conversion of the test-specific test signals into "A1 units".
A=original test kit values B=transformed to A1 units



Legend:

A (left side): Test kit specific result output.

B (right side): Results transformed in units based on the candidate standard A1 (46.4 U/ml).

X-axis: bleed day

Y-axis: test kit specific units (left) and A1 units (right).

Appendix 1: Collaborative study participants.

(In alphabetical order by name)

Name	Laboratory	Country
Dr. Andreas Schmiedl and Mrs Eva Wald	Institut Virion\ Serion GmbH, Würzburg	Germany
Dr. Anna P. Obriadina and Dr. Elena Matveera	RPC Diagnostic Systems Ltd., Nizhny Novgorod	Russia
Dr. David Padley	NIBSC Division of Virology, Quality Control Reagents Unit (QCRU), South Mimms, Potters Bar, Hertfordshire	UK
Dr. Emilio Pereira	Biokit, S.A., Barcelona	Spain
Dr. Evi Struble	Office of Blood Research and Review, CBER/FDA, Silver Spring, Maryland	USA
Dr. Haruhiko Murata	Division of Viral Products, OVRR/CBER, Food and Drug Administration	USA
Dr. Heinrich Scheiblauer and Dr. Sigrid Nick	Prüflabor für in-vitro-Diagnostika beim Paul-Ehrlich-Institut, Langen	Germany
Dr. Kai Hourfar	Institut für Transfusionsmedizin und Immunhämatologie Frankfurt am Main, DRK Blutspendedienst Baden-Württemberg - Hessen	Germany
Dr. Klaus Courault and Mrs Stefanie Schneider	medac GmbH, Wedel	Germany
Dr. Luca Pallavicini	DiaSorin S.p.A., Saluggia	Italy
Dr. Márcia Mitiko Otani	Fundação Pró-Sangue Hemocentro de São Paulo, São Paulo	Brazil
Dr. Sangjan Ban	Ministry of Food and Drug Safety, Biologics Research Division, Chungcheongbuk-do	Korea
Dr. Sheila Dollard	Division of Viral Diseases, Centers for Disease Control and Prevention (CDC), Atlanta, GA	USA
Dr. Shigeharu Uchida	Central Blood Institute, Japanese Red Cross Society, Tokyo	Japan
Dr. Simon Scrimshaw	Trinity Biotech Ltd., Cambridge	UK
Prof. Dr. Thomas Mertens and Dr. Marlies Just	Konsiliarlabor für Cytomegalievirus, Universitätsklinikum, Ulm	Germany

Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
for Quality Assurance of Blood Products and in vitro Diagnostic Devices

WHO 1st First International Standard for detection of IgG antibodies to Cytomegalovirus (anti-CMV IgG) Code number 136616/17

Instructions for use (Version 1, June 2017)

1. INTENDED USE

The 1st International Standard for detection of IgG antibodies to human cytomegalovirus (anti-CMV IgG) was established for the calibration of anti-CMV IgG test kits and for quality control. It may also serve for the determination of the analytical sensitivity of anti-CMV IgG test kits.

A WHO Collaborative Study organized by the Paul-Ehrlich-Institut (PEI) was undertaken to assess the suitability of a candidate international standard (code 136616/17) in diagnostic anti-CMV IgG test kits. Fifteen laboratories from 9 different countries tested the above described material using 16 different test kits.

2. UNITAGE

This material is assigned a unitage of 46.4 IU/ml.

3. CONTENTS

Each vial contains 1.0 ml of freeze-dried anti-CMV IgG positive human plasma.

4. CAUTION

This preparation is not for administration to humans.

The standard is negative for CMV DNA as well as for anti-HIV 1/2, anti-HCV and HBsAg. The material is positive for anti-EBV and anti-HHV-6. The preparation is derived from human plasma material and should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
Each ampoule should be **reconstituted with 1.0 ml distilled water**.

6. STABILITY

The standard is supplied lyophilized and should be stored at or below -20°C. It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended. Stability of the standard nevertheless is monitored by PEI at regular intervals. The results obtained so far indicate long-term stability at or below -20°C.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

7. REFERENCES

N. Wissel, K. Hanschmann, H. Scheiblauer; Report of the WHO collaborative study to establish the First International Standard for anti-CMV IgG.

WHO Report, WHO/BS/2017.2322.

8. ACKNOWLEDGEMENTS

We thank the participants of the collaborative study for their expertise and contribution.

9. FURTHER INFORMATION

Further information for this material can be obtained as follows: pei-ivd@pei.de or WHO Biological Reference Preparations: http://www.who.int/biologicals/en/

10. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to whoccivd@pei.de or pei-ivd@pei.de

11. CITATION

In any circumstance where the recipient publishes a reference to PEI materials, it is important that the correct name of the preparation, the code number, the name and the address of PEI are cited correctly.

12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)	
Physical appearance: Lyophilized powder	
Fire hazard: None	
Chemical properties	
Stable: Yes	Corrosive: No
Hygroscopic: No	Oxidizing: No
Flammable: No	Irritant: No
Other: none	
Handling: See caution, section 4	
Toxicological properties	
Not established - avoid inhalation, ingestion or skin contact.	
Suggested First Aid	
Inhalation and ingestion: Seek medical advice.	
Contact with eyes or skin: Wash thoroughly with water. Seek medical advice.	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant. Absorbent materials used to treat spillage should be treated as biological waste.	

13. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the

Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.