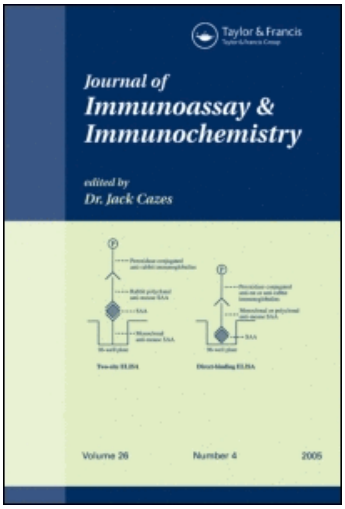

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Development of a Normal Human Immunoglobulin G Standard Curve for Enzyme-Linked Immunosorbent Assay: Use for Comparison of Antigen Efficacy

Christina M. Scheel[ab]; Sukwan Handali[b]; Edneia C. Bueno[c]; Azra Khan[b]; Kathy Hancock[b]; Armando E. Gonzalez[d]; Hector H. Garcia[ef]; Robert H. Gilman[g]; Victor C. W. Tsang[b]

[a] Department of Cellular Biology, University of Georgia, Athens, Georgia [b] Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia [c] Faculty of Pharmacy, University of the Vale do Itajai, Santa Catarina, Brazil [d] School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos, Lima, Perú [e] Department of Transmissible Diseases, Instituto Nacional de Ciencias Neurológicas, Lima, Perú [f] Departments of Microbiology and Pathology, Universidad Peruana Cayetano Heredia, Lima, Perú [g] Department of International Health, John Hopkins University Bloomberg School of Public Health, Baltimore, Maryland

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Christina M. Scheel
Department of Cellular Biology, University of Georgia, Athens, Georgia and Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Sukwan Handali
Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Edneia C. Bueno
Faculty of Pharmacy, University of the Vale do Itajai, Santa Catarina, Brazil

Azra Khan and Kathy Hancock
Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Armando E. Gonzalez
School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos, Lima, Perú

Hector H. Garcia
Department of Transmissible Diseases, Instituto Nacional de Ciencias Neurológicas, Lima, Perú and Departments of Microbiology and Pathology, Universidad Peruana Cayetano Heredia, Lima, Perú

Robert H. Gilman
Department of International Health, John Hopkins University Bloomberg School of Public Health, Baltimore, Maryland

Address correspondence to Christina M. Scheel, Centers for Disease Control, 1600 Clifton Road NE, Mail Stop G-11, Atlanta, GA 30333. E-mail: zsr3@cdc.gov

Victor C. W. Tsang
Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract: Internal standard reference curves are used in enzyme-linked immunosorbent assay (ELISA) plates to control for inter- and intra-assay variance. To compare the diagnostic potential of multiple *T. solium* antigens on an unbiased, universal scale, we have created a standard curve using normal, human immunoglobulin G (hIgG). The hIgG curve is inexpensive and simple to prepare, and remains stable at 5°C for at least one year, with a coefficient of variance of less than 10%. The hIgG standard curve has proven a critical tool for the comparison of several diagnostic antigens slated for assay development.

Keywords: ELISA, Standard curve, Immunoglobulin G, Diagnosis, Assay development, Antigen efficacy

INTRODUCTION

The indirect enzyme-linked immunosorbent assay (ELISA) is currently the most widely used method of detecting and quantifying humoral immune responses. In the past, diagnostic antigens used to develop such assays were derived from crude extracts of pathogens that often contained irrelevant, cross-reactive components. Recent advances in recombinant protein technology have provided a means of circumventing the problems associated with non-specific reactivity in crude antigen ELISAs; development of indirect ELISAs based entirely on recombinant protein antigens is now common practice. The most desirable recombinant ELISA assay format will require only one antigen to detect specific antibody reactivity in an infected population. Often, however, native lysates are comprised of several antigens that elicit sensitive and specific humoral responses in diagnostic assays. In order to determine which native antigen is the most suitable for large-scale assay development, it may be necessary to produce several recombinant antigens for assay against infected material.

Ideally, an internal standard reference curve should be included in each microwell ELISA plate to control for fluctuations in optical density (OD) caused by slight variations in reagent temperature, volume, pH or formulation. The standard curve is assigned arbitrary unit values that remain constant regardless of these deviations in OD. Most often, the standard curve is comprised of pooled immunoreactive samples collected from several donors with proven infections. Because of the rarity of some conditions and diseases, these samples may not be available in sufficient quantities to develop such a standard curve. Even if the immunoreactive material is readily available, it is unlikely that the sources for individual components

of the pool will be available indefinitely. Furthermore, because immune response to a given antigen will likely vary between individuals, the pooled immunoreactive material may be skewed to recognize one antigen over another, and may not be representative of the target population. An unbiased, universal standard curve is necessary to compare the specific activity of several antigens on the same reactivity scale.

We have developed a standard curve for indirect ELISA using normal human immunoglobulin G (hIgG) that allows us to compare the specific activity of many antigens on a universal scale to determine the most efficacious antigen(s) for large scale assay development. Preparation of the curve is simple; hIgG is solubilized, quantified, and titered into several dilutions to represent a reliable curve that falls within the limits of spectrophotometric detection. The chosen dilutions can then be prepared in larger batches and stably stored at 5°C for up to one year. The hIgG standard curve has proven to be a critical tool for the comparison of several antigens slated for large-scale assay development in this laboratory.

EXPERIMENTAL

Normal Human Immunoglobulin G

Normal, human immunoglobulin G (hIgG) was purified by $(NH_4)_2SO_4$ precipitation and ionic exchange chromatography to homogeneity, lyophilized, and frozen in the vapor phase of liquid nitrogen.[1] The hIgG was resolubilized in degassed phosphate-buffered saline (PBS), pH 7.2, 0.1% sodium azide and mixed end over end for 15 min. The solubilized hIgG was then quantitated using the standard Bradford Assay.[2]

Preparation of hIgG Standard Curve

The hIgG solution was diluted in PBS, pH 7.2, 0.1% sodium azide, 0.01% bovine serum albumin (BSA) Fraction V to a working concentration of 20 μg/mL. A pilot curve of ten standards (10 mL each) was prepared in the following concentrations (μg/mL): 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1.0. The standards were assigned unit values of reactivity that corresponded to their respective concentrations, shown above, with the maximum value of 1.0.

Enzyme-Linked Immunosorbent Assay (ELISA) Evaluation of hIgG Standard Curve

The FAST-ELISA method used to evaluate the curve has been described previously.[3] All reagents were dispensed at 150 μL/well. Briefly, the ten

standards were dispensed into 96-well flat bottom incubation plates (cat # 269620, Nunc™, Nalge Nunc International, Denmark) and adsorbed to the polystyrene sticks on lids of the NUNC-TSP transferable solid phase screening system (cat# 445497, Nunc™), at room temperature for 2 hours, with gentle shaking. This step is, hereafter, referred to as sensitization. The sticks were then rinsed with a wash buffer of PBS/0.3% Tween® 20 (polyoxyethylene sorbitan monolaureate, Calbiochem®, San Diego, CA) using a pressurized garden sprayer. Goat anti-human heavy and light chain IgG conjugated to horseradish peroxidase (GAHG-POD)[4] was diluted in wash buffer to a non-rate-limiting concentration (previously determined by titration) and used to detect the bound antibodies. The GAHG-POD was incubated for 5 min with gentle shaking, and sticks were rinsed with wash buffer as before, followed by substrate development using SureBlue™ TMB Microwell Peroxidase Substrate (1 component) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Absorbance at 650 nm was read by a THERMOmax microplate reader using SoftMax Pro 4.7.1 software (Molecular Devices Corp., Sunnyvale, CA). All units of the curve were tested in triplicate two times to ensure that the standard values were representative of a comprehensive curve that fell within the quantifiable absorbance range of the spectrophotomer.

Antigen Comparison using the hIgG Curve

A large batch of the hIgG curve was prepared in 50 mL quantities for each standard. Vials stored at 5°C were later assayed against freshly prepared hIgG curve to determine shelf life. For each assay ($n = 92$), the standard curve was dispensed into wells along the top row of a Nunc 96-well plate. The remaining wells of the plate were loaded with repeats of one of four different synthetic antigens derived from purified *T. solium* cyst homogenate, TS14, TS18var1, TSRS1, and TSRS2var1, at a concentration of 10 μL/mL each.[5–9] Sticks were sensitized with hIgG or antigen for 2 hours with gentle shaking, and then rinsed with wash buffer. The hIgG-bound sticks were then incubated in wash buffer, while antigen-bound sticks were incubated in test antibody diluted 1 : 100 in wash buffer/5% nonfat dry milk. All reagents were dispensed at 150 μL/well. Conjugate and substrate incubations were as described above, with a final reading at 650 nm. This procedure is also described in reference [9]. A positive control serum sample was used on all assay plates. Interassay coefficient of variance (CV) for the positive control was calculated to insure validity of the assay. Plates showing a positive control value with a CV of greater than 10% from the inter-assay mean were repeated. Antigen efficacy was determined using Youden's J Index ([Sensitivity + Specificity] – 1).[10]

Determination of Shelf Life

Shelf life was determined by storing a freshly prepared hIgG standard at an elevated temperature to accelerate the aging process.[11–14] A new batch of fourteen hIgG standards (0, 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, and 2.0 μg/mL) was prepared using a 20 μg/mL working solution as before. The standards were assigned unit values of reactivity that corresponded to their respective concentrations, shown above. After pilot testing, a large batch (30 mL) of each of the fourteen hIgG standards was prepared and dispensed into amber glass vials. The vials were stored at 5°C and at an elevated temperature of 37°C, representing an accelerated model for storage.[12] A portion of the 5°C curve was used to sensitize several plates for dry storage at 5°C and 37°C. After a 2-hour sensitization, plates were air-dried for 1 hour, placed in light protected, covered containers, and stored.

The acceleration model for aging is based on the bracket table method described by Porterfield in 1984.[12] The incubation times used to determine shelf life (Table 1) are based on the assumption that the activation energy (E_a) of hIgG is 20 kcal/mole, and are calculated using a modified version of the Arrhenius equation.[12] Aged samples were tested in duplicate for stability using FAST-ELISA as described above. All assay plates included a standard curve stored at 5°C as a control measure. Test curves were read as both unknowns and standards. Long-term stability was also tested using the hIgG standard curve that was prepared 1.5 years earlier and used for

Table 1. Storage time intervals at 37°C for accelerated aging of the human immunoglobulin G (hIgG) standard curve. The storage times listed at the elevated temperature are based on the bracket table method, which assumes the activation energy (E_a) of a substance to be 20 kcal/mole, and is calculated by rearrangement of the Arrhenius equation [12]

Real time equivalent at storage temperature (5°C)	Accelerated aging temperature (37°C)
1 d	—
1 w	4 h
1 m	17.5 h
3 m	2 d 4 h
4 m	2 d 22 h
5 m	3 d 15 h
6 m	4 d 9 h
8 m	5 d 19 h
1 y	1 w 2d

h = hours, d = days, w = weeks, m = months, y = years.

T. solium antigen comparison.[8] Inter-assay CVs of the points comprising the linear portion of the curve (unit values 0.1 to 2.0) were calculated for all standards and unknowns assayed.

RESULTS

Antigen Comparison using the hIgG Curve

The hIgG standard curve reactivity was consistent throughout the duration of the *T. solium* antigen comparison study (data not shown).[8] Although day-to-day variations, such as temperature changes, did cause the OD of the curve to fluctuate slightly, the positive control in each plate ensured that the unit values by which antigen reactivity was measured remained consistent. Interassay CV analysis of the positive control showed that only 14 of the 92 antigen plates tested fell outside of the interassay mean by greater than 10%.

Determination of Shelf Life

Aged hIgG curve aliquots stored at 37°C remained consistent in reactivity throughout the study, which was terminated at the real time equivalent of 1 year (Fig. 1A). The interassay CV for the OD values of linear points on the curve (unit values 0.1 to 2.0) was calculated for the aged standard curve samples after 18 assays; each aged sample was tested in duplicate. Only one of the standard curves tested (1 year time point equivalent) deviated from the mean OD by greater than 10% at linear OD value points. All other standard curves had CVs of less than 10% for all equivalent linear points (units 0.1 to 2.0, $n = 18$ trials for each point), with a mean CV of 7.99%. Standard deviations between OD values for equivalent points of the curve ranged between ± 0.05 and ± 0.21. An interassay CV of the assigned standard unit values of all of the time points tested, including the 1 year standard curve, was calculated at an average of 9.45%. This figure was very similar to the average CV calculated from the standard unit values of the 5°C control curves (9.19%, $n = 18$) (data not shown). The hIgG standard curve also proved stable in real time aging conditions. The OD values of a standard curve stored at 5°C for 1.5 years was compared with the mean OD data of four trials of the same curve tested in the first month of preparation (Fig. 1B). The average CV calculated for the OD values comprising the linear portions (unit values 0.1 to 1.0) of both fresh and aged standard curves was 9.99%. Standard deviations of equivalent points of the curve ($n = 2$ trials for each point) ranged between ± 0.01 and $\pm$ 0.21).

Dry storage of the hIgG standard curve was unsuccessful, as the curve remained stable for only one day at both 5°C and 37°C, thereafter showing a decline in OD of approximately two-fold at one week (data not shown).

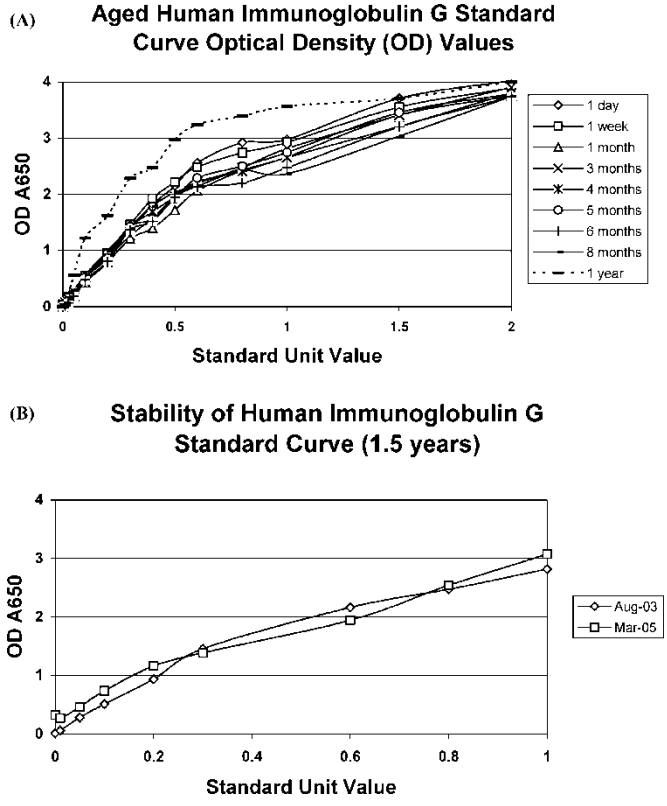

Figure 1. Stability of the human immunoglobulin G (hIgG) standard curve as shown by accelerated aging studies (A) and real time storage tests (B). An hIgG curve was stored at 37°C to accelerate the aging process and tested at time points equivalent to real time aging at 5°C.[12] The legend shows the real time equivalents of the accelerated aging time points tested (A). Real time aging of the hIgG standard curve showing a newly prepared curve (Mar–05) and a curve prepared 1.5 years earlier (Aug–03), both stored at 5°C. Note that only nine standards are shown here, as the unit value "0.4" was masked in all early tests with this curve (B).

DISCUSSION

The hIgG standard curve has played a pivotal role in the selection of diagnostic antigens worthy of large-scale assay development. When we initiated the evaluation of four synthetic *T. solium* antigens, TS14, TS18var1, TSRS1, and TSRS2var1, we realized that the pooled, positive patient serum standard curve that we were using was immunologically biased.[8] By using an hIgG standard curve, we were able to control for variances in all assay components, with the exception of immunological specificity of patient antibodies. Controlling for reagent, conjugate, and substrate variance is *a priori* for all assays that

are comparing antigen efficacy. Additionally, the hIgG curve reflects assay activities that directly correspond to concentrations of hIgG in the analytes, and is independent of antigen-specific antibodies in infection sera.

In our evaluation of the *T. solium* antigens, we were able to eliminate potential diagnostic candidates from further development by utilizing a standard curve independent of immunological bias, saving both time and a great deal of expense. The hIgG standard curve can be also used to test individual sera for reactivity against a panel of antigens representing different infectious organisms to determine a relative level of reactivity to each. In addition, use of this curve frees a diagnostic assay from the need for large quantities of positive sera from which to prepare a pooled standard curve to control for inter- and intra-assay variations. Instead, the standard curve is hIgG, and a positive serum control is included in each assay plate.

The highly purified hIgG used in this study was not from a commercial source, nor was the GAHG-POD conjugate. An attempt to repeat this study using commercially supplied hIgG and conjugate showed that much higher concentrations of hIgG (up to 20-fold) were needed to construct a standard curve that reached the upper limits of spectrophotometric detection (personal communication, Jeff Priest). Because each standard curve is initially prepared as a test pilot batch, little time will be lost in determining the appropriate concentrations of hIgG needed to build a useful curve. It may be useful, however, to verify the purity of commercial hIgG before purchase, and to use hIgG from the same commercial source and lot when preparing new standard curves. For assays that may potentially involve IgM, IgA, or other subclasses of Igs, a standard curve prepared from that ammonium sulfate or alcohol-precipitated serum protein fraction may also be considered. Alternatively, a mixture of purified Igs may be used to construct the standard curve. Albeit, the relevance of this issue is hard to ascertain, given that the 2nd enzyme-labeled antibody that we used here (GAHG-POD) possessed both heavy and light chain specificity and can detect all Ig subclasses equally.

It is unfortunate that the hIgG standard curve cannot survive dry storage, but it may be possible to retain curve sensitivity by storing pre-sensitized, wet plates (sealed) at 5°C, although this has not yet been attempted. In this way, both the antigen and standard curve could be prepared in advance for ease and expediency of use. The hIgG curve should not be frozen as prepared, as a loss of reactivity may result. It is possible that the addition of glycerol to prevent freezing of the hIgG will allow for storage terms beyond those attempted in this study.

The hIgG standard curve for indirect ELISA is a useful method by which to compare the immunodiagnostic potential of any number of antigens on a universal scale. Reliance on pooled immunoreactive material for assay standard development and production is dangerous in two ways; it is unlikely to adequately represent the immune responses of the target population, and is of limited availability. The hIgG standard curve is inexpensive

and simple to prepare, and will retain sensitivity for at least one year when stored at 5°C.

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