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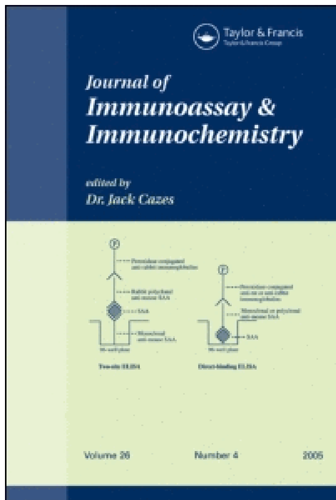

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Assessment of Assay Sensitivity and Precision in a Malaria Antibody ELISA

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Assessment of Assay Sensitivity and Precision in a Malaria Antibody ELISA

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ABSTRACT

Many types of ELISA-based immunodiagnostic test kits are commercially available in the market for specific indications. These kits provide necessary assay components, reagents, and guidelines to perform the assay under designated optimal conditions. By using these kits, any unknown or test sample can be assessed as negative or positive based on the results of referral calibrator (Ref+ve and Ref−ve) samples. It is essential to provide reliable test kits to end-users with adequate quality control analysis. Therefore, it is necessary to check the kit for any variations in its performance. While developing a malaria antibody ELISA test-kit, we optimized assay conditions with chequer-board analyses and developed an assay protocol. We have taken out kits randomly from the assembly line and had them evaluated by operators who are new to the

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test-kits. Assays are performed as per the test guidelines provided. Sera, diluted serially, have shown a clear discriminatory signal between a negative vs. positive sample. A COV is determined by evaluating the Ref−ve calibrator in replicate antigen-coated wells from 6 different plates. This COV is used as a tool to determine S/N ratio of test samples. Besides Ref−ve and Ref+ve calibrators, additional field serum samples are tested with the test kit. Several performance indices, such as mean, standard deviation, %CV are calculated, and the inter- and intra-assay variations determined. The assay precision is determined with large and small replicate samples. In addition, assays are performed concurrently in triplicate-, duplicate-, and single-wells, and the results are analyzed for any assay variations. Different plate areas are identified in antigen-coated 96-well plates and tested blind to detect any variations. The S/N ratio is found to be a very effective tool in determining the assay sensitivity. The %CV was within 10–15%. Variations seen in the assays are found to be due to operator errors and not due to kit reagents. These observations, although, are based upon one type; however, it may as well apply to other line of kits. This is obviously valuable to the end-users of ELISA kits. The operator related error has to be ascertained before lodging any complaint on the kit performance. Based on this data, the test kit has shown acceptable sensitivity and precision and offers compliance on the way the test kits is manufactured. With this, it is concluded that the test kits are suitable for detecting malaria antibody in clinical sample analysis.

Key Words: ELISA; Reference calibrators; Sera samples; Malaria antibody; Assay variation sensitivity; Inter-assay and intra-assay variation; Assay precision; Operator-related errors.

ABBREVIATIONS AND EXPLANATIONS

COV (cut-off-value): a determined mean + 3SD value of negative analyte samples reacting in the assay. An unknown sample is categorized as either negative (if the OD is < COV) or as positive (if the OD is > COV).[18]

Duplicate-well ELISA: assay in two-wells.

ELISA: enzyme-linked immunosorbent assay.

Inter-assay variation: variation seen by testing a sample many times in different assays.

Intra-assay variation: variation seen by testing a sample many times in one assay.

Mean: an average value of replicate readings.

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OD readings: optical density readings of ELISA wells in a plate reader. All readings were uniformly taken with dual filters of 450/620 nm.

%CV: (percentage coefficient variation), calculated as the measure of standard deviation with reference to the mean and expressed as the percentage [$CV = (SD/mean) \times 100$]. This reflects on the assay precision.[18]

PBS/T: phosphate buffered saline supplemented with 0.01% Tween 20.

Precision: an estimate of error in ELISA results. It is expressed as the percent coefficient of variation (%CV, see above) or, less often, as the standard deviation (SD) at a particular analyte level. In other words, it reflects on the consistency of ELISA results.[18]

Ref−ve calibrator: an analyte sample yielding a negative reading (i.e., OD is $<$ COV).

Ref+ve calibrator: an analyte sample yielding a positive reading (i.e., OD is $>$ COV).

S/N Ratio (signal-noise ratio): a differential absorbance of Ref+ve calibrator or a known positive sample, or an unknown sample yielding a positive result divided by the absorbance of Ref−ve calibrator or a known control analyte (reading of an analyte/reading of control negative sample).[14]

Single-well ELISA: assay done in one well.

SOP: standard operating procedure.

SMP: standard manufacturing procedure.

Triplicate-ELISA: assay done in three consecutive wells.

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INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) has undergone considerable improvements since its original description 25 years ago by Engvall and Perlmann.[1] With the development of microtitre format in 96-well plates by Voller et al.,[2,3] the micro-ELISA test has become the industry standard and one of the most popular biomedical methods used for analytical purposes.[4,5] With its robustness and with a provision of testing as many as 96 samples in one assay,[3,6] the micro-ELISA has become the benchmark assay for field purposes. If care is taken in the assay design and construction, as per the need and diagnostic applications, ELISA provides highly sensitive results.[6,7] The ELISA technology is very popular as evidenced by the fact that many types of ELISA kits are on the market. Based on this concept, many diagnostic companies

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have manufactured automated immunoassays.[8] These developments have emphasized the need for quality assurance of manufactured products for use elsewhere.

Assay sensitivity and precision remain the essential quality control tools for assessing the ELISA kits.[9,10] The assay involves multiple steps, some performed manually and some with automated machines; errors occur which invariably reflect on the results. Also, it is important to know how and why errors occur in an assay, although a stepwise or a standardized protocol is followed. If a patient sample is tested in ELISA, the results could have enormous implications in patient management, especially making a decision on drug treatment.[11–13] Therefore, the assay should be flawless, and should yield accurate results for making clinical decisions for the patient. In other words, an ELISA assay should be validated to obtain results with precision.[12,14] Furthermore, it is valuable to understand how assay variations would influence the results.

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These questions are addressed during preparation of test-kits for detection of malaria-specific antibody in serum samples. We have taken certain analytical measures to minimize any variations in the ensuing results. A standard and optimized manufacturing procedure is followed to prepare the kits. Some kits are taken out from the production assembly line and examined for variations in results. Additional serum samples were tested with the kit, besides the calibrator (both Ref−ve and Ref+ve) samples provided in the kit. We use the malaria antibody detection ELISA as an example to report possible sources of assay variation and highlight how they can be minimized.

EXPERIMENTAL

Reagents and Buffers

The buffer salts required to prepare phosphate buffered saline were BDH Analar (BDH Poole, England; Merck Pty Ltd Kilsyth Victoria, Australia). Commercially available high quality water (Baxter water for irrigation AHF 7114, Baxter Healthcare Pty Ltd, New South Wales, Australia) was used to prepare the buffers for the assay.

Antibody Detection ELISA

An indirect ELISA assay was developed to detect malaria-specific antibodies in blood samples. This assay was essentially based on the microplate method originally described by Voller et al.[15,16] The anti-

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body-test kit contained plates coated with *Plasmodium*-specific antigen and other reagents required for running the ELISA assay (see Table 1). These kits were manufactured under GMP conditions with accredited SOPs and having followed established manufacturing procedures (SMPs). A package insert was available with a brief description on the antibody-detection ELISA assay, along with the step-down procedures for performing the assay. It is expected to obtain at least $0.800_{\text{absorbance } 450/620\,\text{nm}}$ for Ref+ve and $<0.22_{\text{absorbance } 450/620\,\text{nm}}$ for Ref−ve sample. Some guidance was available for calculating COV based on the Mean + 3SD value of the Ref−ve sample absorbance.

Assay Performance

The main objective was to examine any significant variation arising in the assay due to the assay material supplied in the test kit and, also, due to the way operators perform the assay. The malaria antibody-detection ELISA was performed based on the standardized step-wise protocol. In short, antigen-coated wells (96 well plates, Greiner Labortechnik, Frickenhausen, Germany) were reacted with diluted serum samples for 1 h at 37°C. Unbound serum components were removed by washing wells with PBS/T (automated wash protocol; Denley Well Wash 04, England) and wells were reacted with monoclonal antibody conjugate (anti-human IgG-HRP) for 1 h at 37°C. After removing unbound conjugate components, the wells were reacted with the substrate, TMB, and colour development allowed for 15 min before the addition of a stop solution. Absorbance readings were taken (450/620 nm) by using a plate reader (Anthos LabTec Instruments 2001). This standard assay protocol was followed in all experiments without any deviation. A total of $n=6$ kits were used in this assay and the assay components are detailed in Table 1. Two operators, without prior knowledge of the assay, performed the assays independently and/or helped the co-operator in performing the assay. The two new operators were used to mimic the situation of the end-user under field conditions. There was some advantage with the plates as the antigen-coated and blocked wells were breakable from the strips in the original plate. According to need and requirement, the wells were remounted onto another frame and assay performed on randomly selected wells. With this, depending upon the assay protocol, Ag-coated wells from any part of the plate were randomly taken out of the original plate and re-fixed onto a new frame and the assay was performed. By this procedure, one can test the possible assay variation in any one of 96-wells in a plate. In addition to Ref+ve and

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Table 1. Assay components and reagents in the malaria antibody detection ELISA test kits.

Total number of kits	$n = 6$
Antigen-coated plates	12 plates
Wash solution (20× strength)	6 bottles
Ref+ve calibrator (strength, neat)	6 vials
Ref−ve calibrator (strength, neat)	6 vials
Conjugate (strength 100×)	6 vials
Substrate and buffers	6 vials each

Note: All kits were marked with a 6 months expiration date and were used within the expiration period. All kits were intact when used.

Ref−ve samples included in the kit, additional reference serum samples were also used for monitoring any assay variations. The assay protocols are detailed under the Results section.

Data Analyses

Replicate tests performed in each assay are detailed under Results. The raw ELISA data (i.e., OD reading at 450/620 nm) from each assay was analyzed by calculating the mean, standard deviation, coefficient variation ($\%CV = SD/mean \times 100$) and signal/noise (S/N) ratio[10,14,17] using scientific calculators and the Microsoft Excel software program. Data were analyzed for statistical significance by using a "two-tailed unpaired T-test" and a "two sample F-test for variances." Assay sensitivity was determined essentially based on S/N ratio, which indicates the higher the ratio, the better the sensitivity. The S/N ratio was calculated from differential absorbance values of test or Ref+ve sera by the Ref−ve sample values.

$$S/N = \frac{\text{OD of test or Ref+ ve sera}}{\text{OD of Ref−ve sera}}.$$

This invariably showed the specificity of the assay as the control (Ref−ve) values were used as the baseline figures. A standard graph has no relevance in indirect antibody ELISA; only the reciprocal serum dilution provides a guide for establishing the titre of antibody present in a given test sample.[13] Accuracy of absorbance was obtained by comparing the absorbance value of one test with that obtained in another test. In

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practical terms, the absorbance raw data were compared between the intra- and inter-assays. Precision refers to how readings from different assays agree with each other in terms of the mean and how individual absorbance values deviate from the mean (i.e., standard deviation). This is essentially reflected in the coefficient of variation. In other words, the percentage coefficient variation (%CV) was considered for the assay precision. The values were plotted and compared using the Microsoft Excel spreadsheet program.

RESULTS

Chequer-board Analyses

The ELISA test involved several steps, as indicated in Fig. 1. A chequer-board assay was performed to determine the optimum concentration of analytes used in indirect ELISA. Based on these

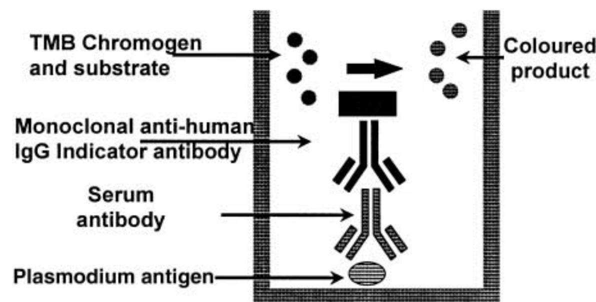

Figure 1. Schematic picture of malaria antibody detection based on the concept of indirect ELISA. In short, the malaria antigens are immobilized on the inner surface of the reactant wells in a 96-well plate. After blocking, the wells are reacted with analyte sera (Ref−ve, Ref+ve calibrator samples; unknown test) samples. If antibody molecules specific to malaria antigens are present in the analyte, then, they bind to the immobilized antigens on the inner surface of solid-phase wells. Unbound serum components are removed by washing the wells with PBS/T. Wells are reacted with an optimal concentration of anti-human IgG-HRP conjugate that binds to IgG antibody molecules. After this, the substrate TMB added which reacts with the enzyme molecules present on the second antibody. The color product is stabilized by addition of a stop solution and readings taken at 450/620 nm. The intensity of color is reciprocal to the amount of antibodies bound to the malaria antigens. With this a qualitative concentration of antibody present in unknown samples can be detected.

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experiments, each well in a 96 well plate was coated with a known amount of antigen, blocked, and the wells were air-dried and sealed in an aluminum foil bag containing silica. The required concentration of serum was also investigated and suggested in the assay protocol. Ref−ve and Ref+ve calibrators were included as reference standards for running the assay. A 100× strength conjugate was developed, based on the chequer-board analysis and included as an assay component in the test-kit. This conjugate was shown to generate the lowest absorbance (< 0.1 $OD_{450/620\,nm}$) (i.e., minimum background) reading in control wells when control serum diluted 1:100 in the sample buffer was used in the assay. The required concentration of TMB for color developed was also developed as 20× strength. A stop solution was supplied to stabilize the color for obtaining consistent absorbance readings.

Assay Parameters for Distinguishing Negative vs. Positive Samples

The main objective was to demonstrate the discriminatory ability of the indirect ELISA in recognising negative vs. positive samples. Four serum samples, diluted 1:100 to 1:12,800 in sample buffer were assayed by following the assay protocol. The plates were read at 450/620 nm and the OD readings were plotted as a line-graph (Fig. 2). The Ref−ve calibrator generated low absorbance readings, as opposed to the higher readings with the Ref+ve calibrator. The serum samples (M1 and M3), from individuals infected with *P. falciparum* during a visit to the malaria endemic country, showed OD readings above the Ref−ve calibrator. A clear discriminatory ability existed in the test between positive and negative samples.

Test Kits and Their Components Used

Altogether, six prototype kits were used in this evaluation. Table 1 shows specific detail of kit components.

Determination of Cut-off Value

The Ref−ve calibrator, which tested negative for hepatitis B and HIV, was used in 1:100 dilution in randomly selected 48 replicate antigen-coated wells from 6 plates (Table 1). Reactivity varied from

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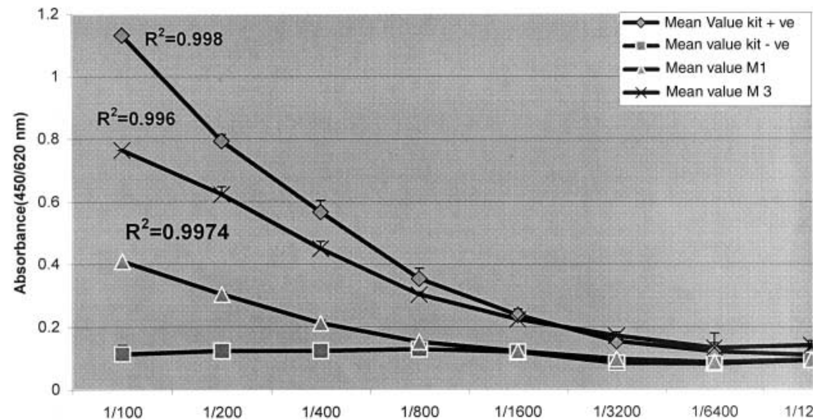

Figure 2. Reactivity of defined sera samples in Malaria IgG-ELISA. Serum samples were diluted in sample buffer from 1:100 to 1:12,800 dilutions and reacted in antigen coated wells for 1 h at 37°C. After washing out unbound serum components, the wells were reacted with anti-human monoclonal IgG-HRP conjugate for 1 h at 37°C, unbound conjugate was washed and the substrate, TMB, was added to initiate the color development for 15 min at RT. The plates were read at 450/620 nm absorbance after stopping the color reaction.

0.064 to 0.132, with a mean value of 0.09 and SD 0.015. Based on this, a mean cutoff value with 3 SD was (mean + 3SD) determined to be 0.135 $OD_{450/620\,nm}$. This COV was used as the basis for determining the negativity of a serum sample.

Titration of Ref + ve Calibrator

All 96 wells in a microtitre plate were coated manually with antigen by using a multichannel pipette; they were subsequently blocked and each plate was preserved in a sealed aluminum foil with silica gel. It is necessary to check any variability occurring in wells due to manual coating. The required number of antigen coated wells was randomly selected from 6 kits and arranged columnwise on a fresh frame. Altogether, $n = 6$ wells received Ref+ve calibrator sera in serial dilutions from 1:100 to 1:6,400. Other steps of ELISA were performed. Data in Fig. 3A show the reactivity absorbance of Ref+ve calibrator at each dilution in wells assembled from six different ELISA plates. When reactivity absorbance was compared, 3 sets of absorbances (A1 to A3

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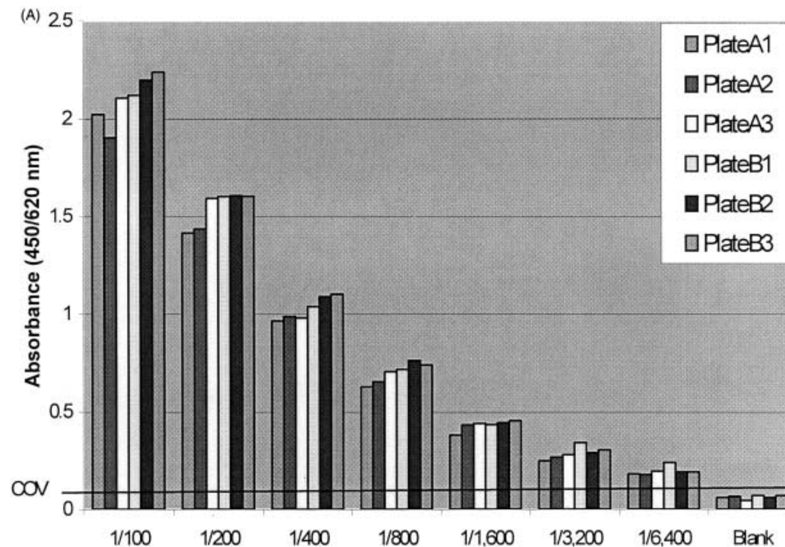

Figure 3. (A) The absorbance of Ref+ve calibrator reciprocally diluted in replicated antigen-coated wells from two batches of plates. The serum was diluted 1:100 in buffer diluent, transferred 100 μL/well, incubated for 1 h at 37°C, and unbound serum components washed. A diluted anti-human IgG-HRP conjugate was added to the wells and incubated 1 h at 37°C, and the unbound conjugate removed by serial washings. A substrate TMB was added to develop the color for 15 min. The color intensity is reciprocal to the amount of antibody bound to antigen in each well. The wells were read at 450/620 nm and the data plotted as a bar chart; (B) The spatial presentation of optical density readings of different dilutions of Ref+ve calibrator. Other specifications as in Fig 3A.

vs. B1 to B3) did not differ significantly (F-test) at each dilution, indicating no variation existed between the antigen wells within the two batches of plates. A cut-off value at 0.135 was inserted to identify the end-point activity. Based on this tool, the Ref+ve calibrator showed a COV at 1:6,400 dilution, suggesting that any unknown sera can be detected as "positive" in this assay, even though the antibody titre of that sample could be 32× less than the Ref+ve calibrator. This range of sensitivity is useful for testing unknown sera. The SD and %CV at each dilution are shown in Table 2. The SD was highest at 1:100 dilution, i.e., nearly 20× less than the mean value (SD of 0.111 for a mean value of OD 2.098) which is insignificant. The CV values were <10% throughout the plate. The data are also presented in the spatial-field format (Fig. 3B) to show the relative spread of optical density readings.

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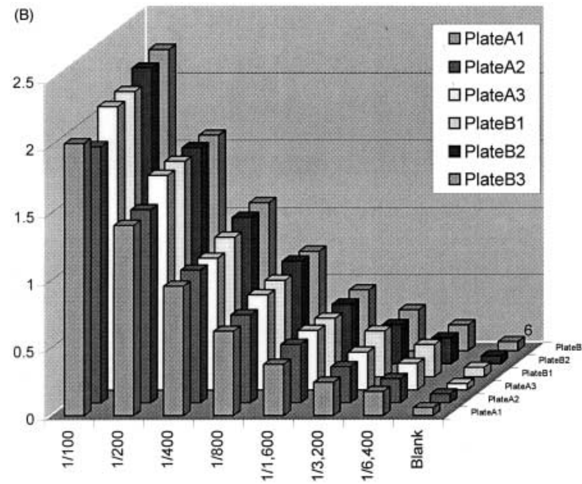

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Table 2. Reactivity of Ref+ve calibrator in ELISA when used in varied dilutions in replicate wells used from two batches of antigen-coated plates (A and B).

Dilution of ref+ve calibrator	Absorbance (450/620 nm) range	Mean of replicates	SD	%CV	Statistic analysis of replicate data from plates A and B
1:100	1.903–2.23	2.098	0.111	5.29	$P > 0.05$ NS
1:200	1.414–1.6	1.542	0.083	5.38	$P > 0.05$ NS
1:400	0.964–1.1	1.026	0.053	5.16	$P > 0.05$ NS
1:800	0.628–0.760	0.702	0.047	6.69	$P > 0.05$ NS
1:1600	0.381–0.45	0.431	0.024	5.57	$P > 0.05$ NS
1:3200	0.249–0.340	0.289	0.029	10.03	$P > 0.05$ NS
1:6400	0.178–0.230	0.196	0.020	10.20	$P > 0.05$ NS

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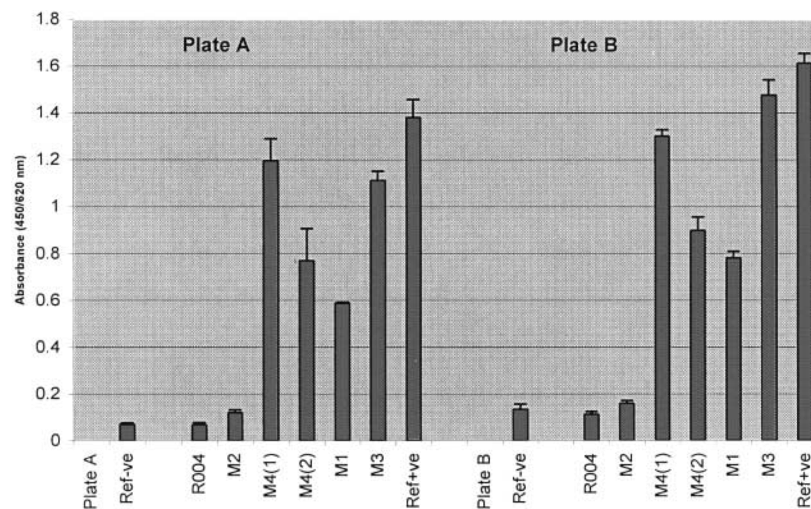

Figure 4. A set of $n=8$ serum samples were tested at $n=12$ replicates in each plate by two independent operators. Mean $\pm$ SD was calculated and the data plotted. In the two tailed unpaired T-test, the absorbance values were not significantly different ($P>0.05$).

Intra-assay Variation in Large Replicates

This assay was performed in two plates (plates A and B); $n=8$ serum samples were assayed, across the row, in 12 replicate wells in each plate, following the assay procedure as per the package insert. In addition to the Ref+ve and Ref−ve calibrator samples, six additional serum samples were used in the assay. Relative optical absorbance was determined by reading the plate with a set up of dual filters (450/620 nm). Altogether, data were available from 24 replicate wells for each serum sample. Data from one column was excluded due to cross contamination while transferring samples. With this, 12 and 11 replicate values (raw data) were available for each plate, respectively. Mean + SD values were determined from the raw data and were plotted as histograms (Fig. 4). Except for one serum sample (M4-2°), the CV values of other samples remained less than 10%. Upon a close look into the raw data of M4-2°, it revealed that the value 0.768 ± 0.138 (mean + SD) due to one of the edge wells showed an OD of 0.682, whereas the data in all other wells were fairly consistent. When this edge well data was excluded, the mean + SD of the remaining wells ($n=10$) were 0.821 ± 0.053 with %CV of 6.5. Here, the variation can be ascribed to its placement in the ELISA plate.

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All other wells showed consistent data. On the whole, no significant variation occurred within (i.e., intra-assay) the assay when a large number of replicate wells was included.

Intra-assay Variation in Small Replicates

The above experiment has shown that a large number of replicate wells within a plate generated no significant variations in the reactivity absorbance. Here, the objective was to examine (a) the variation in the antigen coated wells (plates A and B) and (b) the variation occurring due to the working style of the operators (Operator-1 and -2). We divided each antigen-coated plate (96 wells) into 8 equal areas (designated the plate areas) of 12 wells each for testing $n = 4$ serum samples in triplicate. In total, two antigen coated plates (batch A and B) containing 16 plate areas were used in this experiment. Wells from 4 plate areas (i.e., 48 wells from each plate) were randomly transferred from the original frame into a new frame (designated assay plate) so that the wells were intermixed from two batches in each assay plate (*As each antigen-coated well is breakable from the original strip and can be fitted into a new frame*, *the random assembly of wells from different parts of the plate formed the basis for testing the inter-assay variation*.). Each operator (Operator-1 and -2) has used one assay plate in blind containing plate areas from two batches of plates. Four serum samples, viz., Ref−ve, Ref+ve, M3, and M1, were used in this assay. Each serum was diluted 1:100 by individual operators and has been tested in 8 plate areas in triplicate and all steps of the ELISA test have been performed independently.

Absorbances were analyzed in two ways: (a) based on the plate areas and (b) based on the individual operators. Mean, SD, and %CV values of the replicate $n = 24$ well readings of each serum sample in $n = 8$ plate areas of each plate were analyzed. Figure 5A shows a plot of the mean $\pm$ SD absorbance analyzed on the basis of plate areas and on the basis of operators. No significant differences were observed with the analysis on two sets of mean $\pm$ SD absorbance data from Plate A vs. B and from Operators 1 and 2. They agreed with each other.

Assay precision was examined between the plates and the operators. The %CV data is presented in Table 3 and Fig 5B. When data were analyzed based on the plate, %CV were within acceptable level, indicating that two batches of plates were with antigen-coated wells with no significant variation. However, Plate B has shown the lowest %CV with the M3 sample and almost similar %CVs with other samples. The Ref−ve sample showed a high level of %CVs in both plates A and B.

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Table 3. Assay precision by analyses based on the batches of antigen coated wells and the working style of the operators in blind: The %CV values obtained by the analysis of replicate data are tabulated below.

	Analysis based on two plates			Analysis by two operators		
Sera	No. of replicates	%CV values Plate A	%CV values Plate B	No. of replicates	%CV values Operator 1	%CV values Operator 2
Ref−ve	Used 24 wells in each plate	15.9%	12.9%	Performed $n = 24$ replicate assays in each plate	16.9%	16.9%
M1	Used 24 wells in each plate	9.6%	10.6%	Performed $n = 24$ replicate assays in each plate	9.2%	11.1%
M3	Used 24 wells in each plate	10.5%	7.7%	Performed $n = 24$ replicate assays in each plate	12.2%	8.1%
Ref+ve	Used 24 wells in each plate	10.5%	11.0%	Performed $n = 24$ replicate assays in each plate	8.5%	13.1%

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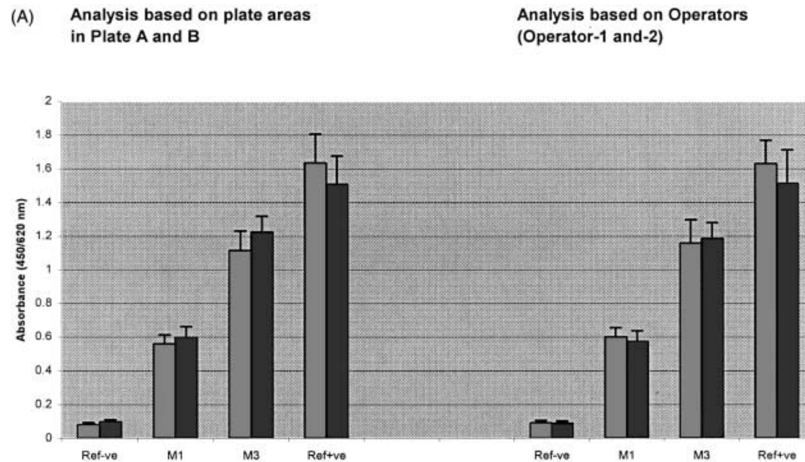

Figure 5. (A) Four serum samples (Ref−ve, M1, M3, and Ref+ve) were tested each in 24 replicate wells in 8 plate areas with two operators (Operator-1 and Operator-2). Mean ± SD absorbance readings are plotted. The paired data plots are not significantly different from each other (F-test $P > 0.05$); (B) Percentage coefficient variation seen when $n = 4$ samples were tested in 24 replicate wells in each plate (Plate A and Plate B) and by two operators (Operator-1 and -2). The range of %CV remained within 15% in all samples except in Ref−ve sample where a higher %CV is seen due to its basal low OD readings. Even a small deviation resulted in a dispropotionately higher %CV. Both plates A & B indicated %CV within a close range, whereas the operators differed from each other, especially with Ref+ve sample and M3 sample, where antibody titre was high.

(*continued*)

Some variations were apparent with %CV of individual operators. The %CV values of individual operators were, however, within the acceptable level of 15%, except with Ref−ve serum samples where both operators have generated higher %CV (i.e., 16.9%). This may be due to the lower OD values generated by the Ref−ve serum samples and the same higher %CVs were also seen when analyses were done in a plate-wise manner.

Operator-1 generated lower %CV values with M1 and Ref+ve samples, whereas slight higher values with M3 sera sample. Operator-2 had generated higher %CV values with M1 and Ref+ve sera and slightly lower with the M3 sample. This shows that Operator-2 had some imprecision while performing the assay that fell within the limits. Careful examination of the operator's style indicated that wells used by the Operator-2 had generated slightly higher SDs in the replicate wells,

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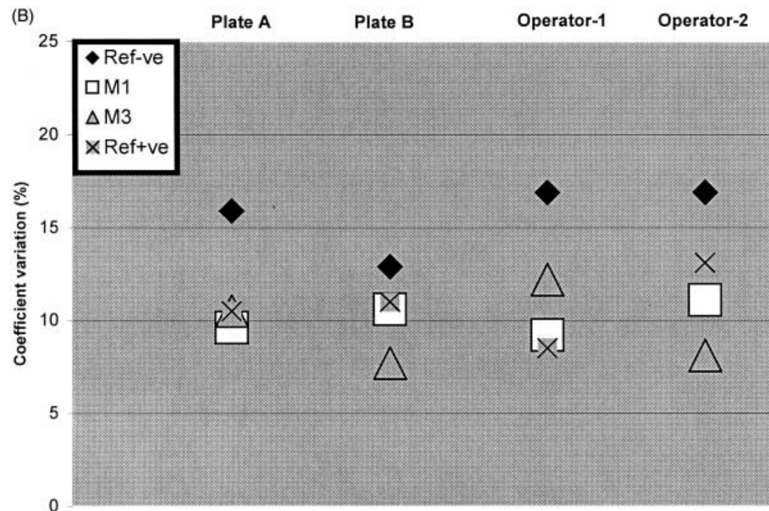

Figure 5. Continued.

confirming some degree of imprecision. As reagents are dispensed through a multi-channel pipette, a minimal variation was expected. Among the two operators, variation was observed in plates operated by one of operators. No such variation was apparent in the comparative areas operated by another operator and also in other triplicate wells by the same operator. In short, the variation, although observed, is not due to the reagents supplied in the kit but was caused due to a particular operator. It was perhaps a dispensing error, but this variation was not due to inconsistency in antigen immobilization on solid surface nor the reactivity of serum samples, as reflected by the results of the other 7 areas where such variation was not observed. It appeared that, in one of the steps, some error occurred while dispensing the analytes.

Inter-assay Variation with Single-, Duplicate-, and Triplicate-Well ELISA

The above experiment showed no significant variation with the antigen-coated plates. However, some variations occurred in the assay due to the working style of individual operators. In this experiment, we examined the possible variations in the assay performed in single vs. replicate wells. Individual well readings in 1-well, 2-well, and 3-well

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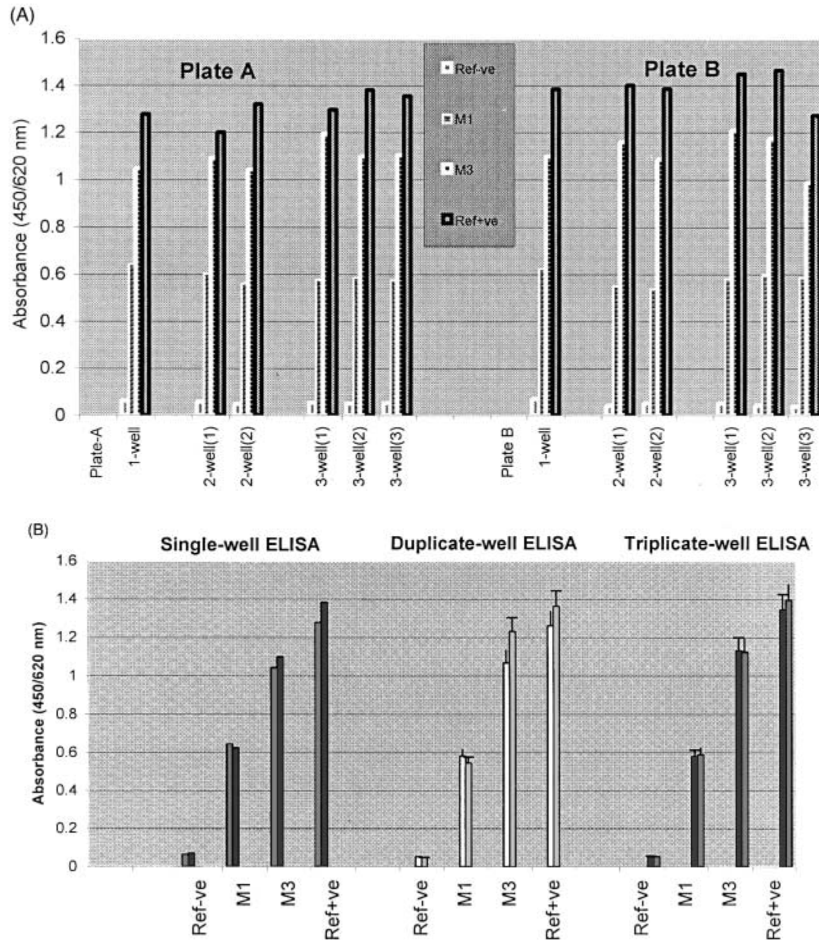

Figure 6. (A) Individual well reading of four serum samples in 1-well, 2-well, and 3-well ELISAs. Ref+ve sample showed the lowest reading in 2-well ELISA in Plate A and in 3-well ELISA in Plate B. M3 sample showed lowest reading in 3-well ELISA. No appreciable variation was seen with M1 and Ref−ve samples; (B) Four serum samples (Ref−ve, M1, M3, and Ref+ve) were tested in single-well, duplicate-well, and triplicate-well ELISAs by using antigen-coated wells from plates A & B.

ELISAs are shown in Fig 6A. Some variations in readings were observed in Ref+ve (2-well in Plate A and 3-well in Plate B) and M3 (3-well, Plate B). There were no significant differences seen between data of Plate A and B apart from the M3 sample in triplicate-well

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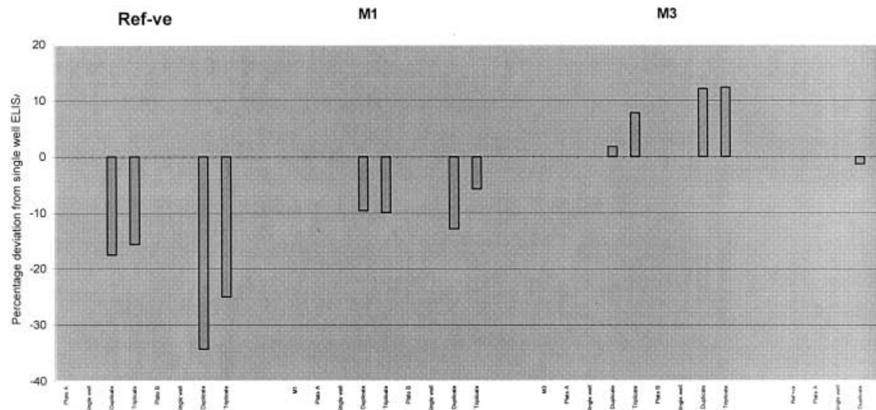

Figure 7. Four serum samples (Ref−ve, M1, M3, and Ref+ve) were tested in single, 2-well, and 3-well ELISA assays. The absorbance of single-well is compared with the mean absorbance of 2-well and 3-well assays. The percent deviations seen with 2-well and 3-well assays are plotted by taking the absorbance of single-well assay as reference (0 on the *x*-axis). Results showed that a negative deviation is applicable to samples containing a low antibody titre (M1 or Ref−ve), whereas with a positive deviation is seen with the sample containing a high antibody titre (M3). No significant variation is seen between the plates ($P > 0.05$).

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ELISA (F-test $P > 0.05$). It is interesting to see why and where M3 data differed.

One of the triplicate wells in Plate B showed a lowest OD of 0.987. When data from this particular well was excluded from the analysis, the plates A and B data were not different ($P > 0.05$, F-test). The lowered reactivity in that particular well may be due to the operator error in transferring an accurate volume of reactant to this particular well. The matching mean ± data are plotted in Fig 6B.

Altogether, $n = 6$ replicate data were available from each sample and from each plate. The mean absorbance of single well assay was considered as the reference and any deviations occurring in duplicate and triplicate well assays were examined. The percentage deviations with duplicate and triplicate well assays shown in Fig. 7 indicate that the deviation is negatively driven with the sample containing low level or no antibody, whereas a positive deviation is seen with the sample having a high antibody titre. Further analysis was done based on the calculation of S/N ratio. Figure 8 shows the S/N ratio calculated for assays performed with single, duplicate, and triplicate wells. Data were not significantly different between the plates and between the matching samples.

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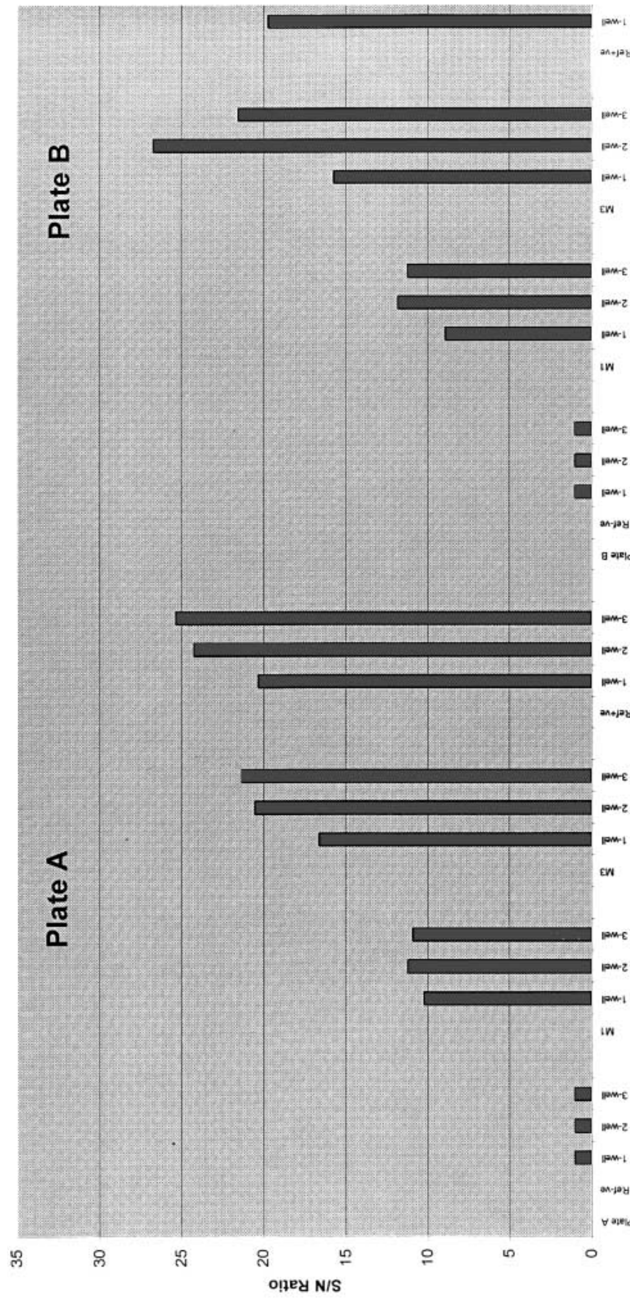

Figure 8. S/N ratio of sera (M1, M3, Ref+ve) samples resulted during 1-well, 2-well, and 3-well ELISA from plates A & B. Matching data of individual serum samples as a whole series of data from plates A & B did not differ significantly (F-test, $P > 0.05$).

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However, there was some positive trend: the samples with high antibody titre tend to show higher S/N ratios in replicate wells. When the %CV were considered, Plate B showed higher %CV than the triplicate well data of Plate A. This observation confirms earlier discrepant data with some wells in Plate B. Plate A provided data with high precision. In other words, both triplicate well assays and duplicate well assays were within the limits (< 10%).

DISCUSSION

The ELISA test, being a sensitive tool, is ideal for detection of very low levels of specific analytes in clinical samples. Based on the detection sensitivity, the ELISA test, *per se*, is valuable for making a decision on clinical samples for determining appropriate patient treatment. Therefore, the ELISA results should be accurate and reliable.[18] Several factors regulate the outcome of an assay.[9] Our evaluation has shown that its outcome is entirely dependent upon the reagents used in the test-kit as well as on the assay performed by the operators. It is, therefore, important to provide validated reagents in the test kit. Reagent validation is, in turn, based on its quality and its optimal concentration as worked out in a chequer-board analysis. In addition to this, stability of reagents is also an important criterion for obtaining consistent results. In other words, assay result is dependent upon several factors and, therefore, should be checked for any error, variation, inconsistency, and for lack of repeatability.

We designed our study by using multiple number of kits randomly selected from the production line, and involved two operators to perform the assay. Results are analysed to single out any error in the assay due to the kit reagents or due to the two operators.

Assay Kit Components and Variation

None of the assay components showed any valid variations in intra- and inter-assays. No significant variations are accounted due to antigen-well plate, Ref−ve and Ref+ve calibrators, conjugate, substrate, and stop solution. Besides the reference calibrators, we have also examined $n = 6$ additional sera samples. No significant variation is seen with the kit components.

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Operator Generated Assay Variation

The kits have been manufactured by experienced production staff, tested by QC analysis, and then used in this evaluation. The two operators have evaluated kits and served as an alternative to end-users of the kit. Our analysis has indicated that operators can introduce variations. Most of the errors seen in this assay are of ''random'' type than ''systemic'' ones.[19] Random errors cause the absorbance readings to scatter about a mean value, assuming the assay system to be ''unbiased.'' The extent to which results scattered around the mean is represented by a low level of SD and lower percentage of CV of replicate measurements in double-well ELISAs than the triplicate-well ELISAs or large replicate ELISAs.[19] This reflects on the ''precision'' of the assay.

Assay Parameters Influence Specific Reactivity

Any non-specific binding of reactants should be avoided in the assay. Here, we did this by developing appropriate assay conditions. We observed minimal binding of the Ref−ve calibrator in ELISA wells coated with *Plasmodium* antigens. Non-specific binding of other reactants was minimal in the assay. For example, if any non-specific serum components bind to the immobilized antigen, then there is a possibility that the conjugate also binds non-specifically. Therefore, antigen used for coating should be free from any non-specific components and should be specific to the targeted antibody in the clinical sera. Also, the indicator enzyme-conjugate (i.e., anti-human IgG-HRP) should bind specifically to the immobilized antibody + antigen complex. If any of these reactions leads to non-specific binding, then it reduces the assay sensitivity. In the present assay, the Ref−ve calibrator sample has shown a low absorbance, clearly indicating absence of any irrelevant or non-specific reactivity. On the other hand, the Ref+ve calibrator has shown a higher absorbance, indicating a specific binding occurred in this test. In other words, it reflected upon the quality of the kit and its components.

If non-specific reactivity is minimal, then a clear differentiation between signal (reactivity of Ref+ve calibrator or unknown test sample) and noise (reactivity of Ref−ve calibrator) would be apparent, which invariably enhances the assay sensitivity. An optimal condition, therefore, has to be set for obtaining a higher S/N ratio.[14] An ELISA test should consider these points and evaluate reagents and assay components for obtaining high S/N ratio. The COV is determined and used to distinguish a sample as negative or positive. Although ELISA

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wells are coated manually, no significant variation occurred between the plates tested in the intra-and inter-assay variation experiments. In an assay intended for field use, the kit should consist of validated assay reagents along with a recommendation to use the test sera in appropriate dilutions for obtaining sensitive results. By using such validated assay reagents, the test kit would provide reliable results.

Assay Sensitivity

Four samples are titrated in this assay, which showed different antibody titres and Ref−ve being minimal. The Ref+ve calibrator reacted more strongly than the M3 and M1 samples. This established that the assay reagents, such as diluent, conjugate, substrate, and stop solution, are found to be optimal for obtaining differential results. A clear difference between a negative vs. a positive sample occurred at 1:400 dilution. Subsequently, the clinical samples formed two distinct patterns, i.e., sample M3 and Ref+ve in one band and M1 and Ref−ve in another. This showed that the samples at 1:400 dilution provided a distinct difference in the antibody titre. The endpoint is determined to be at 1:3200 dilution.

In another experiment, when the Ref+ve calibrator is titrated in wells from $n = 6$ plates, data once again confirmed the consistency of antigen-coated wells. Moreover, this data reflected on the sensitivity of the malaria antibody test kit. Based on this, any unknown/test (clinical) sample can be tested positive even though the sample may contain antibody 32× less than the level seen in Ref+ve calibrator. Such a level of sensitivity is suitable for field use.[13]

Assay Precision

Assay precision is essentially based on the %CV. In a sensitive assay such as ELISA, even a small amount of fluctuation in the reactant volume may result with variable absorbance in replicate wells. This invariably reflects on the assay precision. Routine variations among ELISA results are usually in the 5–20% range.[3] The Ref−ve calibrator serum showed no significant variation in intra- and inter-assay experiments. However, it showed high %CV because minor fluctuations with lower absorbency appeared exaggerated. Higher %CV with Ref−ve calibrator is, therefore, negligible due to absence of antibody.

No antibody detection system is perfect in providing absolutely error-free results. However, test kits should provide reliable results for

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making decisions on a patient's clinical condition. Here, we used a limited number of control samples as reference predictors. These predictor samples are shown to be crucial in judging the results for reliability, consistency, and precision. All our analyses indicated no inherent problems with kit reagents; however, assay variation occurred due to operator's working style. The operator related error has to be ruled out by the end-user before questioning the performance of a kit. Here, our test kit has withstood the assessment and found to be reliable in its performance. This kit is expected to provide reliable results under field conditions. With these quality parameters assessed, the generic antibody detection test-kit analyzed here is found to be suitable for a prospective field study with large number of samples.

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