Should Calibration Curve Retire From Real-Time PCR Assays?

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Research

Keywords: real-time PCR, calibration curve, retrospective review, cycle threshold, copy number

Posted Date: September 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-827761/v1

Abstract

Real-time polymerase chain reaction (real-time PCR) is a biological technique that collects data of target nucleotides as PCR occurs by integrating fluorescent dyes as visual indicators into the amplification cycles. This enables the detection and quantification of the DNA segments in a sample through measurements of the fluorescent's intensity. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to pass a specified threshold and is inverse to the copy number, the initial number of nucleotides in the sample. Calibration curves are commonly used to approximate the copy numbers of experimental samples using standards with known copy numbers. This study is a retrospective review of historical data to help evaluate the efficacy and accuracy of calibration curves in a real-time PCR assay which have been used for screening of a genetic disorder in laboratories. The hypothesis is that including calibration curves in real-time PCR assays may decrease the screening specificity and accuracy, resulting in more false positives and additional retests. Three different scenarios were designed to replay the historical data and evaluate the relative accuracy of assays without calibration curves. The outcomes of all the scenarios conclude that calibration curves are not helpful for detecting target DNA fragments with low copy numbers, suggesting a reconsideration of their implantation in real-time PCR assays.

Background

Polymerase chain reaction (PCR) is a biological technique used to produce more copies of a specific DNA segment from a small quantity of nucleic acid molecules, which allows scientists to study the target region in finer detail (Sambrook and Russell, 2001; Wikipedia, 2021). The PCR is initialized by mixing the nucleic acid template with other reagents like a buffer, primers, free nucleotides, and DNA polymerase. The reaction takes place in a PCR thermocycler that heats and cools the mixture in preprogrammed cycles. The quantity of the DNA target segment is doubled at the end of each cycle. Newly amplified segments serve as templates in later cycles, allowing the DNA target to be exponentially reproduced into millions of copies. Real-time PCR is an application of PCR. Through the integration of fluorescent dyes into cycles of PCR amplification, real-time PCR enables the detection and quantification of a specific nucleic acid region through the measurement of the fluorescent's intensity since it gives a visual indicator for the accumulation of the target DNA segment after each cycle. Herein, the cycle threshold (Ct) is defined as the number of PCR cycles required for the fluorescent signal to cross a specified threshold (e.g., background level). This means that Ct values in real-time PCR are inversely proportional to the quantity of the starting nucleotides – the smaller the Ct value, the greater the beginning amount of the target region.

Introduction of real-time PCR to laboratories has facilitated monitoring of infectious diseases like novel coronaviruses (Corman et al., 2020) as well as screening of genetic disorders (Van Der Spek et al., 2015). For monitoring infectious diseases, real-time PCR assays are used to determine whether samples carry a nucleic acid target region with quantities or copy numbers higher than the cutoff. For screening of genetic deficiency, real-time PCR assays are designed to examine whether a target region in samples has lower

copies than the cutoff. Quantification through real-time PCR assays can be either relative or absolute (Dhanasekaran et al., 2010). In general, relative quantification is based on comparisons of target nucleic acid fragments with references of known and standard Ct values. In contrast, absolute quantification relies on a calibration curve of several standard points with known copy numbers to approximate the copy numbers of target fragments based on their experimental Ct values in a real-time PCR assay. However, calibration curves are often unreliable in quantifying the copy numbers (personal communication), especially for samples that have gone through many cycles, because the target approaches the limit of detection for PCR (Sambrook and Russell, 2001). For identical samples, discrepancies within the copy number observed though similar Ct values are observed across laboratories (personal communication).

This study evaluates the efficacy of calibration curves in a real-time PCR assay that has been deployed in public health laboratories for the screening of a genetic deficiency. In this real-time PCR assay, a calibration curve consisting of six standard points has been used to estimate the copy number of a target gene fragment that is linked to the production of proteins vital to the innate immune system. Any absence or significant reduction of this gene fragment would indicate a potential immunodeficiency. Here, a comprehensive evaluation of calibration curves in this real-time PCR assay was conducted through retrospective investigation of all the Ct values generated in the past several years in a public health laboratory. However, all information except the Ct values and the number of positive cases was not shared to the public (including the author) in order to ensure the privacy of the specimens and comply with confidentiality practices. This indeed helps validate the results as the study is designed to provide an outside evaluation on the calibration curve as a double-blind trial of retrospective review.

Data Generation Algorithm

The Ct values (Table 1) presented to the author for the data analysis and retrospective review were generated in the past several years by following certain algorithms as described below (Fig. 1). Each sample was initially screened in the real-time PCR assay. If a sample was determined to be positive in the initial screening, it would be re-tested in duplicate for confirmation. (a) In the initial screening stage, each sample was tested in the real-time PCR assay, and both the Ct value and the copy number were used to determine whether a sample was positive or negative. A sample with a Ct value less than 37.0 and a copy number equal to or greater than 10 based on the calibration curve in the initial screening was considered negative and no confirmatory retests would be needed for this sample. A sample with a Ct value of less than 37.0 but with a copy number less than 10 based on the calibration curve would undergo an initial test again. Finally, a sample with a Ct value equal to or greater than 37.0 in the initial screening would be presumed positive. (b) The initially presumed positive sample was further confirmed via the same real-time PCR assay in duplicate. A sample with a Ct value equal to or greater than 37.7 in the confirmatory tests was presumptive positive in the laboratory. Also, a sample with a Ct value less than 37.7 but a copy number less than 10 based on the calibration curve in the confirmatory test would be presumptive positive as well. (c) The presumptive positive sample in the laboratory would be further diagnosed by other methods to definitively confirm whether they were true positives.

Table 1
Example of Ct value data used in this study

Sample	Ct	Quantity	Y-Intercept	R2	Slope
1	36.563	3.394	38.132	0.986	-2.957
2	36.073	4.969	38.132	0.986	-2.957
3	37.161	5.9	39.744	0.986	-3.352
4	36.033	5.127	38.132	0.986	-2.957
5	36.328	4.074	38.132	0.986	-2.957
…	…	…	…	…	…
…	…	…	…	…	…
718931	36.184	4.556	38.132	0.986	-2.957
718932	31.595	643.25	40.62	0.959	-3.214
718933	32.1	589.403	40.604	0.957	-3.07
718934	32.533	328.474	40.62	0.959	-3.214
718935	31.186	1,169.16	40.604	0.957	-3.07

Table 2
Presumptive positives and their further assessment with other diagnostic method

Cases	Current	Strategy I	Strategy II	Strategy III
Presumptive Positives	189	161	121	162
Further Diagnosis				
• True positive	9	9	9	9
• Others	36	35	29	36
• Negative	144	117	83	117

Current Data Summary

A total of 3084 successful real-time PCR assays were included in this evaluation. All the data of Ct values from these assays were combined into a .csv file for analysis with the Pandas data analysis package (version 1.2.5) in Python (version 3.9.0). The combined data were then split into two categories or subfiles: a file containing all the single entries and another one containing all the duplicate entries. Single entries represent samples that underwent only one test in the initial screening stage and no confirmatory

retest was needed. Duplicate entries are samples that had been tested multiple times either in the initial or confirmatory stage. Description of the data in Pandas summarized that a total of 718,935 samples had been tested either once or multiple times in the real-time PCR assays. These samples were initially screened and 221 of them were finally presented as presumptive positives in the laboratory. Of these 221 samples, 189 had diagnostic results but the rest were not diagnosed due to other reasons. The diagnosis through other methods confirmed that 9 out of these 189 samples were true positives and the other samples were either negative or irrelevant cases (Fig. 2). Ct values for these presumptive positives varied from 35.604–40 (Fig. 3). While the cases for the true positives had Ct values all above 39.0, other irrelevant diagnoses showed relatively larger variation in their Ct values from 36.604–40.

Furthermore, in the 221 presumptive positives, there were 48 samples with Ct values straddling cut-off across multiple tests. In these 48 samples, 9 were not diagnosed due to other reasons, 3 were diagnosed as irrelevant, and the rest as negative. In addition, 5 samples had Ct values all below 37.7. They were considered presumptive positive in the laboratory due to having copy numbers less than 10 inferred from the calibration curves in the real-time PCR assays. For these 5 samples, one was not diagnosed due to certain reasons, but the other four samples were all diagnosed as negative. Below, all these real-time PCR assays were retrospectively reviewed to evaluate how the calibration curves affect presentation and identification of presumptive positives in the laboratory. The 32 presumptive positives that were not assessed through other diagnostic methods because of certain reasons are not included in the review process due to the lack of final diagnostic results.

Calibration Curves in Presumption of Positives

The data analysis revealed that a total of 2474 out of 718,935 samples were further repeatedly tested after initial screening. Out of the 2474 samples, 221 were presented as presumptive positives for further assessment as described above. The major question is how calibration curves included in the real-time PCR assays for inferring the target copy numbers have contributed to the identification of these presumptive positives. A total of 1450 samples had undergone initial tests multiple times solely due to their copy numbers being presumed to be less than 10 in one of the initial tests based on the calibration curves (Fig. 4).

This demonstrates that the rate of undergoing multiple initial tests attributed to calibration curves was relatively high at 58.6% (1450/2474). However, the actual result shown by the lab was the opposite. Out of the 1450 repeated samples, only 28 were identified in the lab as presumptive positives requesting for further assessment. This means that the rate for identification of presumptive positives contributed by calibration curves is as low as 2% (28/1450) and the rate of retest attributed to calibration curves is over 98% ((1450-28) / 1450). Further, all these 28 presumptive positives do not have the true positive phenotype according to further assessment, in which 26 were diagnosed negative, and 2 were diagnosed as irrelevant. The root cause for the high rate of false retest is the variation of the standards themselves. Each standard varied on the Ct values about almost 2 cycles, e.g., standard 1 (S1) from 35–37 and standard 3 (S3) from 32–34 (Fig. 5), which is flawed for accurately inferring reliable copy numbers. In

contrast, the other 193 presumptive positives (221 − 28) were presented after repeated testing of 1024 samples (2474 − 1450) solely based on their Ct values. The rate for identification of presumptive positives is 19% (193/1024), much higher than 2% in the retest based on standards.

Calibration Curves in Presumption of Positives

All the initial and confirmatory tests were virtually replayed retrospectively to evaluate how the omission of calibration curves in real-time PCR assays would affect the data presentation. Three replaying strategies were presented in this study to cover the possible impacts and solutions after omission of calibration curves in the real-time PCR assays. Also, in the replay process, the possible impact on data presentation after omitting calibration curves was maximized by using the following criteria.

(1) Any samples with a Ct value less than the screening cut-off (37.0) in the initial assay or confirmatory retest would be categorized as "negative" and no confirmatory retest would be required. It should be noted that among these "negative" samples, a very small portion had been identified as "presumptive positives" in the lab either due to their target copy numbers (< 10 derived by the calibration curves) or Ct values (≥ 37.7 in some of the repeated tests). The samples that would be categorized as "negative" in the replay process but were identified as presumptive positive in the lab would be queried with the other diagnosis results to determine if they were false negatives.

(2) Any samples with all Ct values ≥ 37 and at least one Ct value ≥ 37.7 in the initial screening or confirmatory retest would be categorized as presumptive positive according to the current testing and follow-up procedure. The other diagnosis results would then be queried to determine if there were any false positives.

(3) Samples that all had Ct values between 37–37.7 in the initial screening and confirmatory retest and were presented as presumptive positives due to the derived target copy numbers (< 10) would be categorized as "negative" in the replay process. These samples would be also checked with the other diagnosis results to determine if there were any false negatives.

Strategy I

In this strategy, the data replay would follow the actual order of the lab testing for each sample, which means that the initial screening would be considered as the first run and the retests would be repeated runs. The omission of calibration curves in the initial screening would impact 109 samples. These 109 samples had Ct values below 37.0 in the initial screening but were retested because of their target copy numbers being less than 10 based on the calibration curves. The retests determined that 106 of them were negative and 3 of them were presumptive positive. The further assessment confirmed that these 3 samples were also negative. In the scenario of this strategy replaying data without considering calibration curves, these 109 samples would have been reported as negative in the initial screening and thus not trigger the retest. This would result in reducing 4% (109 / 2474) of samples that had required retests due to calibration curves.

As described earlier, 1450 samples had been repeatedly tested at least once due to one of the calibration curves showing their target copy numbers to be less than 10. Therefore, aside from the above 109 samples affected in the initial tests, the other 1341 samples (1450 − 109) would be affected in the confirmatory retests with omission of calibration curves. With calibration curves in the real-time PCR assays, 25 presumptive positives were identified from these 1341 samples after one or more times of retests. The query with further assessment results confirmed that 23 out of these 25 presumptive positives were negatives, and that 2 were false positives with other irrelevant diagnosis results. In other words, if calibration curves were omitted in the real-time PCR assays, these 1341 samples would be retested only once and then reported as negative – a significant improvement.

Strategy II

In this strategy, the data replay would not consider whether Ct values for a sample were from the initial screening or confirmatory retest. For example, if a sample had an initial Ct value of 37.875 and confirmatory Ct values of 37.825 and 36.538, it would just be reported as negative and would not require a confirmatory retest. This is because, when replaying the data for this sample, the author would consider the possibility that the Ct value 36.538 could be from the initial screening. In this way, it would be able to maximize the possibility of influence on the data presentation if the calibration curves would be removed.

Compared to the current data presentation with calibration curves, a total of 2335 samples would have been possibly screened as negative, avoiding the confirmatory retest in this replay strategy because at least one of their Ct values was below 37.0; therefore, they might have been screened as negative in the initial test without triggering the request for confirmatory retest. This replay strategy would also possibly lead 68 presumptive positives to be reported as negative without requesting the further assessment because one of their Ct values was below 37.0. Indeed, these 68 samples were all false positives according to the query with further assessment results. They included 7 samples which were diagnosed as irrelevant and 61 samples which were diagnosed as negative (Figs. 6 & 7).

Strategy III

As discussed above in strategy II, 68 presumptive positives would be possibly identified as negative without requesting further assessment because there was at least one Ct value below 37.0. In these 68 samples, 61 were negative and 7 were diagnosed as irrelevant. These 7 irrelevant samples had Ct values above 36.0 in all of the tests. Therefore, in the principle of not missing other irrelevant cases, strategy III would lower the initial screening cut-off to 36.0 as compared to the replay in strategy II. If the Ct value 36.0 was used as the initial cut-off, the retest rate would be 0.33% ((2474-89) / 718935) based on the available data in this study. Compared to the current data presentation in the lab, a total of 2138 samples would be possibly screened as negative, avoiding confirmatory retests in strategy III, because at least one of their Ct values was below 36.0; therefore, they might be screened as negative in the initial test without prompting a confirmatory retest. Strategy III would also possibly lead 27 presumptive positives to be reported as negative without requesting clinical assessment because one of their Ct values is below 36.0.

These 27 samples had been presented as presumptive positives in the lab (due to straddling cut-off across multiple assays), and the query with further assessment results confirmed that they were negative.

Discussion & Conclusions

High variability in an assay should not be expected in a lab over time. The data replay process in this study suggests that the real-time PCR assay had a relatively high rate of false positive (95% = 180/189) results. Even after considering irrelevant cases, the rate of false positives is still very high (76% = 144/189). In the retrospective replays of this study, there are two essential questions needing to be answered regarding assay sensitivity and specificity. They are: (1) "Would there be any false negatives out of the known true positives in the replay strategies where calibration curves are omitted from the real-time PCR assays?" and (2) "Would there be any false positives out of the negatives in the replay strategies where calibration curves are removed from the real-time PCR assays?" The replay process has shown that the answers to both questions are NO in all three replay strategies. The retrospective reviewing of the real-time PCR assays either, blindly in strategies II and III or not blindly in strategy I, was able to identify all (100%) of the true positive cases (Table 1). While irrelevant cases besides the true positives have also been detected by the real-time PCR assays, the omission of calibration curves might result in less irrelevant cases being identified. This is because, relative to the true positives, irrelevant cases might show low or undetectable copy numbers in the assays. Adoption of the initial cut-off at Ct value 36.0 as reviewed in strategy III might increase the chance of identifying irrelevant cases other than the true positives in the real-time PCR assays. However, if the focus of the laboratory is primarily on the true positives, the current initial cut-off at Ct value 37.0 as reviewed in the strategy II is sufficient.

Additionally, retrospective investigation of the data generated in the past several years has revealed that samples have been frequently requested to retest due to a low copy number derived from calibration curves. Notably, all the true positives identified in the lab in the past years were attributed to the Ct values, not the copy numbers derived from calibration curves. Instead, all the cases that had Ct values (< 37.7) but were considered positive based on calibration curves were further diagnosed negative. This suggests that the false derivation or misrepresentation from calibration curves is very high. This likely occurs due to overconfidence in the accuracy and reliability of the calibration curves. The omission of calibration curves in the real-time PCR assays would significantly reduce the false positive results and misrepresentation rate by forcing researchers to use alternative, more reliable methods. Finally, the strategies used in this study would also provide a good model for future retrospective studies.

Declarations

Acknowledgment

N/A

Funding

N/A

Authors' contributions

N/A

Ethics Approval

N/A

Consent for Publication

N/A

Availability of Data and Materials

The author confirms that the data supporting the findings of this study are available within the article.

Competing interests

The author declares that there is no competing interest.

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Figures

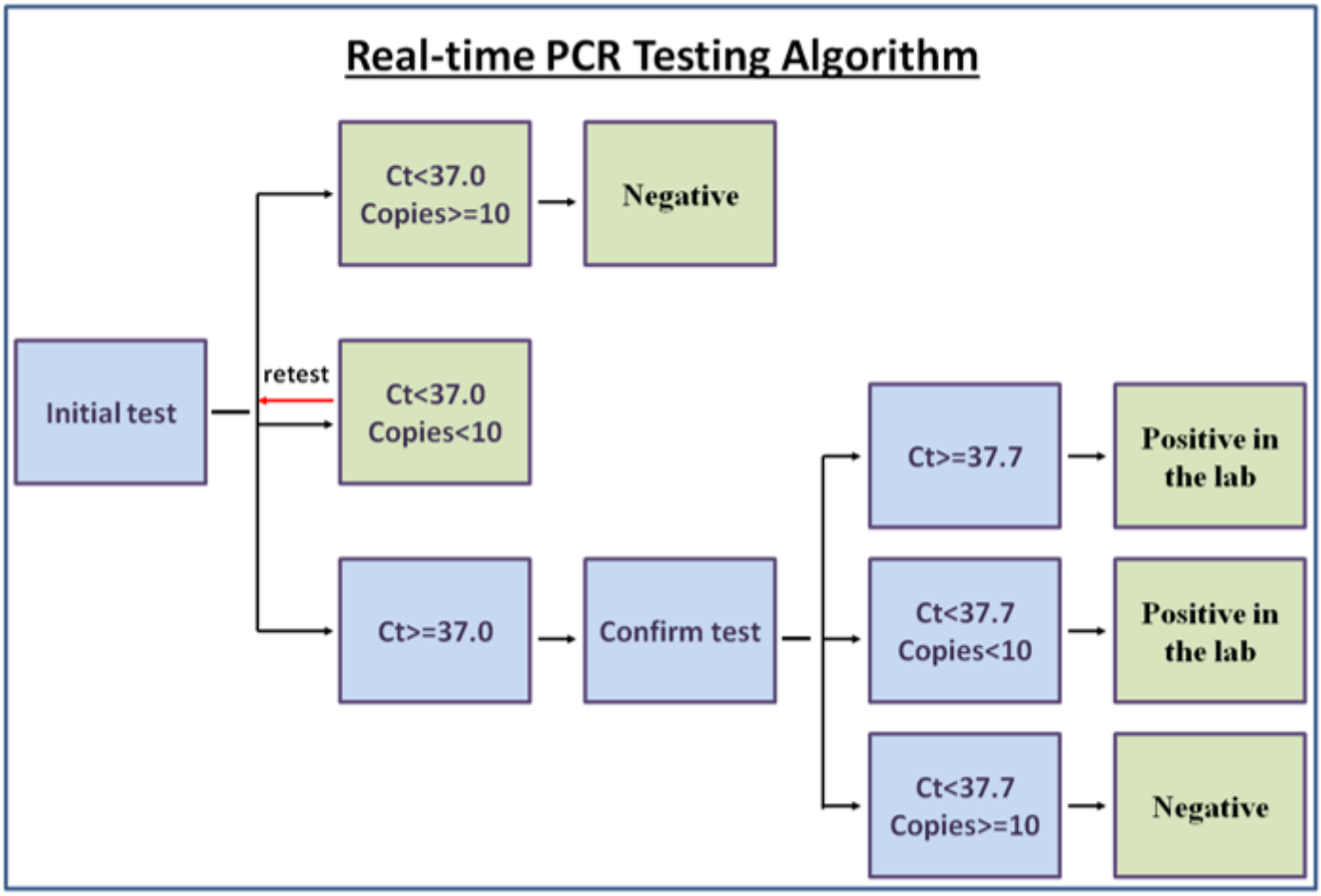

Figure 1

Diagram of the real-time PCR testing algorithm in this study.

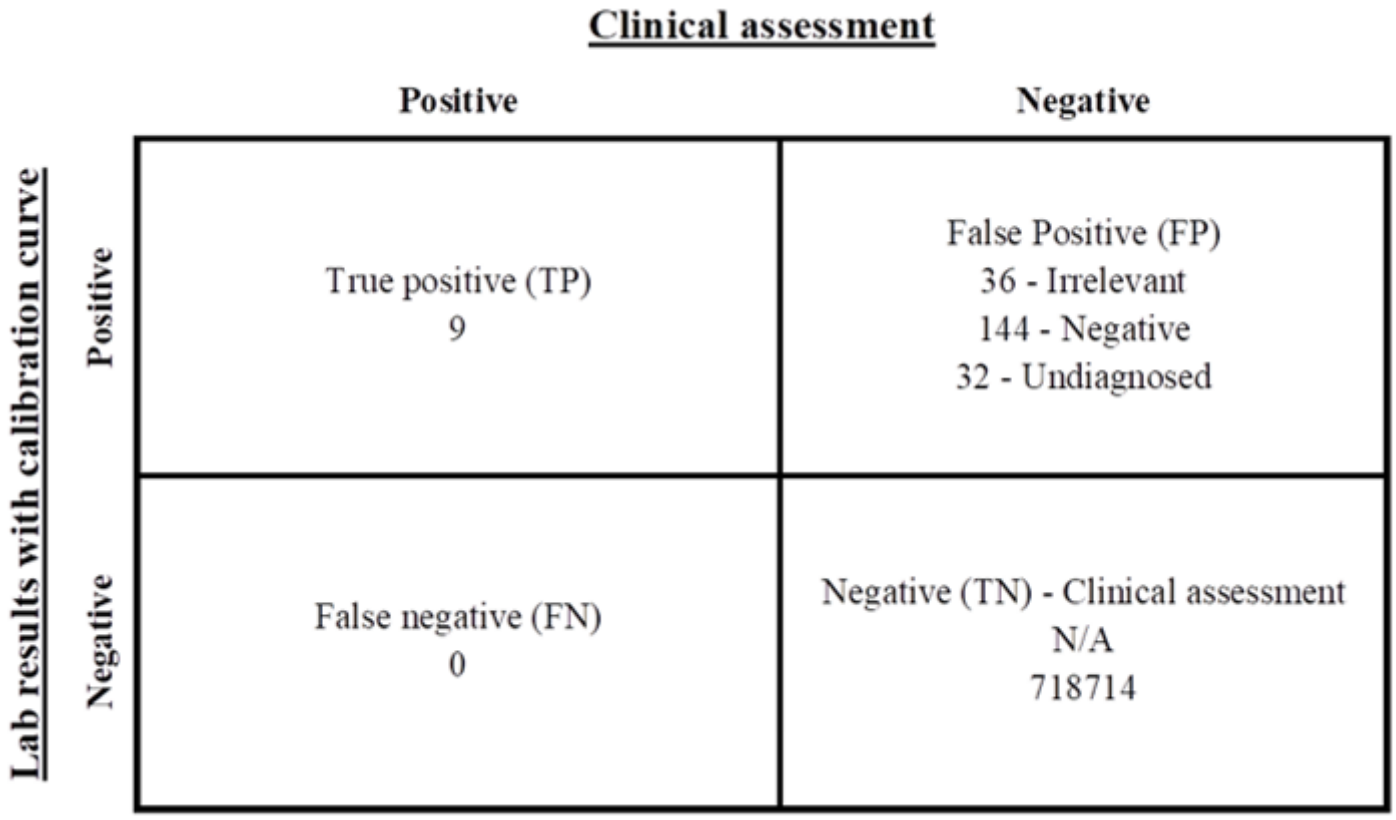

Figure 2

Laboratory identified presumptive positives vs. further assessment results.

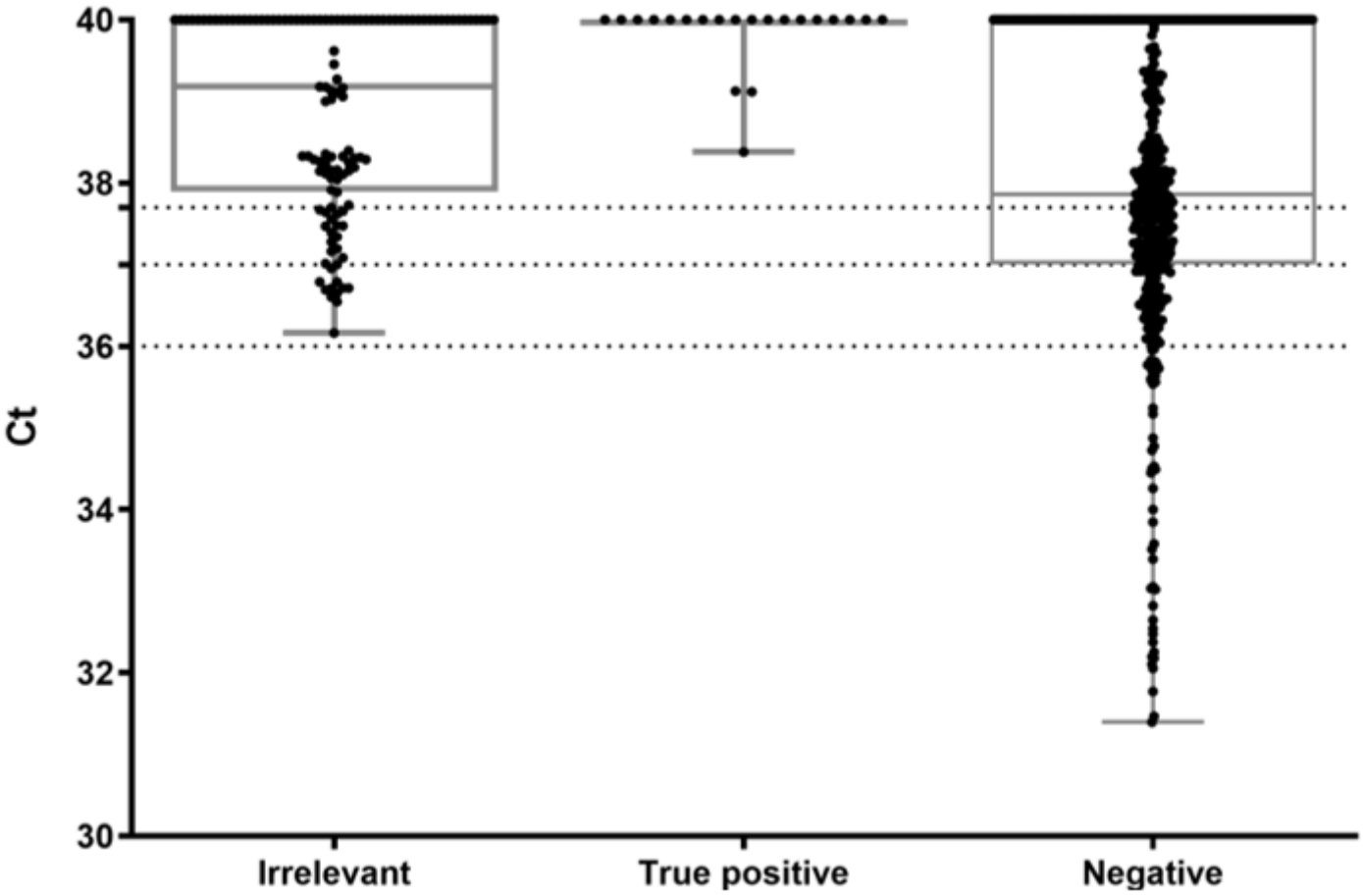

Figure 3

Variation of Ct values for the laboratory identified presumptive positives.

Figure 4

Repeated tests due to calibration curves and Ct values.

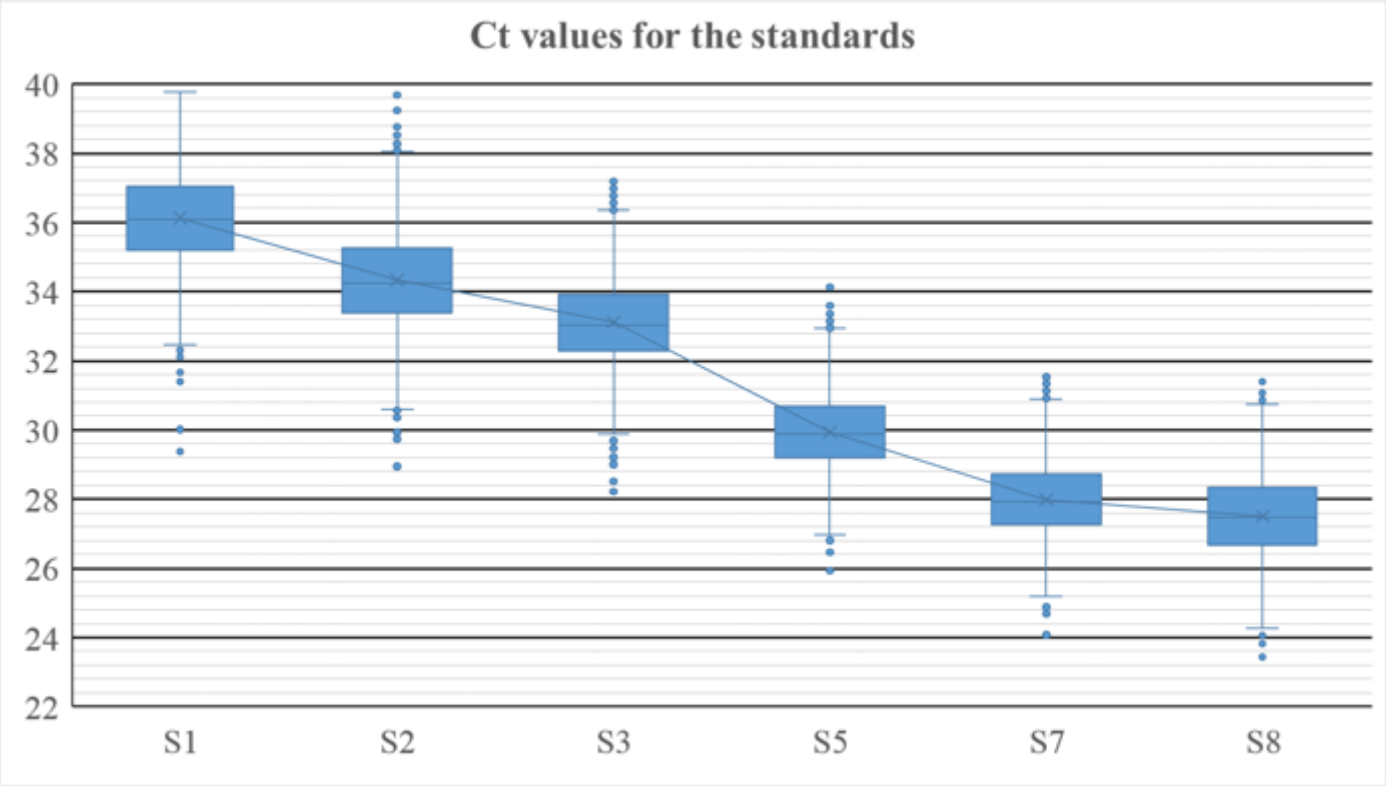

Figure 5

Ct values for the standard points of the calibration curves.

Clinical assessment results

Lab results w/o calibration curve	Positive	Negative
Positive	True positive (TP) 9	False positive (FP) 29 - Irrelevant 83 - Negative
Negative	False negative (FN) 0	Negative (TN) - Clinical Assessment not required 718782

Figure 6

Laboratory identified presumptive positives vs. further assessment results.

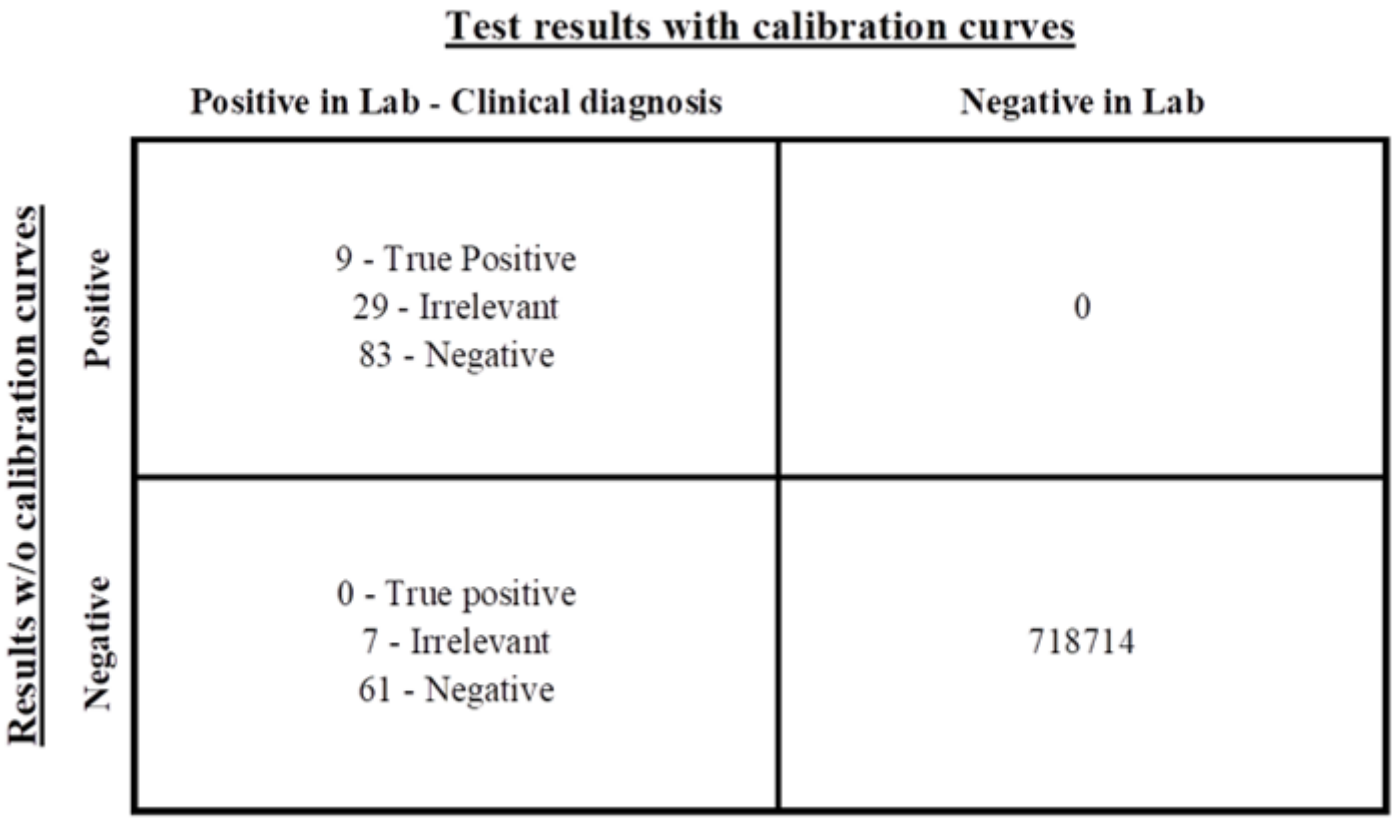

Figure 7

Comparison of test results in the real-time PCR assay with and without referring to calibration curves.