Outline generic protocol for conducting the verification & validation of quantitative molecular amplification/detection assays.

1.0 Background and intended purpose

In order to ensure the reliability and reproducibility of patient test results it is essential that the clinical molecular laboratory conducts appropriate quality control (QC) & quality assurance (QA) measures. These measures are often driven by International and regional regulations and include a requirement for verifying and/or validating the performance specifications of the molecular assay as well as monitoring it's intend use and performance in the routine clinical setting over time.

The purpose of this document is to provide a basic introductory protocol of the minimum recommended requirements for conducting the validation / verification of quantitative molecular assays within the routine clinical laboratory.

The document also outlines requirements for establishing an IQC plan for the continuous monitoring of quality performance over time In line with the requirements of ISO15189:2012 or equivalent, the College of American Pathologists (CAP) molecular microbiology checklists and associated documents such as the Clinical and Laboratory Standards Institute (CLIA) guidelines (EP05-A3 – *evaluation of Precision of Quantitative Measurement Procedures*).

As with any region regulations, the Senior Laboratory Manager / Director is ultimately responsible for ensuring that the verification / validation procedures they conduct meet the laboratory's regional accreditation / certification obligations and this generic protocol should be adapted in order to meeting those specific requirements where necessary.

2.0 Basic verification / validation protocol

The basic protocol assumes that the laboratory is carrying out a V&V assessment within their laboratory and using a single molecular assay platform for the determination of viral load. Although the protocol can easily be adapted to cover multiple platform instruments within a laboratory and across multiple laboratory sites (see section 3.0). Testing is conducted across a 3 to 5-day period (although the days do not have to be consecutive) and provides the laboratory with an indication of its assay linearity, accuracy and precision over time as well as providing a measure of its repeatability and reproducibility when conducted as part of a multi-site study.

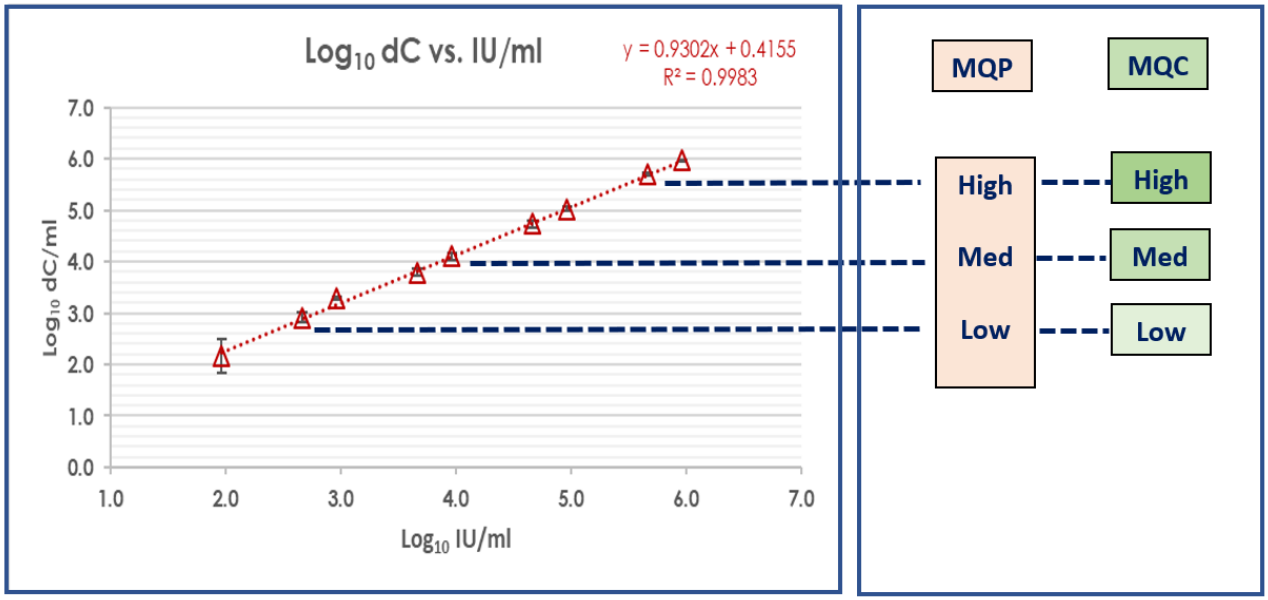
In addition, this V&V protocol also helps the laboratory establish control limits for the continuous monitoring of the assay quality performance over time (see section 4.0).

The following Qnostics products are used to support the V&V study.

Product Description	**Regulatory** (A)	**Format**
Analytical Q Panel (AQP)	CE / IVD / RUO Depending upon the product & market	7 to 10 positive vials of sequential dilution and 1 Negative vial covering the Analytical measurement range (AMR). Units of Measurement in IU/ml, or Copies/ml and/or digital Copies/ml dependant on availability of International Standard (see separate product insert for details and/or relevant technical bulletin – where available).
Molecular Q Panel (MQP)	CE / IVD / RUO Depending upon the product & market	4 vials, 3 positive vials (High, Medium and Low) and 1 Negative vial. Calibrated to the high, medium, and low points within the AQP. Units of Measurement in IU/ml, or Copies/ml and/or digital Copies/ml dependant on availability of International Standard (see separate product insert for details and/or relevant technical bulletin – where available).
Molecular Q Control (MQC)	CE / IVD / RUO Depending upon the product & market	Single level controls, (High, Medium and Low). Linked and calibrated to the high, medium, and low points within the AQP. Units of Measurement in IU/ml, or Copies/ml and/or digital Copies/ml dependant on availability of International Standard (see separate product insert for details and/or relevant technical bulletin – where available).

NOTE: *In addition to the protocol provided the laboratory may want to test 'known' clinical / patient samples along side the V&V materials provided in order to verify the clinical performance of the assay in parallel. Those specific requirements are not covered in this document.*

The Relationship between Analytical Q Panel (AQP), Molecular Q Panel (MQP), and Molecular Q control (MQC)



Day 1: Schematic diagram of laboratory test requirements

	AQP Format Panel member	Target Conc Log10 IU/ml	Number of replicates			Day 1 total tests runs
	1	6	1	1	1	3
	2	5.7	1	1	1	3
	3	5	1	1	1	3
High	4	4.7	1	1	1	3
	5	4	1	1	1	3
Med	6	3.7	1	1	1	3
	7	3	1	1	1	3
Low	8	2.7	1	1	1	3
	9	2	1	1	1	3
	10	Negative	1	1	1	3
					Total tests	**30**

2.1 Verification of the Linearity / Analytical Measurement Range (AMR).

Testing the Qnostics AQP panel in triplicate allows the laboratory to demonstrate linearity across the dynamic range of the assay in line with criteria outlined by the manufacturer.

- Before the AMR testing is undertaken it is important that the laboratory ensures that the assay is calibrated to the manufacturers requirements and there are no recalibration / maintenance events scheduled.

- In this example all tests are performed within a single day usually with a minimum of 2 hours between each run and a run is defined as testing each of the AQP panel members at the same time. However, if this is not practical for the laboratory the time period can be extended accordingly, such that 1 full AQP panel is tested once a day across three days in order to obtain 3 replicate datasets for analysis.

- It is important that a different aliquot is used for each replicate within each run

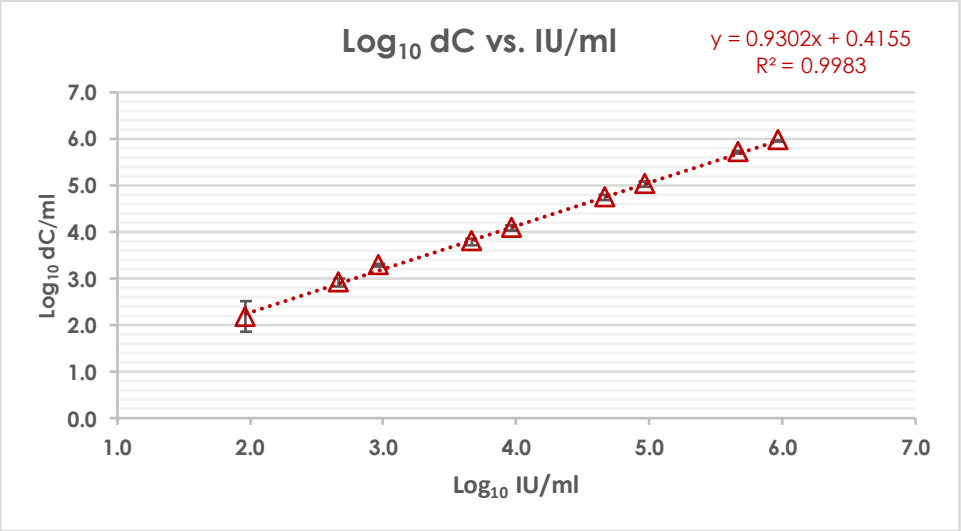
- The values obtained for each test replicate are recorded by the laboratory and reported 'on-line' through the web portal provided.

- Any missing points failed runs should be repeated and reported so that they can be investigated accordingly.

- Within the verification report, a plot of the measured values from the actual laboratory test against the relative concentrations in IU/ml and digital PCR (dC) will be provided (figure 1). This also takes into consideration potential outliers which are documented / subjected to outlier test (Grubbs test).

NOTE: *Where available, the assay manufacturer's instructions for verifying the AMR should be followed. The Laboratory Manager / Director must define limits for accepting or rejecting verification tests of the AMR based on the information provided within the V&V report.*

Figure 1: Linearity plot of EBV AQP points in Log digital Copies versus log IU/ml



NOTE: The AMR of the assay must be verified at least every six months after initial calibration. This is in line with the requirements set out in the CAP checklist (section MOL.31360, MOL.33860, MOL.33942, MOL.33983). In addition to this the frequency of calibration / calibration verification is determined in line with the following criteria:

- At changes of reagent lots, unless the laboratory can demonstrate that the use of different lots does not affect the accuracy of patient/client test results and the range used to report patient/client test data
- If QC materials reflect an unusual trend or shift or are outside of the laboratory's acceptable limits, and other means of assessing and correcting unacceptable control values fail to identify and correct the problem.
- After major maintenance or service
- When recommended by the manufacturer of the assay.

NOTE: It is important that the laboratory has a written policy defining the method, frequency and limits of acceptability of calibration verification for the instrument / assay and can fully demonstrate they have calibration records. The V&V report provided helps complement these requirements.

Day 2 & Day 3: Schematic diagram of laboratory test requirements

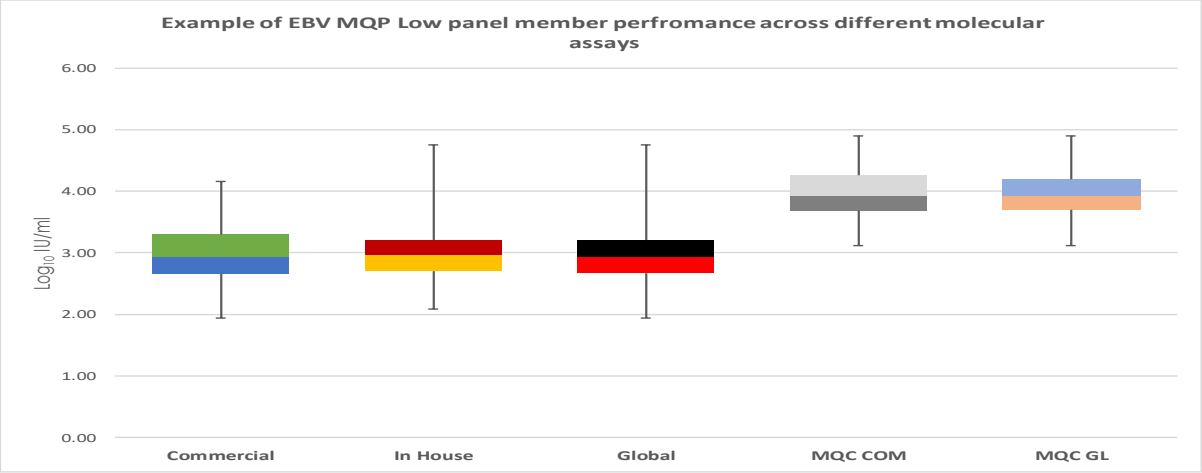
MQP Format												
Panel Member	Target Conc Log10 IU/ml		Number of replicates			Day 2 total test runs		Number of replicates			Day 3 total test runs	
High	1	4.7	1	1	1	3		1	1	1	3	
Med	2	3.7	1	1	1	3		1	1	1	3	
Low	3	2.7	1	1	1	3		1	1	1	3	
	4	0	1	1	1	3		1	1	1	3	
						Total tests	12				Total tests	12

2.2 Verifying Repeatability (within run precision) & Accuracy

Testing of the Qnostics molecular Q panel which consists of a high, medium, low, and negative sample in triplicate allows the laboratory to verify the within run precision and accuracy. The testing of the High, Medium, and Low designated samples from the Qnostics Analytical Q Panel (AQP) and from the Molecular Q panels allow the day to day repeatability to be evaluated within the laboratory.

The accuracy of the assay is established from evaluating the results obtained on the High, Medium, and Low panel members in comparative to the overall consensus of all Siemens users (within the multisite evaluation) and the consensus 'all' as determined against the data that Qnostics has compiled for the MQP panel samples across multiple labs and technology / assay groups (see figure 2 below). This enables the laboratory to comply with the CAP guidelines (MOL.31130) or equivalent.

Figure 2: Example of EBV MQP low sample accuracy across different molecular workflows



Molecular Workflow	Total Datapoints (n=)	Average Log IU/ml	Standard Deviation	95% CI
In-House	155	3.93	0.29	3.88-3.98
Commercial	315	3.97	0.40	3.92-4.02
Global	470	3.96	0.37	3.92-3.99

In figure 2, the "commercial" workflow group consists of all assays provided by a molecular IVD manufacturer. The "in house" group consists of laboratories using laboratory developed assays and "global" is the combination of both in house and commercial. The results were obtained across a 4-week period.

The testing is conducted over two separate days. This does not have to be consecutive days but should generally be in line with the routine testing timeframes within the laboratory. For example, if the laboratory only carries out the test once a day then the V&V testing plan should follow this timeframe.

Hence the above protocol can be modified or expanded in order to meet specific additional criteria and provides a means for estimating the repeatability and within lab precision and associated variation representative of that user assay in the routine clinical setting and relevant to the clinical utility of the assay – run to run, day to day and replicate to replicate. The repeatability and within lab precision established on the MQP panel will be reported accordingly (see example in figure 3).

Figure 3: Example table representing within lab precision across the MQP panel.

		Repeatability (within lab precision)	
Sample	**mean**	**SD**	**%CV**
High	4.61	0.20	4.30%
Medium	3.62	0.25	6.81%
Low	2.68	0.24	8.83%

3.0 Reproducibility across multiple sites using the same molecular platform and quantitative assay

In order to assess site to site variability (as well as operator to operator, instrument to instrument) as well as provide an estimate of reproducibility the testing outlined in the sections above would be conducted across a minimum of 3 sites over a minimum of 3 days. These do not have to be consecutive working days and the sites do not need to start or finish testing on the same days. Allowing laboratories 4 weeks in which to test and report their results back through the web-based portal should provide each laboratory with sufficient time to conduct the testing.

NOTE: As a general rule the same assay reagent lots should be used across sites when evaluating the assay in order to reduce the potential impact of variation introduced through different lots / batches of assay reagents.

NOTE: Each site will receive the same Qnostics materials for conducting the V&V evaluation

NOTE: Any scheduled calibration cycles within a site should be identified, recorded, and conducted in accordance with frequency designated by the manufacturer and/or regional regulatory authority.

3.1 Key points related to the multi-site reproducibility study (minimum 3 sites)

- Qnostics AQP and MQP products selected as they are representative of the types of sample and the spectrum of concentration encountered in routine clinical practice.
  - Consisted of whole pathogen in a clinically relevant matrix.
  - AQP includes distinct measurand levels spanning the assay analytical measurement range / dynamic range.

- At each of the 3 sites, aliquots of each of the AQP were assayed as unknowns in the same manner as patient samples on each of the specified days.

- Study involved a different instrument at each of the three site and different operators at each site, but all sites used the same assay reagent lots / calibrators.

- The manufacturers internal control was run in parallel to the control material provided for the V&V study. The values obtained for the manufacturers internal control were also collected during the V&V study.

- The assay was calibrated before the study and then again after the study.

- Data processing / V&V report generation – labs reported their data on-line when they obtained each result. Qnostics' collect and collate the data and establish site specific mean, SD and %CV for each sample tested at each site.
  - For each sample ANOVA is used to evaluate the variation and provide a measure of precision in relation to within-run (residual error) within day run to run, day to day sources of variation.
  - to allow for site specific estimate of repeatability and within lab precision across sites.
  - Estimate of reproducibility from a combined dataset using two-way ANOVA.

4.0 External Quality Control / Internal Quality Control (IQC)

Variability of results from molecular assays comes from a variety of different sources. Some of the more common sources of variation include run, reagent lot, calibrator lot, calibration cycle, operator, instrument, laboratory. Having established the extent and where known the clinical relevance of the variation it is important that the laboratory continues to monitor the variation to ensure that it remains within acceptable levels. This is done through the implementation of a quality control plan.

Figure 4: Source of variation within the molecular laboratory.

Device Type	Potential important source of variation	Comments
Automated molecular test	Run Day Reagent lot	Calibrator lot to lot variability is included in between lot component
Manual / semi-automated molecular test	Run Day Reagent lot Operator	Calibrator lot to lot variability is included in between lot component
Laboratory developed molecular test	Run Day Calibration cycle Calibrator lot Reagent lot Operator	Operator is important if test is manual or semi-automated. Reagent lots are important when reagent lots are laboratory developed.

As a result, many regulatory regulations (CLIA, ISO) include the requirement for the laboratory to run external and/or Internal Quality Controls on a regular basis for both quantitative and qualitative molecular assays. This is in addition to the 'built-in' controls provided by the assay manufacturer of the molecular assay. Many laboratories document their requirements

within their quality control plan. The quality control plan includes the monitoring of the extraction and amplifications phases of the molecular assay in line with the manufacturer's instructions.

In general, for qualitative tests a positive and negative control are included in each assay as specified in the laboratory procedure which takes into consideration the assay manufacturer's instructions. For multiple assay targets some laboratories may rotate different positive controls over a given time period at a frequency defined within their laboratory procedure (MOL.34229).

For quantitative tests such as EBV the laboratory should include controls at or near clinically relevant levels, where known (MOL.34270). The laboratory establishes the performance of the external control materials after the initial verification / validation phase. The protocol in section 4.1 below provided an outline for establishing external control limits.

4.1 QC Protocol Guidelines (20 Day QC Study)

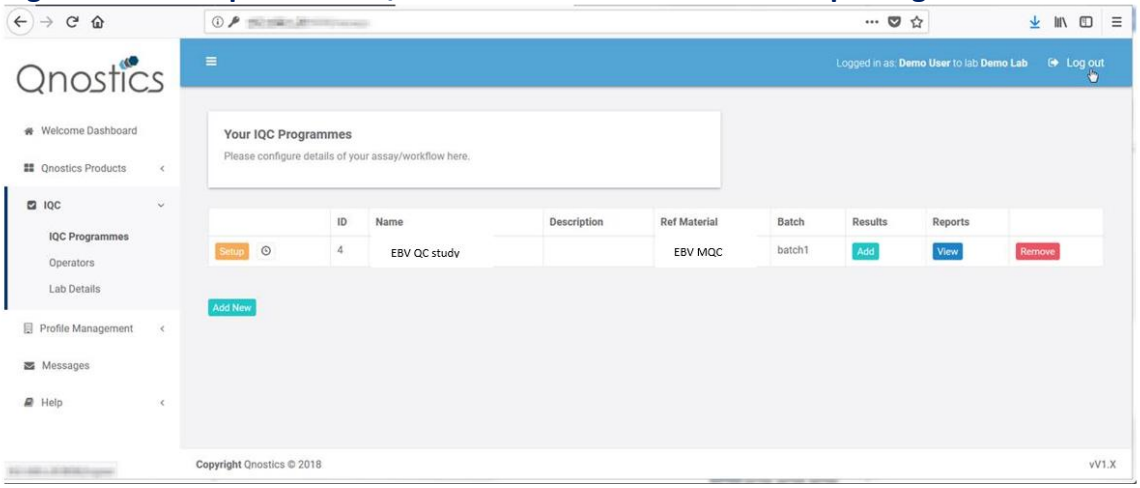
In order to establish the performance of the QC material within the individual laboratory it is standard practice to test the QC material over a period of time usually 20 days in order to obtain sufficient data points.

For regular monitoring of EBV quantitative performance we would initially recommend running both the high and low Q control (see figure 5) along with the negative control. Depending on the results obtained over time the laboratory may then decide reduce the frequency of testing of the high control or to only run the low control with each run.

NOTE: It is also important that the QC material should be from the same source as material used during the V&V study. This also means that the laboratory will already have established 3 to 7 data points for the QC material and will only need to complete the remainder of the 20 data points.

- *On each day of the QC study the laboratory tests the 3 QC controls. The management of the QC results can be facilitated through the on-line IQC software provided by Qnostics. Further details can be provided on request.*

Figure 5: On-line profile area / dashboard with access to IQC reporting software module



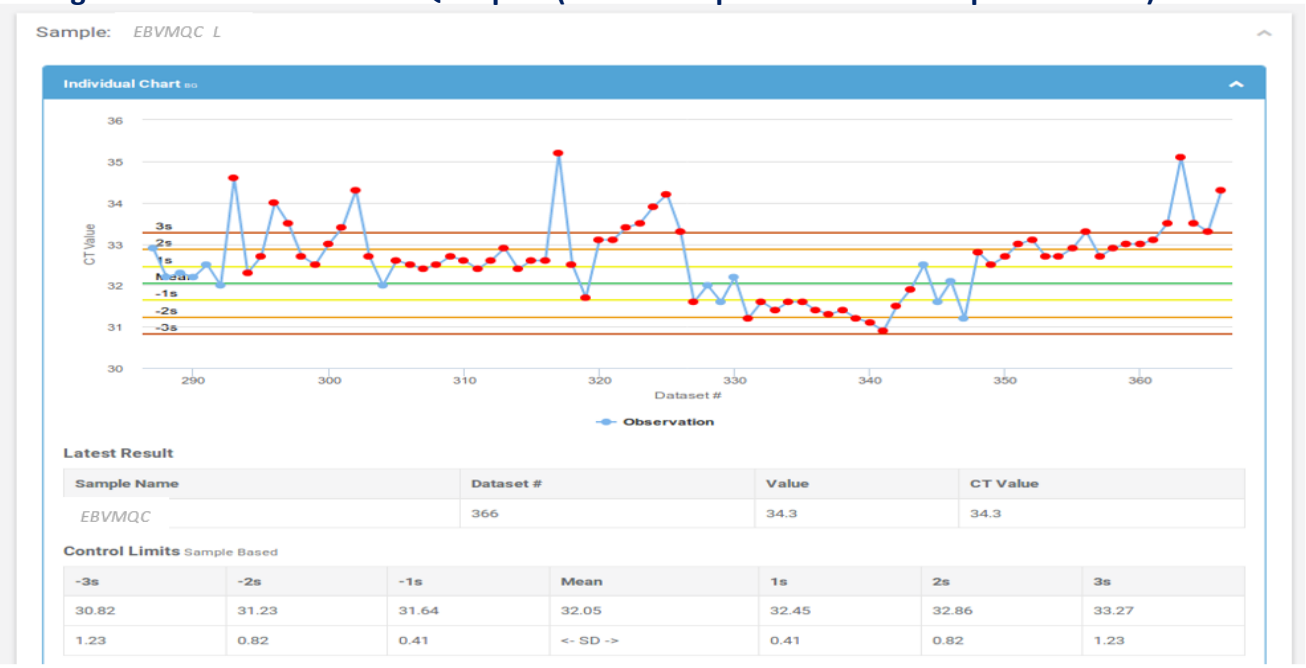
NOTE: Laboratories using more than one molecular instrument / platform should perform the 20 day study on one instrument and then perform comparison testing to confirm performance

on the other instruments. Alternatively, the laboratory could opt to run alternate QC runs across platform instruments. The laboratory director is responsible for determining the extent of the comparison studies and/or approach taken.

- The laboratory repeats the QC testing in order to obtain the 20 datapoints and entering them into the on-line dashboard. Once this has been achieved the software will automatically calculate and apply the control limits and acceptance criteria which the laboratory director can then approve.

- The results & QC reports can be viewed directly through the laboratories individual profile area, where the user has the option to select the time period / parameters and visualise the report on-line (see figure 6). The user also has the option to download the QC report or view on-line in real-time. The report setting can be further customised (Date range, operator, instrument, etc) dependant on user specific requirements.

Figure 6: On-line 'real time' QC report (note: example result values expressed in CT)



Latest Result

Sample Name	Dataset #	Value	CT Value
EBVMQC	366	34.3	34.3

Control Limits Sample Based

-3s	-2s	-1s	Mean	1s	2s	3s
30.82	31.23	31.64	32.05	32.45	32.86	33.27
1.23	0.82	0.41	<- SD ->	0.41	0.82	1.23

The interactive Individual laboratory control chart for each QC sample is used to display a laboratory's results against the established control limits (mean, ±1Standard Deviation (SD), ±2SD and ±3S lines are displayed). Data points are 'flagged' and colour coded in order to clearly show broken rules – which the laboratory can then use to monitor any potential issues such as reagent lot changes etc. Further parameters can be overlaid on the chart to enable further interrogation of specific parameters / events over time. These include but are not limited to the Operator, Specific reagent lot, instrument etc.

4.2 Post QC study - laboratory QC management solution

Participation in a high-standard quality control system is important for laboratories to help ensure reliability and precision of testing systems. The inclusion of the Qnostics online web-based solution supports the seamless transition from V&V study to routine QC and makes it simple and intuitive for the end user laboratory and provided a QC audit trial so that the laboratory can demonstrate their compliance to their regulatory organisation.