Original Article

Implementation of internal quality control program for monitoring of enzyme-linked immunosorbent assay performance at a blood center

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Abstract:

BACKGROUND: Internal quality control (IQC) samples may be incorporated in enzyme-linked immunosorbent assay (ELISA) routinely for detection of errors occurring due to change in environmental conditions, test system, or operator performance. We have described methodology for preparation of IQC samples, monitoring of results using Levey–Jennings (LJ) charts and their interpretation. We have also described our experience of quality control in ELISA using IQC samples, identification of errors and corrections applied.

MATERIALS AND METHODS: IQC samples for anti-HIV, hepatitis B surface antigen (HBsAg), and anti-HCV ELISA were prepared "*in-house*" using standard methodology. After validation of run, E-ratio of IQC sample was calculated and plotted on LJ chart. Further interpretation was done to detect the errors. LJ charts illustrating the performance of IQC samples on 180 runs for each ELISA were drawn and analyzed.

RESULTS: For anti-HIV ELISA, violation of warning rule was found in 2 runs (1.11%). Only one run (0.55%) was rejected due to violation of rejection rule. For HBsAg ELISA, violation of warning rule was indicated in two runs (1.11%). Two runs (1.11%) were rejected due to violation of rejection rules. For anti-HCV ELISA, violation of warning rule was indicated in two runs (1.11%), whereas two runs were rejected due to violation of rejection rules. Comprehensive checks were performed for the evaluation of equipment calibration, handling, and storage temperature of reagents and operator's technique. A thorough investigation was undertaken according to the type of error.

CONCLUSION: Inclusion of IQC with each ELISA run is valuable to check the assay performance, ensuring reliability and reproducibility of test results.

Keywords:

Enzyme-linked immunosorbent assay, E-ratio, internal quality control, Levey–Jennings charts

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Introduction

Inclusion of quality control measures in a testing laboratory facilitates validation of test results in terms of accuracy and precision. Monitoring day-to-day performance of assays increases the probability of detecting the deviations at the earliest. Quality control of assay may be performed using control sera supplied with the kit, known standard sera available through national and international agencies or pools of sera prepared "*in-house.*" Till date, most transfusion-transmitted infections (TTI) testing laboratories rely solely on commercial kit controls provided with the kit for test validation. These controls have a high positive value and provide only single-point calibration.[1] They are incapable of monitoring batch to batch variation in test kits or gradual faltering of equipment or deterioration of test reagents.

Internal quality control (IQC) using "*in-house*" samples of borderline positivity offers a low cost and flexible option for the objective evaluation of test procedure on a day-–-to-day basis. The purpose is to (a) detect immediate errors occurring due to change in environmental conditions, test system or operator performance; (b) monitor the test performance over time, influenced by variance in environmental conditions, test system, or operator performance.[2]

IQC samples detect errors which may be systematic or random. Systematic errors indicate a change in accuracy or stability of assay whereas random errors point to decreased precision. The identification and analysis of the errors which occur in different phases and components of testing process help in establishing and implementing the trouble shooting and corrective action protocol.

In this article, we have described the methodology for preparation of IQC samples, monitoring of results using Levey–Jennings (LJ) charts and interpretation through the application of rules for interpretation of LJ charts. We have also described our experience of quality control in enzyme-linked immunosorbent assay (ELISA) using IQC samples, identification of errors and the corrections applied.

Materials and Methods

This study was based on the analysis of results of IQC samples on routine ELISA for TTI testing, performed from January 2017 to December 2018, after obtaining approval from institute ethics committee. ELISA for anti-HIV (anti-HIV 1 and 2 Microlisa, J. Mitra and Co. Pvt Ltd., New Delhi India), HBsAg (HBsAg Hepalisa, J. Mitra and Co. Pvt Ltd., New Delhi India), and anti-HCV (anti-HCV Microlisa, J. Mitra and Co. Pvt Ltd., New Delhi, India) screening of donor blood samples was performed as per the manufacturer's guidelines. One IQC sample for the corresponding marker was tested in each run similar to any other test sample. After validation of run, E-ratio of IQC sample was calculated and plotted on LJ chart. Further interpretation was done using Westgard (WG) rules.

1. Preparation of IQC samples: The preparation for all IQC samples was based on standard methodology described for HIV ELISA in *Manual for Quality standards for HIV testing laboratories by NACO India*.[3] Positive IQC samples for each marker were prepared for the kit lot in use (Lot 1), and it was repeated after 1 year (Lot 2). The samples had borderline reactivity and hence were capable of detecting even minor errors in assay performance. For preparation, donor sample reactive for test marker (anti-HIV, hepatitis B surface antigen [HBsAg], or anti-HCV)

and nonreactive for other TTI markers was repeat tested with ELISA from alternate manufacturer for the corresponding markers (Erba Sure HIV, Transasia Biomedical Ltd., Daman, India; Erba Lisa Sen HBsAg, Transasia Biomedical Ltd. Daman, India; Erba Lisa Hepatitis C, Transasia Biomedical Ltd. Daman, India). Sample showing E-ratio of 5–10 on both ELISA was selected. Plasma from blood unit was separated, heat inactivated at 56°C for 30 min and then re-calcified to yield serum. It was serially diluted with normal serum and all the dilutions were further tested on routine ELISA considering each dilution as a separate sample. The dilution which had an E-ratio of 1.5–2.0 was chosen to prepare the IQC sample. The reactive serum was diluted to this value with normal serum. It was divided into 25 different aliquots of 1 ml and numbered serially. Sample from each aliquot was run on ELISA for corresponding marker. E-Ratio of each aliquot was calculated and CV was determined to rule out inter-aliquot variation. If CV of the run was <10%, the run was accepted. Outliers were excluded by analysis of data box plot on SPSS software (IBM SPSS STATISTICS Version 23.0.0, Chicago, IL). Acceptable sample aliquots were labeled with date of testing and ELISA kit details. They were stored at −40°C for 1 year. A new aliquot of control was used every week. After thawing, it was stored in a refrigerator between 2°C and 8°C for further use. This positive IQC sample was dispensed in the end row of the plate randomly. In addition, one nonreactive sample with E-ratio <0.8 was dispensed as negative control in the first sample well after the kit controls. Run was accepted if this control tested nonreactive. We have not described this control in data analysis.

Calculations:

E-Ratio = Optical density (OD) of test or control sample/cut off OD

Mean (M) is the arithmetic average of all E-ratios. It is a measure of central tendency.

M = Sum of individual E-ratios (E1 + E2 + E3…. +En)/no. of aliquots (n)

Deviation (D) for each aliquot is the difference between individual E-ratio and the mean E-ratio.

D1 = E1 − M, D2 = E2 − M,………… Dn = En − M

Standard deviation (SD) is a measure of dispersion of observations about mean. It was calculated by summing up all the deviations, then squaring the sum. This was divided by n. The square root of this value yielded SD.

$$SD = \sqrt{(D1 + D2 + D3\ldots + Dn)^2 / n}$$

Control values were calculated mean ± 1SD, mean ± 2SD, and mean ± 3SD.

The coefficient of variation (CV) was expressed percentage and calculated as:

$$CV = SD \times 100 / M$$

It is a measure of consistency and values <10% indicate minimum inter-aliquot variation and hence suitable for quality control of daily run.

2. Plotting of LJ charts:[4]
   LJ charts were plotted to graphically monitor if the control values were falling within the range. Mean was marked on Y axis as a horizontal line. Control limits were marked at appropriate intervals as ± 1SD, ±2SD and ± 3SD. Runs were plotted on X axis. Each day the values of control E-ratio of assay were marked against the run. Each chart was plotted for 30 runs. Charts were labeled with kit details (name, lot number, and expiry date), equipment used (ELISA washer and reader), and operator name. Any modification in the above details was promptly recorded on the chart.
3. The use of statistical methods for interpretation: LJ chart interpretation rules[5] with some modifications [Table 1] were applied to detect the errors.[6] As there are no previous studies to the best of our knowledge on quality control of ELISA for TTI testing in a blood bank, rules were applied so as to maximize the probability of error detection and at the same time to minimize the possibility of false rejection of ELISA. Incorporation of shift and trend was done as per the NACO guidelines for quality control of HIV ELISA.[3]

All the calculations were done manually. Charts were drawn and plotting was done using SPSS Software (IBM SPSS STATISTICS Version 23.0.0, Chicago, IL). Daily analysis of result was done to determine any systematic or random errors. If any rejection rule was violated, it was marked on the chart and the run was rejected. Subsequently, a thorough investigation was undertaken according to the type of error, as described in Table 2.

[7] The IQC sample aliquot in use was discarded and a new vial was used for subsequent runs. If only warning rule was breached with no other rejection rule being violated, the run was accepted. As it suggested that an error may be in development, following checks were performed (a) comprehensive evaluation of equipment calibration, (b) review of sample, reagent and control handling and storage temperature, and (c) operator's sampling technique and test procedure.

Results

Table 3 shows values of E-ratio parameters obtained while running the IQC sample aliquots for determining the consistency and control limits. Controls of Lot 1 of anti-HIV, HBsAg, and anti-HCV were used for preparing charts 1, 2, and 3 of respective markers; whereas controls of Lot 2 of anti-HIV, HBsAg, and anti-HCV were used for preparing charts 4, 5, and 6 of respective markers.

E-ratio of aliquots for determining the consistency and control limits had CV <10%. LJ charts illustrating the performance of IQC samples on 180 runs for each ELISA (anti-HIV, HBsAg, and anti-HCV) are shown in Figures 1-18, respectively.

The violations of LJ chart interpretation rules for each ELISA are shown in Table 4. For anti-HIV ELISA, all runs were accepted in chart 1 [Figure 1], chart 3 [Figure 3], chart 4 [Figure 4], and chart 6 [Figure 6]. W was indicated in 2 runs (1.11%) as shown in chart 2 [Figure 2] and chart 5 [Figure 5]. Only one run (0.55%) was rejected due to violation of S rule as shown in chart 5 [Figure 5]. For HBsAg ELISA, all runs were accepted in chart 2 [Figure 8], chart 3 [Figure 9] and chart 5 [Figure 11]. W was indicated in two runs (1.11%) as shown in chart 1 [Figure 7] and chart 4 [Figure 10]. Two runs (1.11%) were rejected due to violation of R1 and T rules as shown in chart 4 [Figure 10] and chart 6 [Figure 12]. For anti-HCV ELISA, all runs were accepted in chart 4 [Figure 16] and chart 6 [Figure 18]. W was indicated in two runs (1.11%), as shown in chart 3 [Figure 15] and chart 5 [Figure 17] whereas two runs were rejected due to violation of S and R2 rules as shown in chart 1 [Figure 13] and chart 2 [Figure 14].

Table 1: Levey-Jennings chart interpretation rules and their modifications used in the study

Category	Rule	Definition	Type of error
Warning	W	One control is outside±2 SD	Random
Rejection	R1	Two controls are outside±2 SD in the consecutive runs	Systematic
Rejection	R2	One control is outside±3 SD	Random
Rejection	R3	Four consecutive controls are on one side of the mean and further than ±1 SD from the mean	Systematic
Rejection	Shift	Six consecutive points either above or below the mean. results rejected on sixth occasion	Systematic
Rejection	Trend	Six consecutive points either rising or falling. results rejected on sixth occasion	Systematic

SD=Standard deviation

Table 2: List of causes investigated in the events of error

Random errors	Systematic errors	Type
Air bubble in washer tubing or pipette tip	Change in kit lot	Shift
Improper reconstitution of buffer solution	Change in operator	
Pipette tip not fitted properly	Major equipment maintenance	
Clogged pipette	Change in room temperature/humidity	
Imprecise pipette or error in pipetting method	Inaccurate calibration of pipette	
Incubation time/temperature/dark room condition not as per manufacturer's instructions	Modification in testing method	
Interrupted power supply	Deterioration of reagents/control material due to improper storage	Trend
Poor operator technique	Accumulation of debris in washer tubing	
Improper equipment calibration	Deterioration of incubation chamber temperature/light filter integrity	
	Pipette misalignment	
Sampling error (no addition, interchange)	Deterioration of calibration of pipette	

Table 3: Statistical parameters of internal quality control sample lots

Marker	Lot	Charts	Mean±SD	CV (%)
Anti-HIV	1	1, 2, 3	1.77±0.14	7.85
	2	4, 5, 6	1.69±0.16	9.40
Hbs Ag	1	1, 2, 3	1.81±0.15	8.34
	2	4, 5, 6	1.73±0.16	9.24
Anti-HCV	1	1, 2, 3	1.84±0.17	9.29
	2	4, 5, 6	1.68±0.14	8.33

SD=Standard deviation, CV=Coefficient of variation, HIV=Human immunodeficiency virus, Hbs Ag=Hepatitis B surface antigen, HCV=Hepatitis C virus

Discussion

Quality control procedures improve the reliability of a test by providing an objective evidence of the variability arising from preanalytical and analytical sources. Vast majority of blood centers in developing countries are performing ELISA for the screening of blood units. In order to ensure the validity of a run and reliability of results, it is important to incorporate control samples. *In house* prepared borderline reactive control samples are an economic mean to ensure that all required test conditions have been met and there is a consistency in test performance.[8]

In this article, we have described the preparation and validation of IQC samples for anti-HIV, HBsAg, and anti-HCV ELISA. These samples have been incorporated in ELISA runs on a routine basis. Data have been recorded on LJ charts which provide a useful tool for visual monitoring and statistical analysis of results has been done by the application of LJ chart interpretation rules which allow for the differentiation between normal deviations and errors. Several errors have been noted in the assay performance even when the tests fulfilled the manufacturer's criteria for validity based on optical densities of internal kit controls. One previous study[9] has used positive control serum and analyzed the test differences among various laboratories, among the HIV-1 antibody test kits of different manufacturers, among
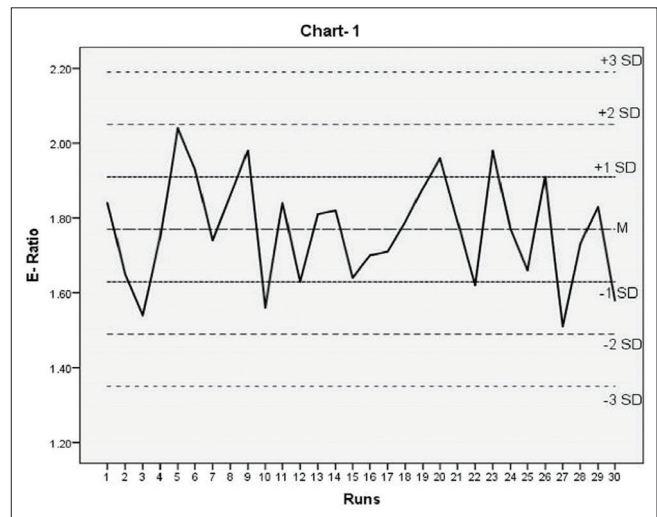

Figure 1: Levey–Jennings Chart 1 for anti-HIV enzyme-linked immunosorbent assay; all runs accepted

different lots of the same test kit, and among pipetting devices and techniques. However, a standardized pool of human sera positive for HIV-1 has been used and authors have found it useful strategy for proficiency testing of laboratory. Another study[10] has proposed a quality control procedure for HBsAg ELISA using pooled HBsAg-positive serum as control. The authors have used selective WG rules to give a high probability of error detection while maintaining low probabilities of false rejection.

Warning rule violation was indicated on anti-HIV run charts 2 and 5, HBsAg run chart 1 and 4, and anti-HCV run charts 3 and 5. In all these events, ELISA run was accepted but an exhaustive comprehensive review was done for sample and reagent handling, equipment calibration, temperature maintenance, and operator technique. The probable cause was identified and appropriate corrective action was taken. However, we could not discern a specific cause in three events. In anti-HIV Chart 5 and anti-HCV Chart 5, the subsequent runs were within control limits nonetheless. In HBsAg
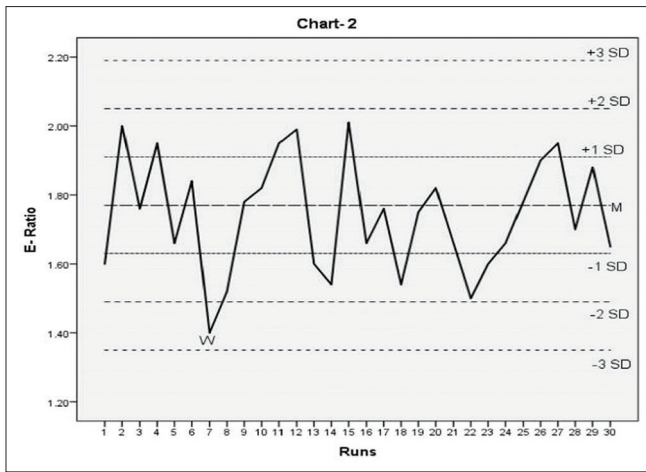
Figure 2: Levey–Jennings Chart 2 for anti-HIV enzyme-linked immunosorbent assay; W indicated in one run
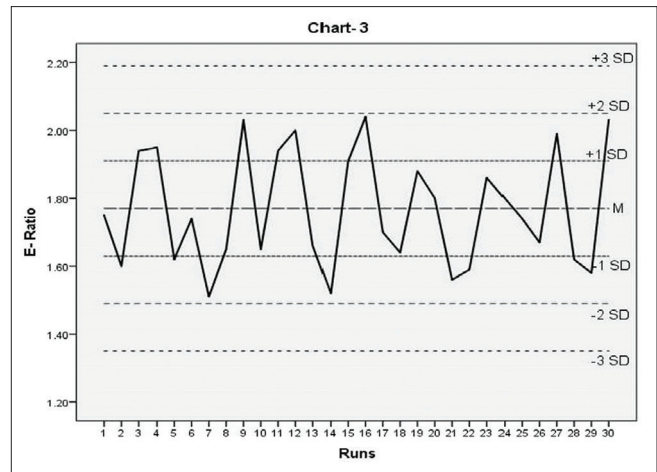

Figure 3: Levey–Jennings Chart 3 for anti-HIV enzyme-linked immunosorbent assay; all runs accepted
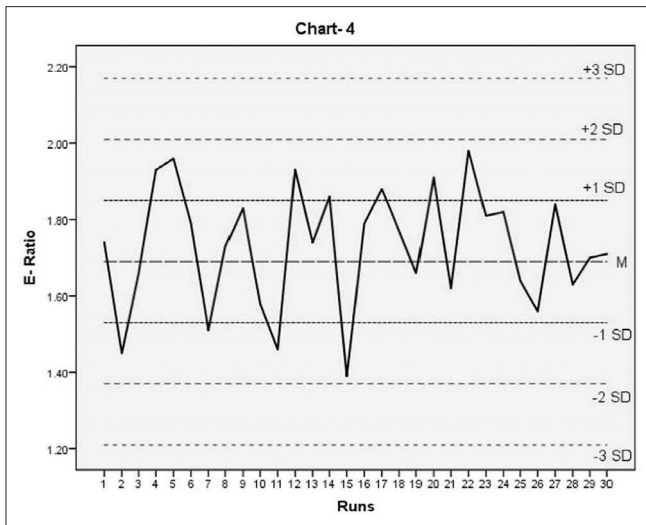

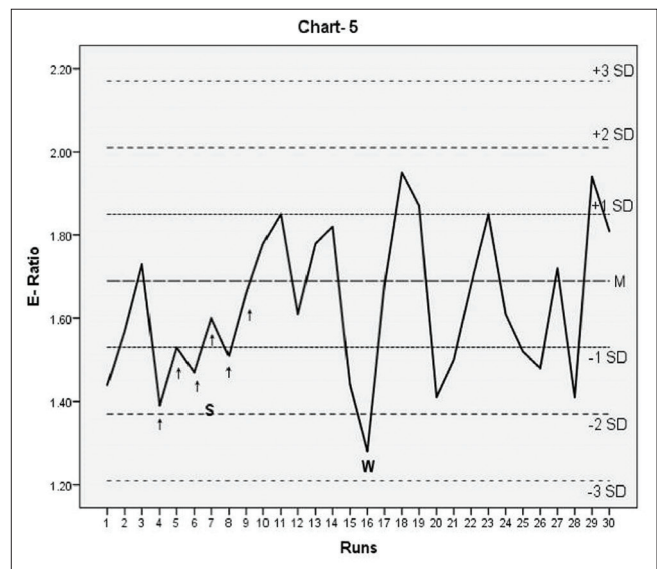**Figure 4:** Levey–Jennings Chart 4 for anti HIV enzyme-linked immunosorbent assay; all runs accepted

Figure 5: Levey–Jennings Chart 5 for anti HIV enzyme-linked immunosorbent assay; one run rejected due to S, W indicated in one run

run Chart 4, we could not discern the exact cause after W which led to R1 subsequently.

Random errors have an unpredictable occurrence in both magnitude and direction. These are not amenable to the application of corrections but reduced by repetition. These can be minimized by proper training of personal, strict adherence to standard operating procedures (SOPs) and careful supervision of the process. We have recorded random error R2 in only one event (anti-HCV ELISA chart 2) where control value dropped below − 3 SD. The cause was sample mix up due to improper sequencing. As this type of error may have significant consequences, we trained our operator for meticulous identity check of samples before dispensing them in the microplate.

Systematic errors are easy to detect as these remain constant if measurements are made in similar conditions.

These create a predictable bias in results of the test and are amenable to application of correction. Systematic errors were recorded on four charts in this study. Inadvertent changes in the system process accounted for these events. To prevent their recurrences, we performed relevant modifications in our SOPs and trained the newly employed staff to abide by the standards.

We have recorded 11 (2.03%) violations out of total 540 runs in the present study. A study in clinical virology laboratory[11] has recorded 3.3% violations on running IQC samples using WG rules. The authors were able to highlight batch to batch variation in the serological assays by inclusion of IQC samples. They have recommended the use of data obtained with assay controls to set the acceptable limits for testing of anonymous samples as a part of quality assessment schemes.
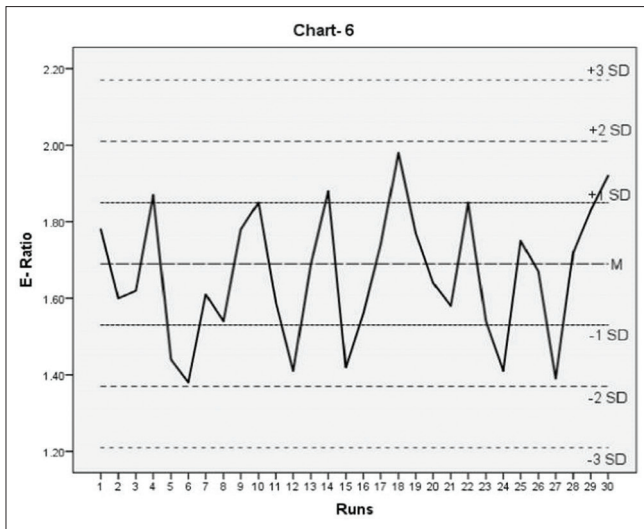
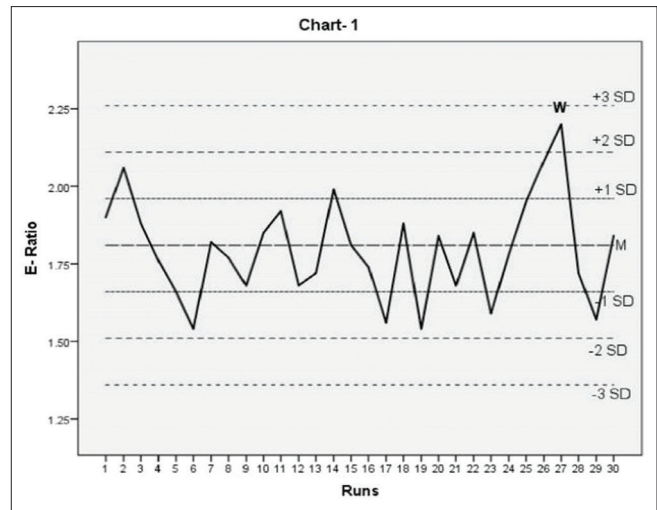**Figure 6:** Levey–Jennings Chart 6 for anti HIV enzyme-linked immunosorbent assay; all runs accepted

Figure 7: Levey–Jennings Chart 1 for HBsAg enzyme-linked immunosorbent assay; W indicated in one run
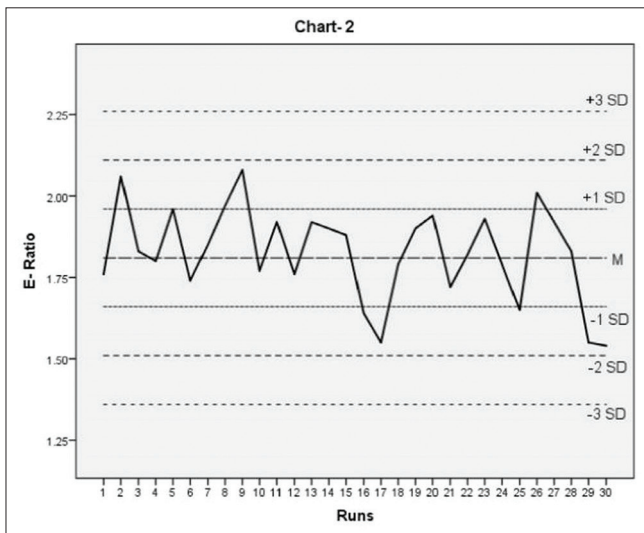

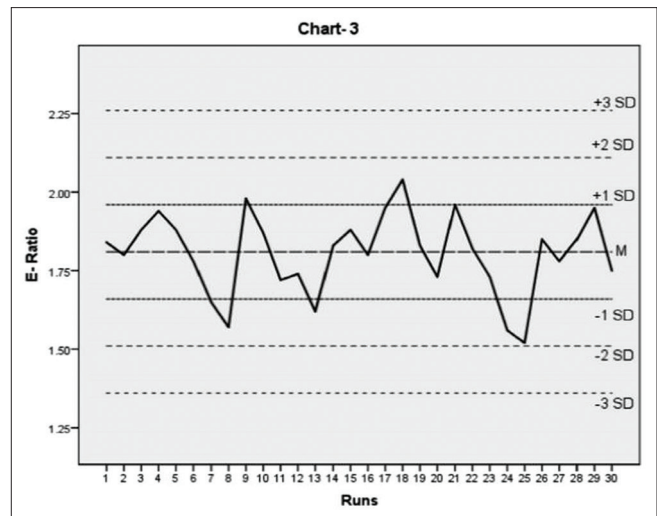**Figure 8:** Levey–Jennings Chart 2 for HBsAg enzyme-linked immunosorbent assay; all runs accepted

Figure 9: Levey–Jennings Chart 3 for HBsAg enzyme-linked immunosorbent assay; all runs accepted

The implementation of proficiency testing program is an integral part of quality assurance in a testing laboratory. Blood centers screening the blood units using manual or automated ELISA should implement and maintain a QC program using low reactivity ICS which have been standardized for daily use and acceptability limits defined according to the assay. Variations in performance may be seen between different lots of the same test from the same manufacturer. If these variations result in significant deviations, IQC samples must be re-standardized for use with that particular lot. Similarly, if any new equipment is introduced or the routine procedure undergoes some modification, there should be re-standardization of IQC samples. Moreover, the laboratory should validate its own IQC program and check the accuracy of its results by participating in external quality assessment schemes.

Quality control data should be periodically reviewed by supervisory staff to assess the validity and to understand the system changes.

There should be clearly written policies and procedures in the laboratory which should define the course of action, interpretation, and corrections. There should be an intensive training of laboratory staff enabling them to detect and analyze the errors in routine assay runs. Whenever, a new procedure or equipment is implemented, SOPs should be revised and operators should be thoroughly trained before using it in routine practice. All the laboratory equipment should fulfill standard specifications and validated before routine use. There should be periodic calibration of sensitive equipment such as micropipettes, multichannel pipettes, incubators, and shakers. Routine maintenance
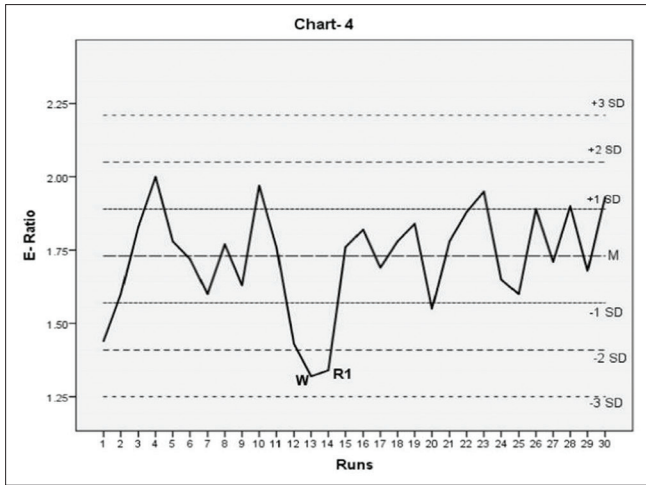
Figure 10: Levey–Jennings Chart 4 for HBsAg enzyme-linked immunosorbent assay; W indicated in one run, one run rejected due to R1
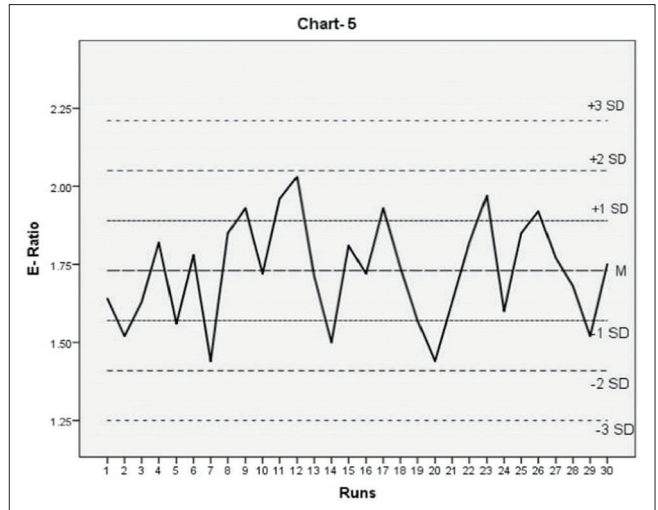

Figure 11: Levey–Jennings Chart 5 for HBsAg enzyme-linked immunosorbent assay; all runs accepted
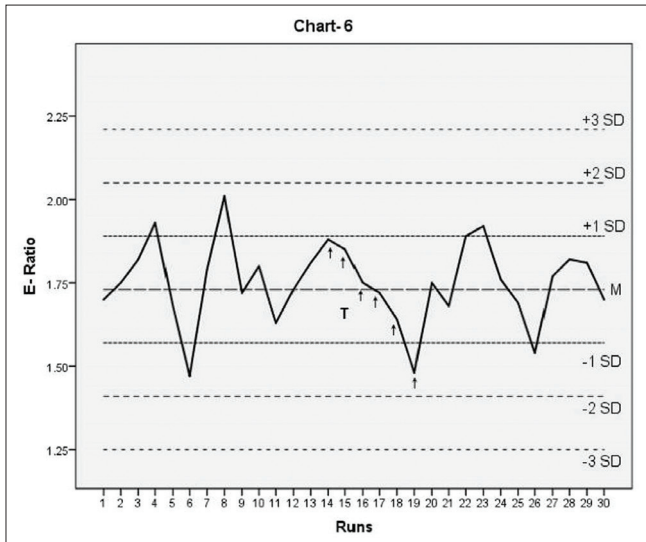

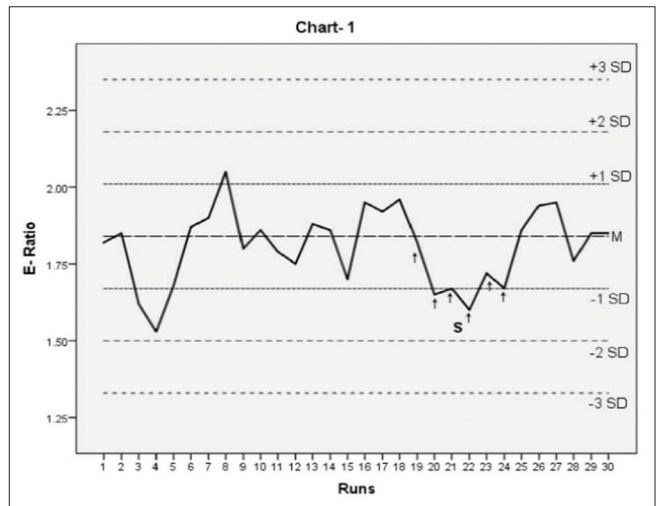**Figure 12:** Levey–Jennings Chart 6 for HBsAg enzyme-linked immunosorbent assay, one run rejected due to T

Figure 13: Levey–Jennings Chart 1 for anti-HCV enzyme-linked immunosorbent assay; one run rejected due to S
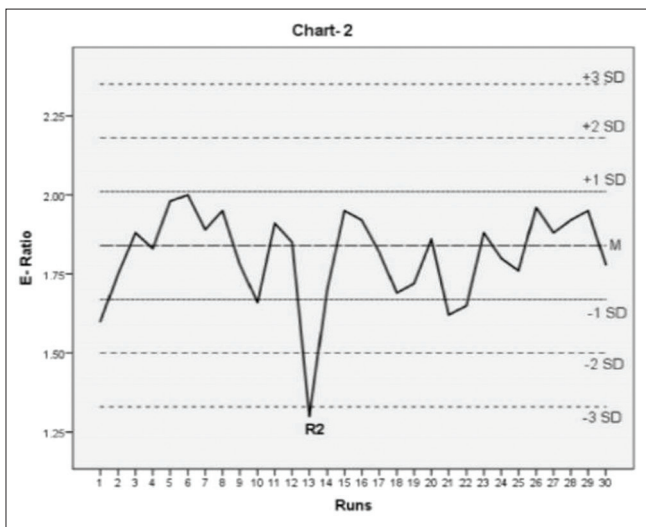

Figure 14: Levey–Jennings Chart 2 for anti-HCV enzyme-linked immunosorbent assay; one run rejected due to R2
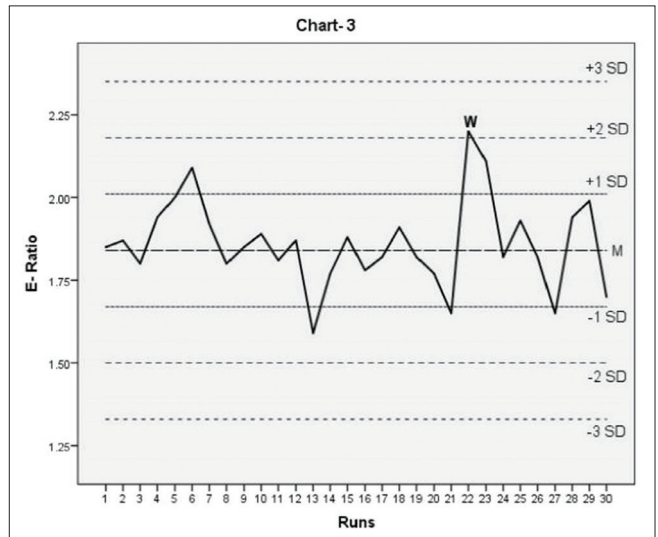

Figure 15: Levey–Jennings Chart 3 for anti-HCV enzyme-linked immunosorbent assay; W indicated in one run

Table 4: Description of errors and remedial actions for the results of internal quality control samples

Marker	Run chart	Error	Action	Probable cause	Correction applied
Anti-HIV	1	None	All runs accepted	-	-
	2	W	Run accepted. Comprehensive review undertaken	Crystals formation in buffer solution due to storage, leading to inaccurate concentration	Re-solubilization of buffer solution by warming at 37°C
	3	None	All runs accepted	-	-
	4	None	All runs accepted	-	-
	5	S	Run rejected	Change in operator	Operator given extensive training
		W	Run accepted. Comprehensive review undertaken	Cause not discernable	Next run performed under strict supervision
	6	None	All runs accepted	-	-
HBsAg	1	W	Run accepted. Comprehensive review undertaken	Incubation time exceeded the prescribed limit	Technician instructed for strict adherence to SOP
	2	None	All runs accepted	-	-
	3	None	All runs accepted	-	-
	4	W	Run accepted. Comprehensive review undertaken	Cause not discernable	Next run performed under strict supervision
		R1	Run rejected	Erroneous calibration of the working pipette	Use of correctly calibrated pipette
	5	None	All runs accepted	-	-
	6	T	Run rejected	Deterioration of control sample aliquot (left at ambient temperature after the test procedure)	Use of fresh control sample aliquot
Anti-HCV	1	S	Run rejected	Use of kit of different lot number erroneously	Kits of same lot number resumed
	2	R2	Run rejected	Arrangement of sample was disturbed leading to sample mix up	Samples rearranged carefully, training of operator
	3	W	Run accepted. Comprehensive review undertaken	Improper functioning of washer leading to overflow of buffer solution	Maintenance repair of washer performed
	4	None	All runs accepted	-	-
	5	W	Run accepted. Comprehensive review undertaken	Cause not discernable	Next run performed under strict supervision
	6	None	All runs accepted	-	-

HIV=Human immunodeficiency virus, Hbs Ag=Hepatitis B surface antigen, HCV=Hepatitis C virus, SOP=Standard operating procedures
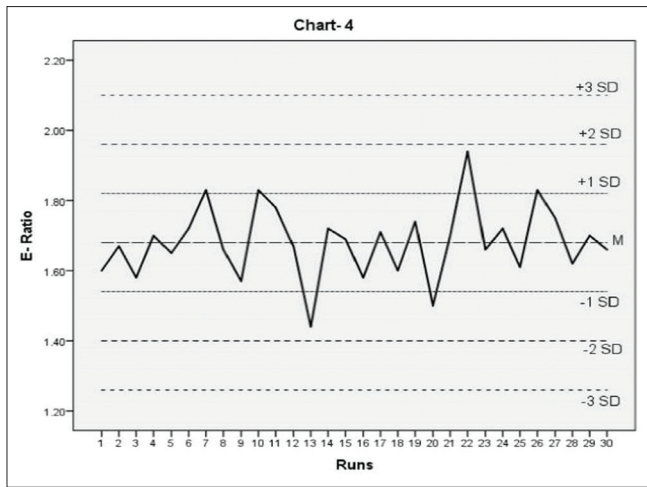

Figure 16: Levey–Jennings Chart 4 for anti-HCV enzyme-linked immunosorbent assay; all runs accepted
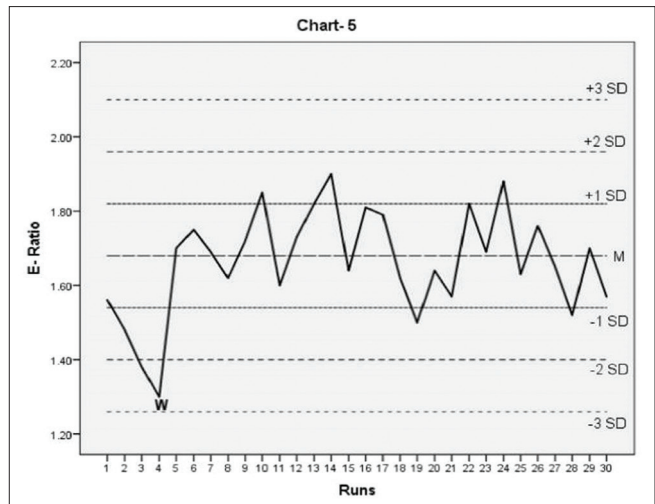

Figure 17: Levey–Jennings Chart 5 for anti-HCV enzyme-linked immunosorbent assay; W indicated in one run

of ELISA washers and calibration of readers should be done every 6 months. Washer should be rinsed with distilled water after use to avoid crusting of salt within the metallic pipes. Light filters in ELISA readers should be regularly checked for moisture and fungal contamination to prevent erroneous OD values. There should be continuous monitoring of storage equipment

to ensure optimum quality of diagnostic reagents and control samples.

To summarize, inclusion of IQC samples provides valuable information on the integrity of the test system, kits, and reagents and also on the performance of the staff. These should be used with each ELISA run to
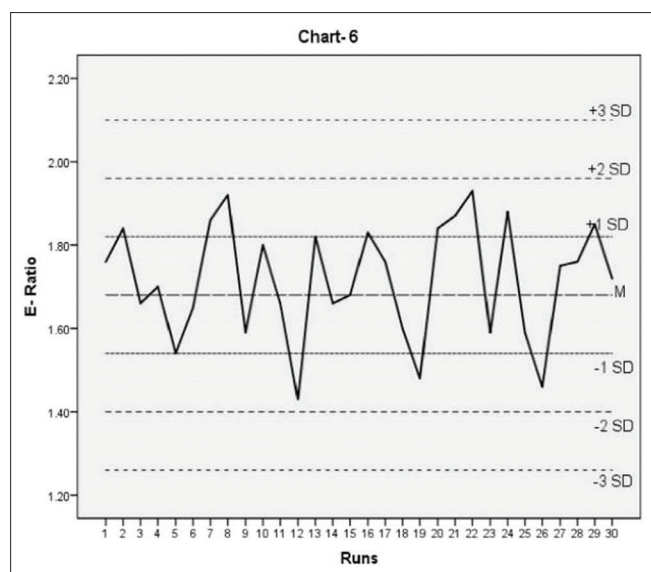
Figure 18: Levey–Jennings Chart 6 for anti-HCV enzyme-linked immunosorbent assay; all runs accepted

check the assay performance, ensuring reliability, and reproducibility of test results. If IQC sample values are out of range, corrective actions, and troubleshooting should be undertaken immediately and problem should be rectified before validating ELISA results.

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Conflicts of interest
There are no conflicts of interest.

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