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DETERMINATION OF DILUTION AND QUALITY CONTROL OF TOTAL AND ANTI-MEASLES IMMUNOGLOBULIN G ANTIBODY ASSAYS
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ABSTRACT

***Objective*: To determine the correct dilution and Quality control commercial ELISA of total and anti-measles antibodies for HIV infected pregnant women.**
***Design*: A laboratory based study**
***Setting*: The University of Nairobi, Department of Paediatrics laboratory.**
***Subjects*: HIV infected pregnant women enrolled and exposed to different ARVs depending on their degree of immunosuppression for prevention of mother-child transmission of HIV-1.**
***Results*: The dilution used in this study, was 1:400000. Tight error bars of +/-0.1 were produced hence testing was done in singles not in duplicates as recommended. Validation steps did not pass for measles ELISA.**
***Conclusion*: Despite the recommendations of the manufacture each laboratory should always optimize an assay before performing tests and reporting the result. Every laboratory should determine the best dilution to use for quantitative TIgG assays and should perform internal and external quality control before reporting the results. These results will give insight on good laboratory practice during trouble shooting while assays are failing.**

INTRODUCTION

The ELISA technique was first introduced between 1971 and 1972. It is based on the practical theories and experiments of Engvall and Perlmann who described a method for the analysis of the concentration of Immunoglobulin G in human sera. The method involves a solid phase where the proposed antigen (or antibody) could be bound followed by the addition of sera for examination (1). Most immunological tests used in clinical laboratories are commercially produced.Sometimes however, tests are developed, evaluated, and validated within one particular laboratory. There are advantages and disadvantages of either of the two sources of the ELISA kits but one of the key disadvantage of commercial reagent kit is the cost and there could be false results (sensitivity, specificity and accuracy).This is because high serological criteria of an ELISA kit cannot be reproduced in another country (2).

Laboratories may quantitatively report results that fall within the reportable range. It is recommended to check for poor repeatability as part of the linearity study by testing two to four replicates at each concentration, depending on the expected imprecision of the assay (2). Analytical sensitivity of an assay is generally determined in one of two ways statistically, by calculating the point at which a signal can be distinguished from background, or empirically, by testing serial dilutions of samples with a known concentration of the target substance in the analytical range of the expected detection limit (3). Precision which is the closeness of agreement between independent test/measurement results obtained under stipulated conditions is measured as repeatability and reproducibility. Repeatability (or within-run imprecision) being the smallest measure of precision and involving measurements carried out under the same conditions (same operator, reagent lots, instrument, laboratory, time) and reproducibility (run-to-run imprecision, day-to-day imprecision) being the largest measure of precision and involving results of measurements under changed conditions (different operators, reagent lots, time, laboratory) (4).The reference interval is usually the last performance characteristic to be studied, since it is used to decide whether a method is acceptable or not (3). The reference interval ("normal range") of a test is simply defined as the range of values typically found in individuals who do not have the disease or condition that is being assayed by the test (the "normal" population) (5).

Although commercial ELISAs have been used for more than two decades and are specific and highly

sensitive for identifying and quantifying analytes, there are several laboratory issues. These issues include; failing assays, determination of correct dilution in a specific population. These issues have significant effect on the results and final conclusions. Validation of assays and general quality control of commercial ELISA remains a key challenge. This is because the assays may be developed and validated in the manufacturing countries without putting into consideration the uniqueness of the population in the application country (2).

Accurate, reproducible and reliable laboratory results are crucial for patient management and care and revaccination of children with insufficient protection following routine childhood vaccination. It is important for evaluation of study results or establishing reference ranges for specific populations or age groups. This study emphasizes the importance of optimization, validation and quality control whenever an assay is performed in order to produce reliable, accurate and reproducible results.

MATERIALS AND METHODS

Study Design: This was a laboratory based study conducted as a sub study of a research carried out at the University of Nairobi, Department of Pediatrics laboratory.

Sample size: Eight samples were randomly selected

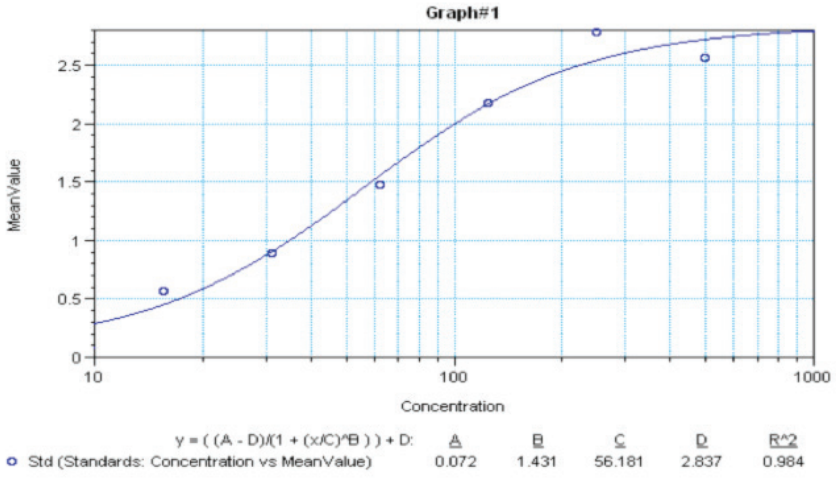
Sampling: Systemic random sampling was carried out by first having a list of the whole population of serum specimen numbers, secondly a random number was chosen at the start of the list. Thereafter every third number from the whole population was sampled. A skip interval had been computed using MsExcelc .

Sample dilution for TIgG Assay: Immunology Consultants' Laboratory ELISA kit recommended dilution of 1:80000 was first tested and found to be concentrated hence produced high ODs. The correct dilution for the samples needed to be determined so that the OD was in the linear part of the standard curve. A serial dilution was conducted at 1:160, 000, 1:80,000,1:60000,1:40,000,1:20,000 for both serum and plasma, HIV positive and negative samples. A dilution of 1:100000-1:800000 was conducted several times. All assays were run in duplicates at the dilution of 1:400000 and standard error was calculated and error bars constructed by SOFT max PRO. Performance of QC for the failing assays was done the variables included incubation time, temperature and pipetting and use of different reagent kits.OD reading at 450nm using Elisa (molecular devices –Emax – precision micro plate reader) was done within 30 minutes.

RESULTS

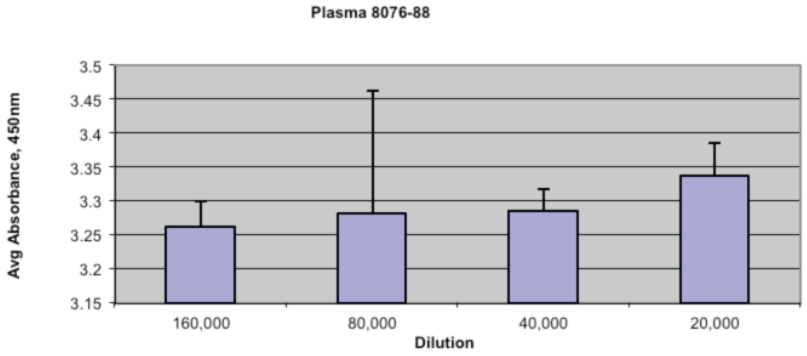
Standard curve obtained using manufacturer standards: Based on this standard curve obtained using the manufacurers' standards the only ODs that can be used to determine the concentration of the samples are the ones that fall into the linear part of the standard curve (approxiamtely between 0.5 and 2.5 OD). ODs standard curve constructed produce OD's precisely 0.3-2.8 (Figure 4) meaning ODs that can interpolate the sample results are the ones that fall in the linear part of the standard curve and within this range.

Figure 4
Stardard curve for manufacturer recommended dilution of 1:80000



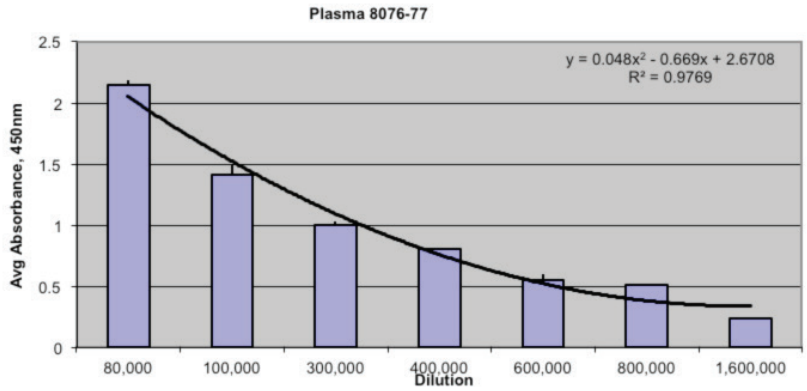
Initial dilution of 1:80000 for TIgG as recommended by the manufacturer for both serum and plasma: From the initial dilution recommended by the manufacturer the samples were too concentrated giving ODs of 2.9-3.6 that were outside the linear part of the curve (Figure 4). With these ODs the sample concentration cannot be interpolated from the standard curve. OD'S of 3.0 and above are above the linear part of the curve, meaning they are high as can be seen from the figure below (Figure 5).

Figure 5
Initial recommended serial dilution of 1:20000-1:160000



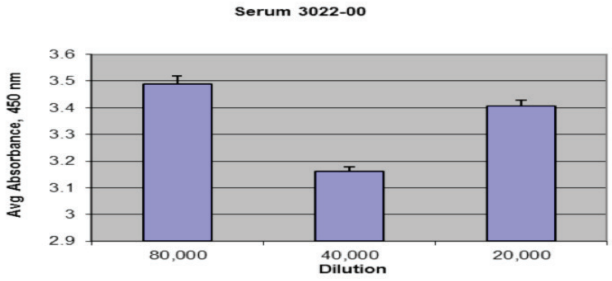
Serial dilution of 1:100000- 1:800000: The sample ODs at a lower concentration of 1:100000-1:800000 were 0.3-2.5 and fall within the linear part of the standard curve (Figure 6) hence can be interpolated to find the concentration from the standard curve constructed (Figure 4).The actual dilution used in this study was1:400000 because at this dilution the ODs all within the linear part of the standard curve.

Figure 6
Sample dilution of 1:80000-1:1600000



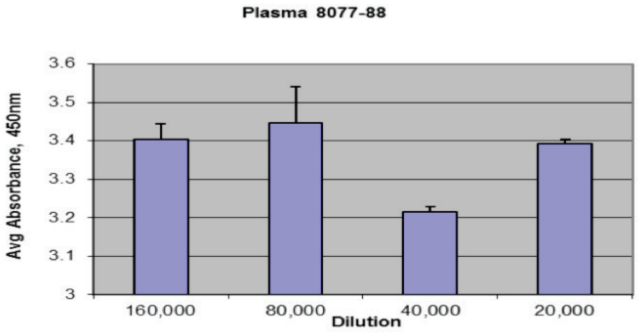
Dilution of HIV negative Serum and plasma: The ODs for HIV negative serum at dilution of 1:20000-1:80000 were 2.9-3.6 and the plasma at the same dilution were 3.0-3.6. meaning they were high despite the sample type hence the ODs did not vary based on sample type, but rather individual sample(Figure 7 Figure 8)

Figure 7
Dilution of serum at 1:20000-1:80000



Dilution of Plasma sample type at 1:80000: The OD's of plasma sample were3.0-3.6 which was high at the manufactures required dilution of 1:80000.

Figure 8
Dilution of plasma at 1:20000-1:160000



Dilution 0f HIV positive plasma: HIV positive samples OD's at a higher concentration of 1:20000-1:160000 were 3.0-3.5 and at a lower dilution of 1:100000-1:80000 were 0.1-2.3 (Figure 9, 10) hence HIV serostatus of the sample did not influence dilution, the dilution was influenced by individual sample not the HIV serostatus of the sample

Figure 9
Dilution of HIV positive samples at dilution of 1:20000-1:160000

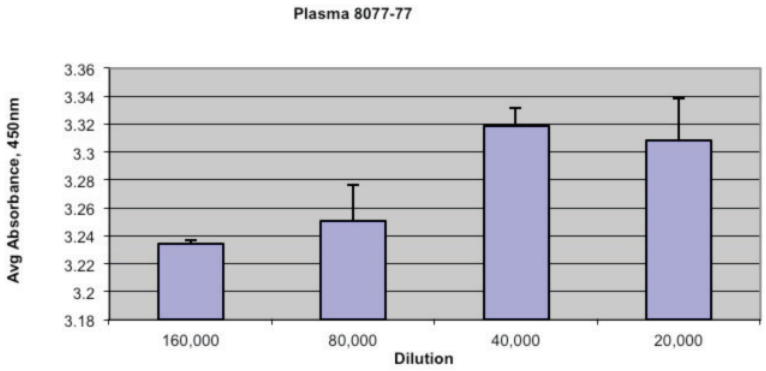
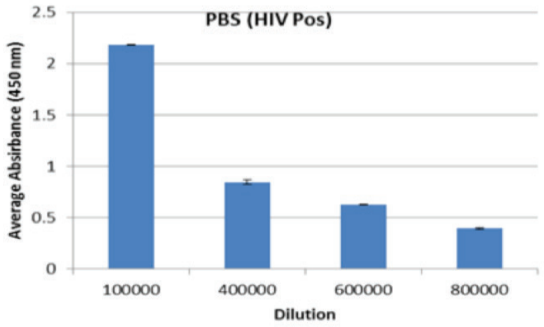
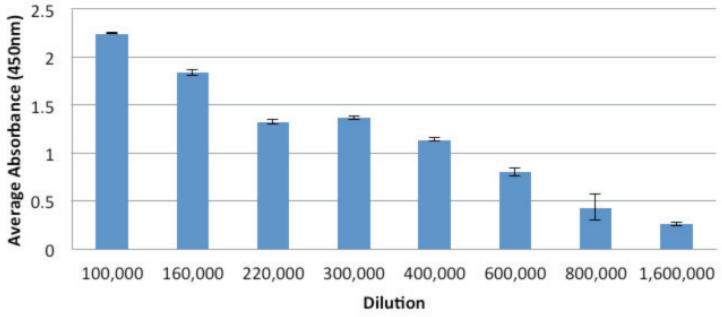


Figure 10
Dilution of HIV positive samples at 1:100000-1:80000



Reproducibility of samples ODs at dilutions of 1:100000-1:1600000: Multiple dilutions of 1:100000-1:600000 were conducted on each sample in duplicate and standard error calculated bySOFTmax PRO .Standard error bars were then drawn and were close with exception of a few as shown below,the close error bars gave confidence to perform the tests in singlets (Figure 11).

Figure 11
Multiple dilution of a sample at 1:100000-1:1600000



Precision to determine the reproducibility: Sample ODs at different dilutions was done using samples with known concentrations of analyte. Test materials were the patient specimens after determining the dilution factor. The samples were tested in duplicates so that the standard error could be determined and the error was+-0.1 meaning it was close and duplicates gave similar results hence the samples were run in singles.

Dilution of Nairobi cohort plasma: Samples from Nairobi site cohort before ARVs at dilution of 1:40000-1:400000 gave ODs of 0.6-2.4 (Figure 12) and after ARVs were 0.2-2.3 so could be interpolated from the standard curve

Figure 12
Dilution of plasma at 1:40000-1:400000 from Nairobi before ARVs

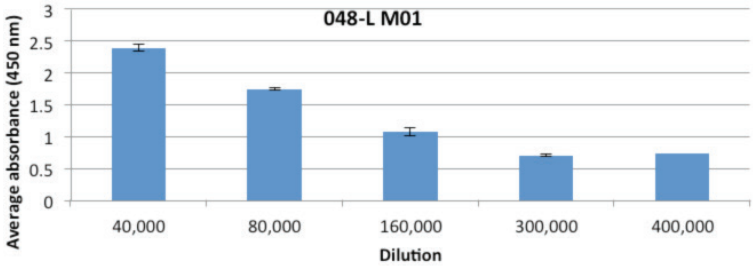
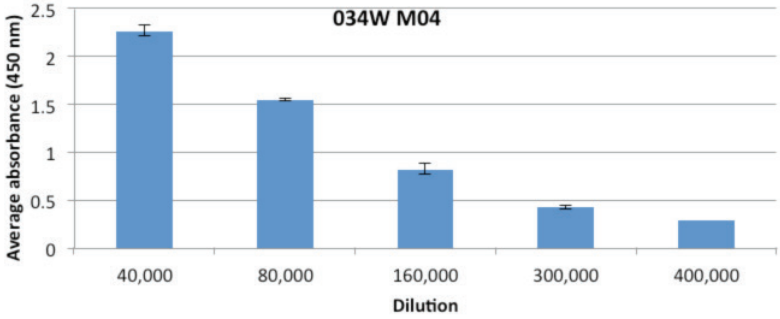


Figure 13
Dilution of plasma at 1:40000-400000 from Mombasa after ARVs



QC variables for measles IgG: Table 1 shows the results for the different variables for QC when the assays were failing. . The first three reagent kits passed the validation criteria before they failed which was the absorbance value obtained from the measurements with measles virus minus the absorbance value of the same sample obtained with measles virus control antigen (OD AG-ODCOAG) and individual ΔA values (anti-measles virus Reference P / N at the start and end of series of measurement or test plate) must not differ by more than+-20% from the mean calculated from the values. Trouble shooting was done on different rot numbers of the failing kits. Samples were run simultaneously in different incubators observing accuracy on pipetting and incubation time. Below is the summary of theresults (Table 1).

Table 1
QC variables for failing measles IgG

QC Variables	Old Reagent Kit	Old Reagent Kit	New Reagent Kit	New Reagent Kit
Incubators	Pass/Fail	Fail/Fail	Pass/Pass	Pass/Pass
Pipeting	Pass/Fail	Fail/Fail	Pass/Pass	Pass/Pass
Time	Fail/Fail	Fail/Fail	Pass/Pass	Pass/Pass

DISCUSSION

The TIgG reagent kits used in this study were optimized but the correct sample dilution needed to be determined to produce OD's 0.1-2.5 which falls on the linear part of the standard curve. The purpose of identifying the correct dilution is to ensure high sensitivity and good linearity of the patient's antibody concentration. This is because an ELISA standard curve expresses protein concentration as a function of spot intensity 6 The manufacturer suggested the sample dilution to be done at 1:80,000 this dilution produced OD's that were between 3.18-3.5 (Figure 5) which were high and the sample concentration could not be interpolated from the standard curves (Figure 4). The required maximum signal (OD) should not be more than 2.0 (Immunology consultant laboratory) although not a strict cut off because some tests were leveling at 2.5 (Figure 6 and 8). From this work we could not identify a clear cut off, but rather an approximate OD range of 0.1-2.5. The actual dilution used was 1:400000 this was much lower than the manufacturer's recommendation of1:80000. Studies have shown that very low dilution affect theresult by lowering the concentration this was not determined in this study. A study carried out on malaria ELISA showed that results change depending on dilution factor selected7.Future studies need to be done to establish that in total IgG ELISA. Although multiple dilutions were done for each sample at the lower dilutions (Figure 11) the results were consistent with Kazutoyo's study.

Dilution of different sample types (plasma, serum)carried out showed that there was no significant difference in the OD's of the various sample types, because at the initial dilution of 1:80000 required by the manufacturer produced OD's that were high (Figure 7 and 8) .Therefore the ODs did not vary based on sample type, but rather on individual sample. Theexpectation was that serum would require a lower dilution than plasma because of the proteins used up in the coagulation process 8. Dilution of HIV positive and HIV negative plasma was compared(Figure 9 and 10). HIV positive samples produced relatively higher OD's as compared to HIV negative samples though both were high at the required dilution by the manufacture this is because HIV infection causes hypergammaglobinaemia9. The high OD's could be because of the physiological and pathological variability of the study population under investigation or due to storage conditions of the samples. There have been reported cases of hyper-gammaglobinaemia in the tropics due to tropical diseases such as intestinal nematodes10Since these assays were imported from the USA the manufacturer maybe they did not perform validation with adequate demographic information for the population to be studied. Therefore if details for dilution were adequately provided, the laboratory needs to experimentally verify the dilution for its unique population (2). There are many other causes of increased OD apart from high concentration of the analyte under study, for instance interference/ cross-reactivity of other antibodies (11).

It is required to always run ELISA samples in duplicate or triplicate in most cases this is not feasible because of the cost of the reagents (3). In this study this was done and because the error margin was small then it is okay to run in singles to safe on the cost of the reagents.

To obtain reliable data, quality control (QC) of data is important. Despite automated processes, rigorous QC measures and improved assays, laboratory testing remains susceptible to errors (11). The reproducibility and reliability of ELISAs are dependent upon proper technique and attention to detail. Relying on routine QC testing alone for error detection could be vulnerable to failure. For instance, in this study the reagent kits had been evaluated alongside other kits and found to be most accurate and reliable (12). It is therefore easy to assume that after running the first assay successfully all subsequent ones would pass the validation criteria. After the first run, the assays were failing (Appendix 4). Troubleshooting had to be done to ensure that the failure was not due to the test conditions. The troubleshooting variables focused on included incubation time/ temp, pipetting, reagents like distilled water, ELISA reader (Table 1).

CONCLUSIONS

In conclusion, the actual dilution used in this study was 1:400000 which was low as compared to1:80000 required by the manufacturer. This dilution was used for all samples in regardless of the type, HIV status of the patient. Failing measles IgG assays were due

to manufactures fault because it was confirmed that all the reagents produced at that time did not pass the validation criteria from other laboratories. Therefore every laboratory should determine the best dilution to use for quantitative TIgG assays and every laboratory should perform internal and external quality control before reporting the results

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