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White Paper

Assessing Quality Using ACCURUN® Controls for Quantitative Viral Load Assays

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Consistent accuracy in reporting quantitative viral load results is critically important to patients, their physicians and to the clinical laboratory. In clinical practice, quantitative viral load results are used both to diagnose the severity of disease and are crucial for guiding patient management and treatment. Such clinical decision-making relies on the accuracy of clinical laboratory testing. With the help of these assays, many viral infections that were fatal or untreatable just a few years ago can now be effectively managed with potent antiviral therapies.

Clinical laboratories have many choices between assays and platforms to quantify viral burden. In today's cost conscious environment, every laboratory must ask what are the appropriate QC goals and what are the most cost effective ways to achieve these goals. Assay performance specifications should reflect the intended use of a procedure and should be supported by the laboratory's quality management system.[1]

ACCURUN® controls from SeraCare Life Sciences are developed and manufactured as third-party controls. These products are designed to support the quality control procedures within clinical labs. When used properly and as part of an overall QC program, ACCURUN® controls will monitor assay accuracy and precision, help identify performance issues and improve the work efficiency of a clinical lab. The ultimate goal of the QC program is to provide added confidence in the reliability and validity of the reported results.

SeraCare Life Sciences has been manufacturing quality control materials for over 25 years. Our products include individual and multiplex controls for antibodies, proteins and nucleic acids for infectious diseases and genetic disorders. SeraCare has nucleic acid positive (quantified across the linear range) and negative controls that can be used for viral load assays for HIV, HBV, HCV, HPV, Ct and Ng.

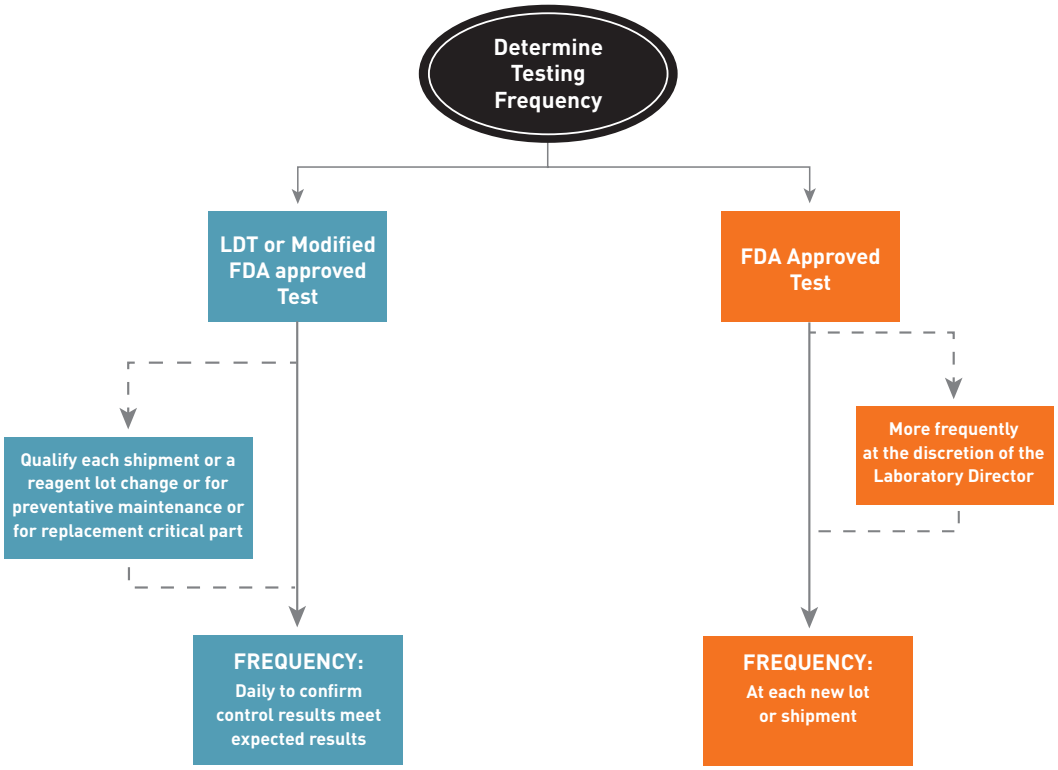
Frequent testing of independent, third-party nucleic acid controls provides the laboratorian with a means of monitoring the performance of quantitative viral assays. Routine use enables the lab to monitor inter and intra assay variation, lot-to-lot variation, operator variation, environmental changes, and can assist in identifying either isolated or systematic errors.

Viral load measurements (virus copies/ml) are performed to monitor the disease progression in a patient over a period of several months to years and thus accurate, precise, reproducible and sensitive assays are essential. For example, the medical decision point to treat chronic HBV infection is $>10^4$ copies / ml of HBV DNA for HBe antibody negative patients with normal ALT / liver cirrhosis status.[2] HIV-1 RNA assays are used to diagnose acute infection, demonstrate the effectiveness of antiretroviral therapy, and guide patient care[3]. For HCV, quantitative RNA assays are used to monitor patients who have been diagnosed with HCV and to determine sustained virological response leading to end of treatment[4].

Testing Frequency and Third-Party Controls: Control testing frequency is recommended by CLIA regulation, guidelines from recognized laboratory organizations and by manufacturer's instructions. Manufacturers of FDA cleared NAT assays generally provide both positive and negative controls that should be run as specified by the manufacturer's instructions. CLIA regulations also dictate control testing frequency for a laboratory developed test (LDT) or FDA cleared test.[1,5] Figure 1 is a decision flow chart that outlines whether a control should be run daily or run with any lot change per CLIA regulations.

Figure 1.

Recommended Frequency of Running Assay Controls at CLIA Certified Labs for Laboratory Developed Tests and for FDA Approved Tests



Once a lab has decided when to run controls, the next decisions are what types of controls and how many replicates to run to establish a mean. As stated earlier, third-party controls like ACCURUN are designed for use with laboratory testing for purposes of monitoring assay performance. One unique advantage of ACCURUN controls is that the products are manufactured in large volumes in a patient-like commutable matrix. In addition, the ACCURUN virology controls have very good stability which is usually >2 years from date of manufacture. These features can yield significant benefits to maintaining quality in today's cost conscious environment. For patient samples, CLIA regulations mandate running two levels of control for quantitative assays.

Replicates and eQuality™ Peer Review

Statistical guidance for clinical assays typically suggests running 20 replicates for any new control lot in order to establish a stable mean concentration value.[6] However, with qPCR assays, the assay expense to run this many replicates can be cost prohibitive. Whenever possible, SeraCare recommends following the 20 replicates guidance, as was recently reported[7] by measuring 2 duplicates for 10 consecutive days to establish an HBV control value. With 20 replicates, the data is statistically significant and can be used to establish the initial mean and standard deviation for QC trend analysis. If the laboratory cannot run 20 replicates, eQuality™ Peer Review Analysis is a useful resource to assist the laboratory in running fewer replicates.[8] eQuality™ is a user-shared, dynamic database where laboratories can post control measurements and review their results versus other laboratory results for lot-specific controls over the lifetime of the product. By accessing eQuality, laboratories can immediately view results for old and new control lots. The eQuality data becomes a reference point to compare means. Standard deviations may be used to benchmark performance between different laboratories. eQuality does not substitute for a laboratory running its own replicates and establishing its own values for a control. For example, if there was a lot where 10 or more values were posted, the laboratory can calculate the mean and standard deviation for comparison to their

own data. In this instance, the laboratory could run duplicates for just three consecutive days, n=6, and average them to establish their own laboratory value. The laboratory can then follow their own QC guidelines and use a one-way ANOVA analysis to compare their own value to the eQuality averaged value. If the data sets are similar enough, then the laboratory has established an initial mean and standard deviation and has reduced testing expenses.

Sigma-Metrics and qPCR Virology Assays

Continually evaluating random error (ordinary standard deviation) and bias (systematic errors inherent to an analytical method or method calibration) that arise from diagnostic tests is critical to the delivery of confident analytical results upon which medical decisions are based. An important objective of this tutorial is to provide instructive examples of sigma-metrics applied to quantitative DNA and RNA viral load assays. Levy-Jennings charts that show trends over time and normalized OPSpec charts that show assay performance are two sigma metric tools that are simple to construct, convenient to use and applicable to help ensure the quality of quantitative viral load assays. Levey-Jennings charts are available on eQuality. QPCR data from HBV, HIV-1 and HCV ACCURUN controls are used as examples.

Materials and Methods

ACCURUN controls are designed to run with *in-vitro* assay procedures for the purpose of monitoring test performance but do not have assigned values. The controls are supplied in a plasma-like matrix with minimal processing for commutability to patient blood samples. As described, it is recommended that each laboratory validate the use of the ACCURUN control with each specific assay system prior to routine use as specified by CLIA regulations.[1,6] ACCURUN 315 Series 300 is a Low Positive HIV-1 RNA positive quality control and is prepared by diluting a cultured HIV-1 type B virus (8E5) in HIV-1 negative defibrinated plasma. ACCURUN 325 Series 370 is a low positive Hepatitis B Virus DNA control and is manufactured from HBV DNA positive human serum or plasma. ACCURUN 305 Series 300 is a low positive Hepatitis C Virus RNA quality control and is manufactured from HCV RNA positive human serum or plasma.

Assays: FDA cleared qPCR assays for HIV-1, HBV and HCV were used on an automated qPCR instrument. Microsoft Excel is used here for statistical analysis. Any other statistical package such as JMP is also suitable.

Results and Discussion

Six Sigma lays out a basis for statistical quality control and Dr. James Westgard[9,10] has extended these concepts for diagnostic laboratories by helping to describe the probability of error detection and of false rejection. Measurement observations are comprised of true value plus random error plus measurement bias.

When evaluating the performance of a diagnostic test, one needs to know the total allowable error, TE_a, that meets the criteria for medical decision-making, the analytical random error, and the analytical bias, i.e., the systematic error that offsets the measurements from the "true" concentration. The traditional methods used here assume that the random error is Gaussian distributed, which may or may not be true for PCR-based analyses, but is a useful first-level assumption. Pertinent viral loads may vary over many orders of magnitude of concentration, so for convenience, and to stabilize the error distribution to be more Gaussian-like (assuming percent concentration variation is more likely than absolute concentration variations), the log10 transformation of viral concentrations, c, is used. It is further assumed that the medically allowable error, TE_a, for all viral assays is half a log cycle, $\log_{10} c \pm 0.5$[7]. The random error for any assay is taken as the standard deviation, s. This standard deviation is attributed to the procedure assay alone, assuming that there is no variability in the analyte or reagent concentrations. Bias, $\langle x \rangle^b$, becomes more complicated. Ideally, bias is simply the systematic error from the "true" quantity, but truth might be elusive. Therefore, bias might be the systematic deviation from benchmark assay procedures, or might be the systematic variation introduced by variations in reagent kits.

The sigma statistic, Σ, describes the overall quality of a procedure. A higher value for the statistic indicates a higher quality for the procedure. This may be calculated by

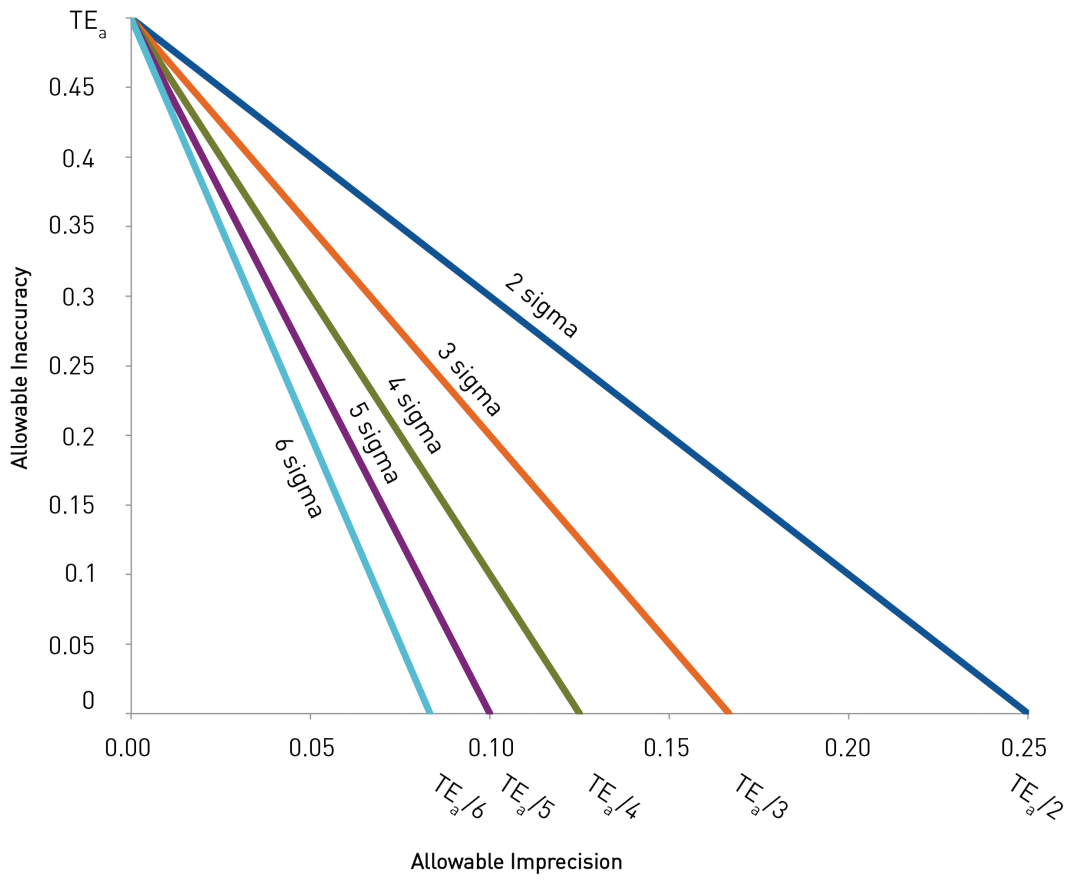
$$\Sigma = \left| \frac{TE_a - \langle x \rangle_b}{s} \right|$$

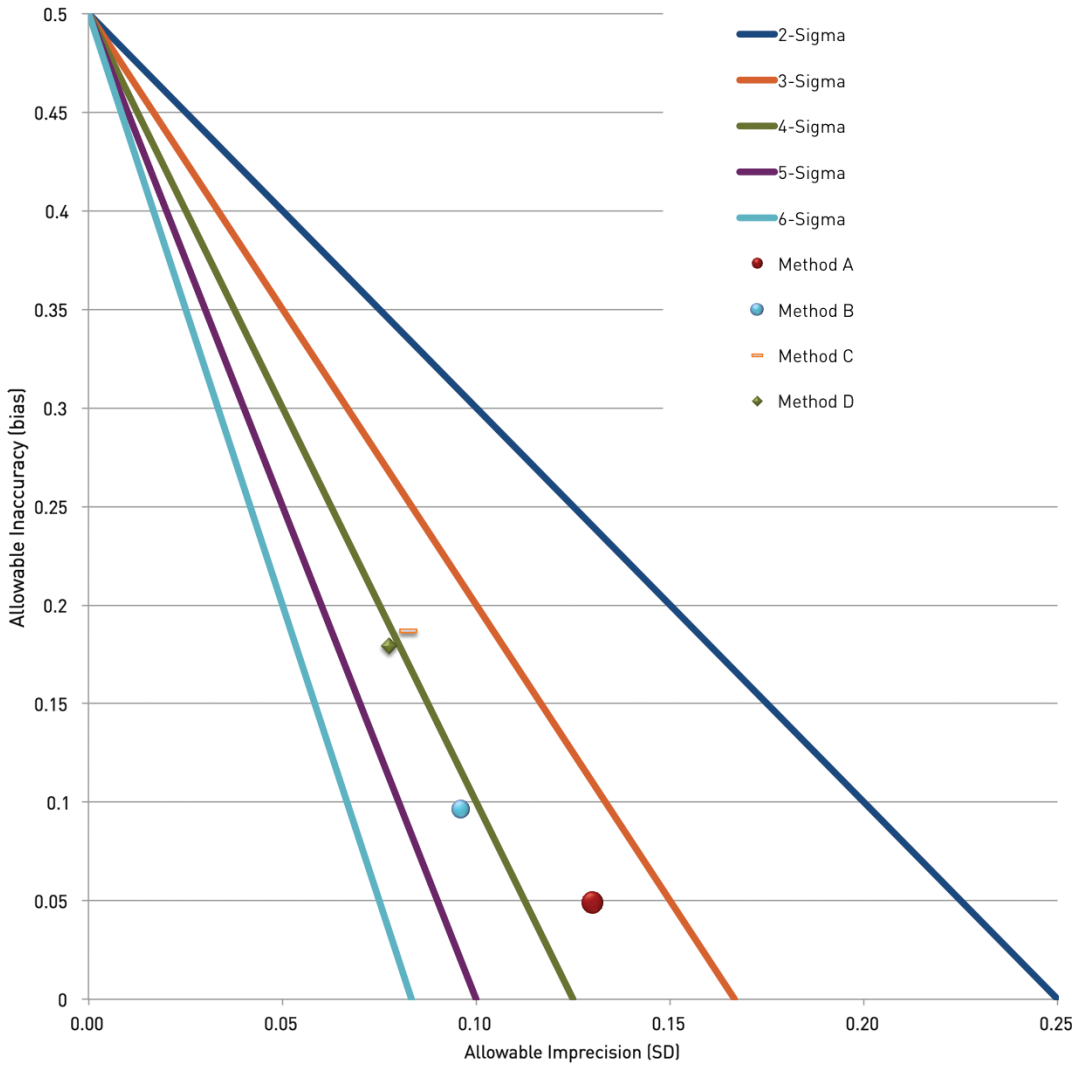
Alternatively, performance can be viewed graphically by plotting a procedure's bias and standard deviation on a Method Decision Chart.[10] In Figure 2, qPCR procedure Method A was compared with 3 other quantitative procedures to obtain a bias from these procedures.[12] The bias was determined as the deviation of the mean measurement from Method A from the average mean measurement from Methods B, C and D. Replicate runs from the procedure were used to establish the assay's random error. The Method Decision Chart is constructed as follows:

1. The vertical axis, Allowable Inaccuracy, is drawn with a span of TE_a.
2. The horizontal axis, Allowable Imprecision, is drawn with a span of $TE_a/2$.
3. Σ Levels are drawn from (0, TE_a) to (TE_a/Σ, 0), i.e. from TE_a on the y-axis to TEa/2 on the x-axis for Σ=2; from TEa on the y-axis to TEa/3 on the x-axis for Σ=3; etc. through Σ=6.This is shown in Figure 2a.
4. The procedure performance is plotted as (s, $\langle x \rangle_b$), i.e., s on the x-axis and $\langle x \rangle_b$ on the y-axis. This is shown in Figure 2b.

Figure 2.

Method Decision Chart. Graphical Tool to evaluate the quality of a laboratory test.





For monitoring assay quality control and showing when an assay is out of control in real-time, the Levey-Jennings Chart is a useful tool. Levey-Jennings creates a pictorial view of a procedure's history by plotting control measurements versus time. The procedure's control limits are depicted on the chart to identify when a procedure is out of control. Typically, the limits are based on the procedure's standard deviation. A normalized Levey-Jennings Chart plots the observation minus the expected value versus time, with the control limits at ±2s and ±3s depicted. This centers the observations around the y-axis value of zero. Figures 3a, 3b and 3c show the normalized Levey-Jennings Charts for HBV, HIV-1 and HCV assays.

Figure 3a.

HBV Levey-Jennings Plot

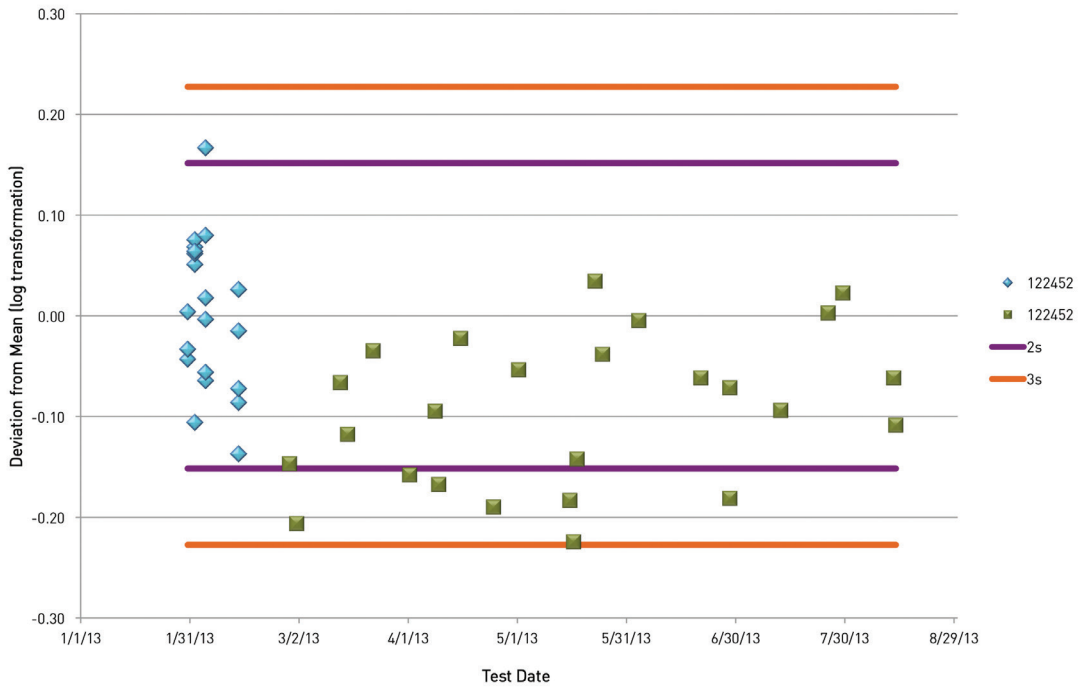


Figure 3b.

HIV-1 Levey-Jennings Plot

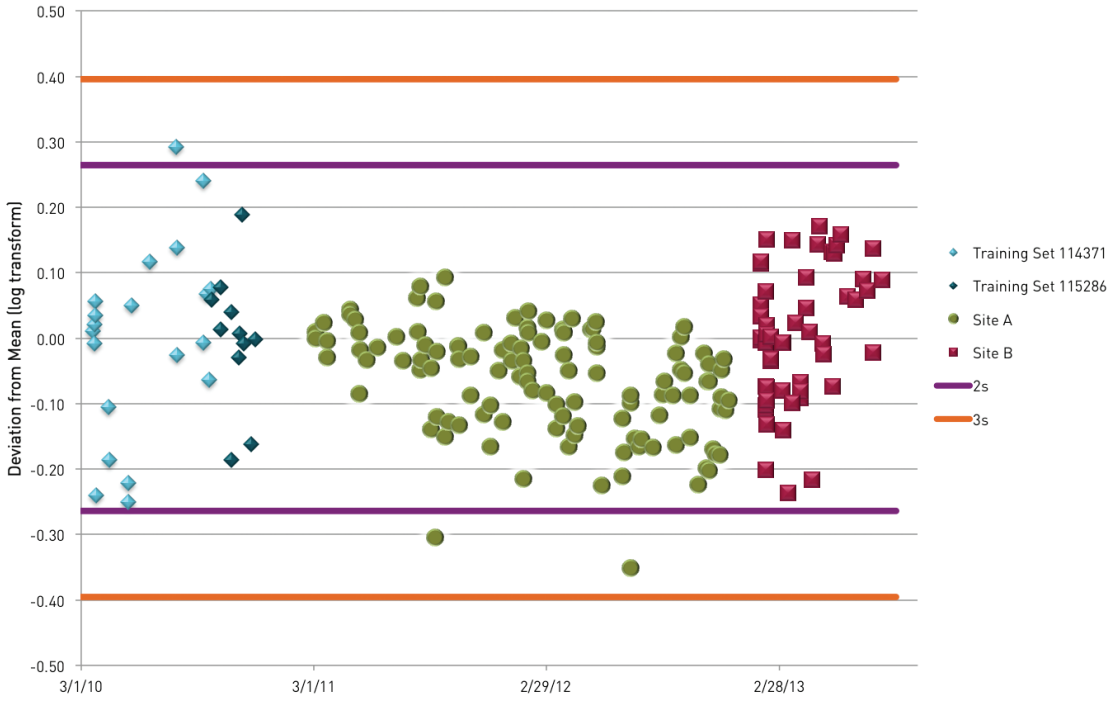
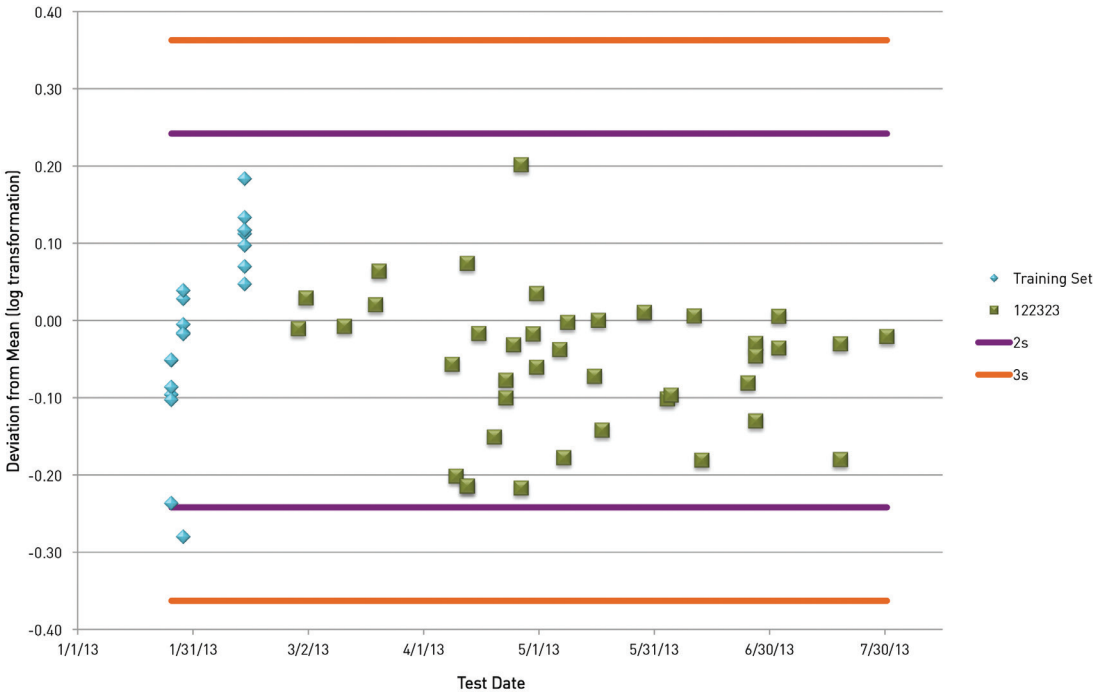


Figure 3c.

HCV Levey-Jennings Plot

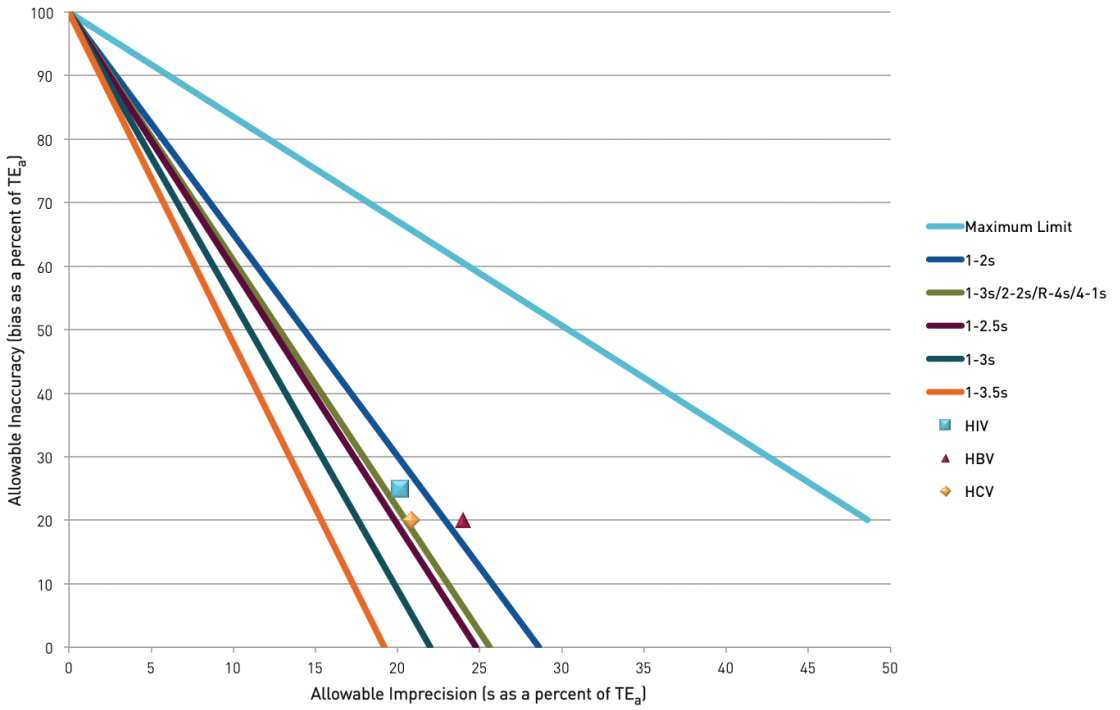


For charts 3a, 3b and 3c, we used 20 measurements to calculate the measurement mean and standard deviation, s. These data are labeled "training set". The remaining observations were plotted, along with the 2s and 3s control boundaries. Alternatively, an external or historical measure of standard deviation, and a published value or derived value for the true concentration can be used. SeraCare recommends that once a control sample's reliable mean is established by the lab, the laboratory personnel closely monitor every run by critically examining every prospective data point by comparing it to the established mean and observe if any drift in the assay's performance is evident.

The Levey-Jennings Chart is a historical representation of a process, and does not overlay any particular acceptance or rejection criteria. Statistical analysis of qPCR assays has not been rigorously evaluated in the literature as routine clinical chemistries have. Dr. James Westgard has been a proponent of multi-rule procedures that can identify when a process becomes out of control while minimizing the number of required control tests. For this, Westgard proposes the use of an OPS Chart, which plots a procedure's performance versus various QC protocols for identifying when a process is out of control. Figure 4 shows the OPS Charts for the viral assays under study.

Figure 4:

OPS Chart (N=4)
Total Allowable Error=100% with 90% Error Detection



OPS Charts are generated using tools from the web (for example www.westgard.com) or from the literature. The charts may be absolute, i.e., plot assay bias and standard deviations directly, or can be normalized where all values are divided by the total acceptable assay error, TE_a. The operating lines on the OPS Chart represent the various possible QC rule sets that efficiently identify when a process becomes out-of-control. Figure 4 shows the OPS Chart for a TE_a of 100%, i.e., a total allowable error that equals the medically tolerable variation of a half $\log_{10}$ cycle. The AQA (analytical quality assurance) of 90% means that 90% of processes in an out-of-control condition will be identified. The Westgard rules can be interpreted as follows for N=4, i.e., 4 control measurements are needed to interpret the process state.

- 1_{2s} means that one of the control observations exceeds 2 standard deviations from the expected mean value (as observed, for example, on the Levey-Jennings Chart)
- $1_{3s}/2_{2s}/R_{4s}/4_{1s}$ means if any of the following conditions exist for 4 control observations:
  - one observation is greater than 3 standard deviations from the mean
  - two observations are greater than 2 standard deviations from the mean
  - two observations span a 4 standard deviation range, e.g., they traverse from -2s to 2s
  - all four observations are within 1 standard deviation from the mean but lie on one side of the mean
- $1_{2.5s}$ means that one of the control observations exceeds 2.5 standard deviations from the mean
- 1_{3s} means that one of the control observations exceeds 3 standard deviations from the mean
- $1_{3.5s}$ means that one of the control observations exceeds 3.5 standard deviations from the mean

One needs to locate the most efficient QC rule by locating the nearest operating line that lies above the process performance point.

In Figure 4, the assay performance points were plotted using an estimated assay bias, $\langle x \rangle_b$, (plotted as $100\%\langle x \rangle_b/TE_a$) versus the observed assay standard deviation (plotted as $100\%s/TE_a$). The bias was estimated by observing the historical mean variations in measurements of a single control standard using different assay reagent kits. From Figure 4, we identify that the 1_{2s} (N=4) QC rule is the most efficient for these viral assays based on these data, with the caveat that the HBV assay will perform at slightly lower than a 90% AQA or that a lower apparent bias can be justified. In the legend of the OPS Chart, Westgard gives a probability of false rejection, pfr, for the control rule. In the case of the 1_{2s} QC rule, it is 18%.

When external control results are outside of the 2 S range, laboratories may want to investigate further to determine whether the results point to a pending problem. Investigation could include such questions as:

- Are there adverse trends in the external control data? For example, are the last few data points all trending lower or higher than the established mean value?
- Were the proper reagents, calibrators and controls used and were they within their expiration dates?
- Are instrument calibrations and maintenance up-to-date?
- Is the technician's training current and are all aspects of the test protocol being followed precisely?
- Are samples and external controls being stored properly? Are they stored at the correct temperature and with no unnecessary freeze-thaw cycles?
- Are the environment and laboratory conditions acceptable? (Ambient temperature, humidity, air quality, dust, etc.)

Finally, it is always up to the laboratory team members and the lab's quality systems to adhere to CLIA regulations and provide quality results. ACCURUN Controls and the eQuality Peer Review Analysis from SeraCare are important tools available to any laboratory and can help ensure quality qPCR results are reported for quantitative virology.

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About SeraCare

SeraCare partners with IVD researchers and manufacturers as well as clinical laboratories to bridge the gap between today's diagnostic solutions and tomorrow's emerging technologies for molecular diagnostics, next-generation sequencing and companion diagnostics.

Our extensive collection of high-quality biological materials and specialized expertise help optimize diagnostic performance, reliability, and repeatability across the IVD lifecycle—from the research bench to the manufacturing site to the clinical laboratory or point of care.

With more than 25 years of diagnostic industry experience, SeraCare is uniquely positioned to ensure a high level of consistency, confidence, and compliance as we partner with IVD leaders to shape the future of medical diagnostics.

Strategic areas of focus

- Women's Health
- Infectious Disease
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- Next Generation
- Cardiovascular
- Allergy
- Neurology

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