Uncertainty of Reported Results in Molecular Diagnostics

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Abstract
With qualitative PCR tests, there are two types of reported results: target not detected and target detected. With quantitative PCR tests, there are four types of reported results: target not detected; target detected, but below the lower limit of quantitation; quantitative; target detected, but above the upper limit of quantitation. The number of copies of target nucleic acid varies randomly in randomly drawn samples. Formulas based on cumulative Binomial and asymptotic cumulative Poisson probabilities, quantifying the uncertainties of the above types of reported results as functions of expected numbers of copies in test samples, are derived and graphed. It is shown that Poisson approximation errors are small, and simplified Poisson-based formulas can be used for quantifying the uncertainty of the types of reported results. The uncertainty of the quantitative results is characterized by the repeatability and reproducibility standard deviations along with the respective numbers of degrees of freedom estimated with data. This work helps in clear understanding of the uncertainty of reported results, which in-turn helps laboratorians interpreting and QC test results, clinicians diagnosing and monitoring treatment of disease, blood banks evaluating the risk of transmission of infectious agents from donors to recipients, pharma evaluating the efficacy of new medications, etc.

Key Words: Qualitative and Quantitative Tests, Types of Reported Results, Probabilities of Various Types of Reported Results, Repeatability and Reproducibility

1. Introduction

The purpose of this paper is to explain the uncertainties of two types of results reported with *qualitative* PCR tests, described in Section 2, and four types of results reported with *quantitative* molecular diagnostic tests described in sections 3, as well as of the quantitative results described in section 4. Clear understanding of the uncertainties of reported results is important for correct interpretation of those by the users – clinical laboratory, medical and research personnel. An explanation with a graph of the probabilities of reported results of a PCR-based quantitative molecular diagnostics test was published by Bryan Cobb et al[1]. Three types of reported results were discussed: (1) target not detected (*TND*), (2) target detected, but below the lower limit of quantitation (< *LLoQ*), and (3) quantitative. The probabilities of the three types of reported results were illustrated with a graph for the case of a single copy per PCR detectable. The method of calculation of the probabilities was not described. The fourth type of reported result, target detected, but above the upper limit of quantitation (> *ULoQ*), was not considered. The uncertainty of quantitative results was not discussed. The uncertainty of the

reported results with qualitative PCR test was not discussed either. Described in this paper are derivation of the formulas for calculating probabilities of each of the two types of reported results obtained with qualitative PCR-based assays and of the four types of reported results obtained with quantitative PCR-based assays in the general case of the number of copies required for detection, $v \geq 1$. Also described is method of characterization of uncertainty of the quantitative results.

TND result is reported when the number of extracted / reverse transcribed copies of target nucleic acid per PCR, obtained from a randomly drawn test sample, is insufficient for detection. In case of assay with no restriction on the number of amplification / detection cycles, *Ct*, the number of cycles is sufficient for detection of a single copy per PCR. Subsequently, the *TND* is reported when there are no extracted / reverse transcribed copies of the target nucleic acid available for amplification and detection. In case of assay with a restriction on the number of amplification / detection cycles (*Ct* cutoff), *TND* is reported when the number of extracted / reverse transcribed copies of target nucleic acid per PCR is less than *v*, insufficient for amplification and detection.

Less than the lower limit of quantitation result is reported when the number of extracted / reverse transcribed copies of target nucleic acid from a test sample is larger than the minimum number, *v*, required for detection, but smaller than the lower limit of quantitation, *LLoQ*. The *LLoQ* is defined as the lowest analyte concentration meeting the goal for the total analytical error, and it cannot be lower than the limit of detection, *LoD*[2], and the lower limit of the linearity interval[3].

Quantitative reported result, calculated using calibration math model from *Ct*, is reported when the number of extracted / reverse transcribed copies of target nucleic acid from a test sample is within the measuring interval [*LLoQ*, *ULoQ*].

The upper limit of quantitation, *ULoQ*, is defined as the highest concentration of the target nucleic acid that meets the goal for the total analytical error[2], and it does not exceed the upper limit of the linearity interval[3]. It is reported when the number of extracted / reverse transcribed copies of target nucleic acid at PCR input exceeds *ULoQ*.

The qualitative PCR assays have two types of reported results: target detected (TD) and target not detected (TND).

Table 1 summarizes the relationship between the number of target copies per PCR, extracted from a sample tested, and the type of reported result for a molecular diagnostics test; $v \geq 1$ is the minimum number of copies per PCR required for detection.

Table 1. Types of Reported Results with PCR Tests

Number of Target Copies per PCR, x	Type of PCR Test	Type of Reported Result
$x \geq v$	Qualitative	*TD*
$x < v$	Qualitative & Quantitative	*TND*
$v \leq x < LLoQ$	Quantitative	$< LLoQ$
$LLoQ \leq x \leq ULoQ$	Quantitative	Quant
$x > ULoQ$	Quantitative	$> ULoQ$

2. Probabilities of two types of results reported with qualitative PCR assay

With sufficient number of amplification cycles for detection of a single copy of target nucleic acid per PCR, a single copy of a target nucleic acid extracted from a test sample, and if needed reverse transcribed, is always sufficiently amplified and detected. In some cases, the clinical utility of a PCR assay improves with $v > 1$ copies required for detection. This is achieved by restricting the number of amplification and detection cycles, Ct. Such are the cases with some microbiology tests when it is desirable to reduce effect of detection of dead bacteria and when the rate of false positives, caused by cross-contamination, needs to be reduced. The target is not detected, and the test result is reported as *TND* only when there are less copies of target nucleic acid, extracted / reverse transcribed, available at the PCR input than the minimum required number, v. With:

- samples randomly drawn from large pool with mean number, μ, copies of target,
- probability of extraction/reverse transcription, θ,
- $v \geq 1$ minimum number of copies required for detection,

the probability of the test samples having $x < v$ target copies per PCR is the probability of non-detection described with the cumulative Poisson distribution[2], and it is the expected proportion of *TND* results:

$$P(TND) = P(x < v) = \sum_{x=0}^{v-1} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \qquad (1)$$

Probability in (1), cumulative Poisson, is easy to calculate, e.g., with Microsoft Excel® function poisson($v - 1$, $\mu\theta$, true). Formula (1) is simplified when $v = 1$:

$$P(TND) = e^{-\mu\theta} \qquad (1,a)$$

The expected proportion of the target detected, *TD*, results is the probability of detection complementary to the probability of non-detection in (1). Graph of probability of detection (the expected proportion of 'target detected' reported

results) vs. concentration – mean number of target copies per test sample volume – for several minimum numbers of target copies required for detection is given in Fig. 1. The curves in the graph from left to right are for v values listed in the legend from top to bottom.

3. Probabilities of four types of reported results with quantitative PCR assay

The expected proportion of results reported as target not detected, *TND*, with quantitative PCR assay is the same as with the qualitative assay calculated with formula (1). The expected proportion of test results reported as "$< LLoQ$" is the probability of the of the test samples with x target copies per PCR on the interval $[v, LLoQ)$, asymptotically (for large sample pool volume) described with the Poisson distribution:

$$P(< LLoQ) = P(v \le x < LLoQ) = \sum_{x=v}^{LLoQ-1} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \qquad (2)$$

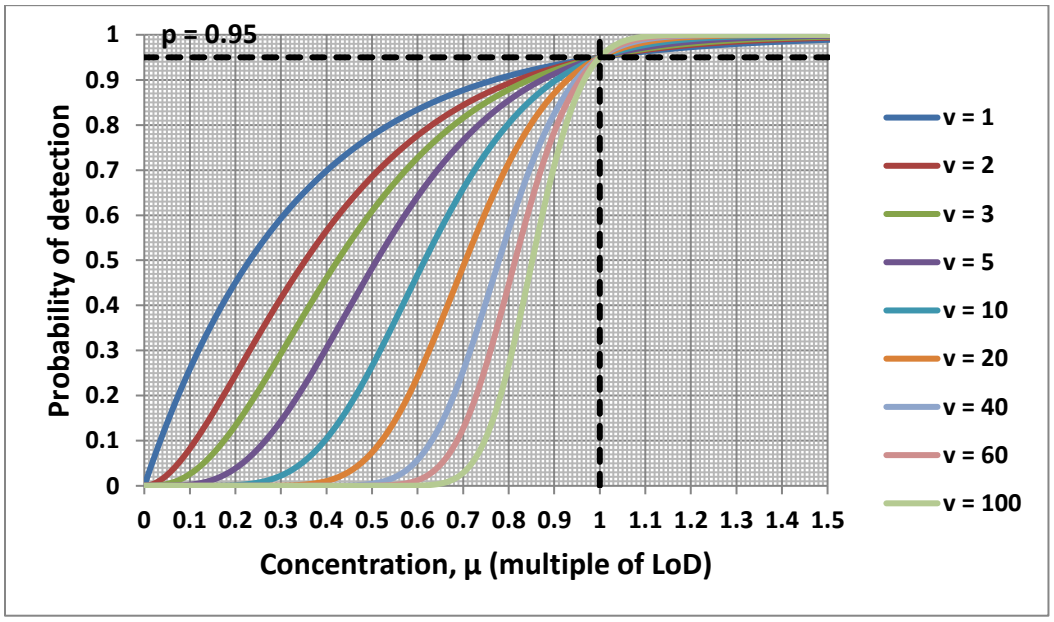

Figure 1: Probability of detection vs. concentration as multiple of LoD

The probability of "$< LLoQ$" reported result (2) can be easier calculated as:

$$P(< LLoQ) = \sum_{x=0}^{LLoQ-1} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} - P(TND) \qquad (3)$$

The first term in (3) is cumulative Poisson that is easy to calculate in Microsoft Excel® using function poisson($LLoQ - 1$, $\mu\theta$, true). The expected proportion of

test results reported as quantitative is the probability of the number of x target copies per PCR to be on the interval [$LLoQ$, $ULoQ$]:

$$P(Quant) = P(LLoQ \le x \le ULoQ) = \sum_{x=LLoQ}^{ULoQ} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \quad (4)$$

The probability of *Quant* reported result in (4) is easier to calculate as:

$$P(Quant) = \sum_{x=0}^{ULoQ} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} - P(< LLoQ) - P(TND) \quad (5)$$

The first term in (5) is cumulative Poisson that is easy to calculate in Microsoft Excel® using function poisson($ULoQ$, $\mu\theta$, true). The expected proportion of test results reported as "> $ULoQ$" is the probability of the number, x, of target copies per PCR in test sample to be greater than $ULoQ$:

$$P(> ULoQ) = P(x > ULoQ) = \sum_{x=ULoQ+1}^{\infty} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \quad (6)$$

The probability of "> $ULoQ$" reported result in (6) is easier to calculate as:

$$P(> ULoQ) = 1 - \sum_{x=0}^{ULoQ} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \quad (7)$$

The second term in (7) is the cumulative Poisson that is easy to calculate in Microsoft Excel® using function poisson($ULoQ$, $\mu\theta$, true).

The sum of the proportions of the four types of reported test results for the same target concentration equals to Poisson probability of the number of copies per PCR, x, to have any integer value in the range from 0 to infinity, which equals 1.

The extraction (and reverse transcription) efficiency in (1) to (7) is[3]:

$$\theta = \frac{\ln(20)}{LoD} \quad (8)$$

LoD = the limit of detection in case a single copy per PCR is detectable, $v = 1$.

Figure 2 shows the probabilities of the four types of reported results for $LLoQ = LoD = 20$ cp/mL with $v = 1$ - no restriction on the number of cycles, and single copy per PCR detectable. Figure 3 shows the probabilities of the four types of reported results for $LLoQ = LoD = 504$ cp/mL with $v = 62$. $ULoQ = 10^7$ cp/mL in both examples.

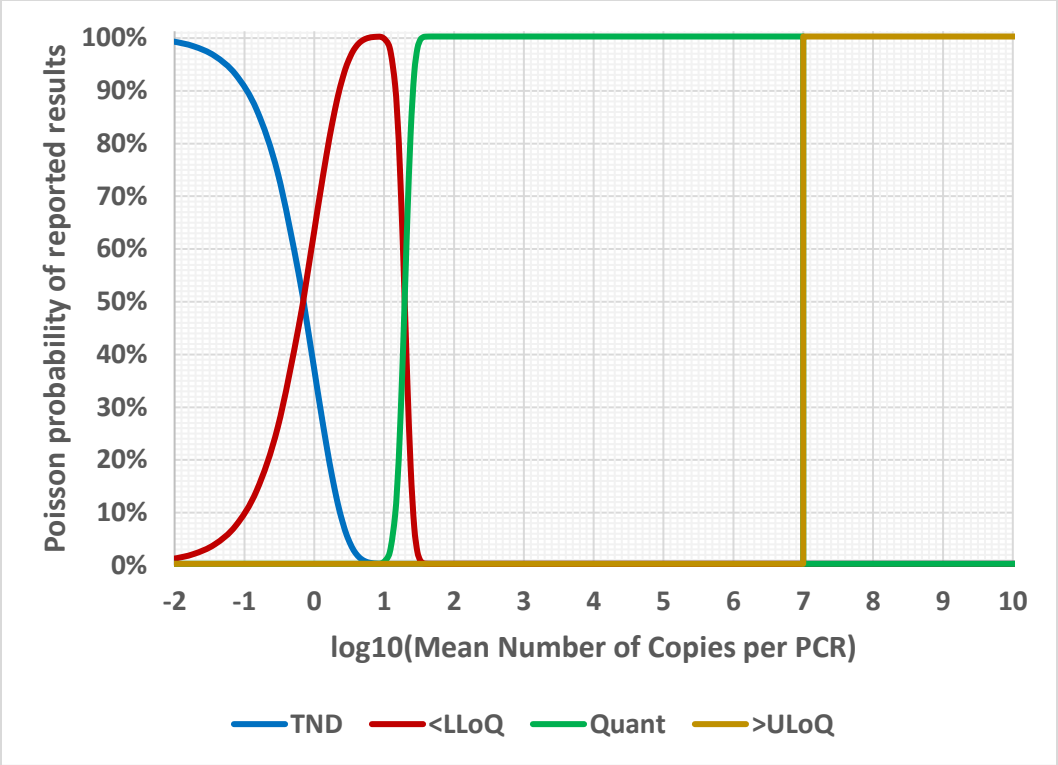

Figure 2. Probabilities of four types of reported test results vs. target concentration, $v = 1$

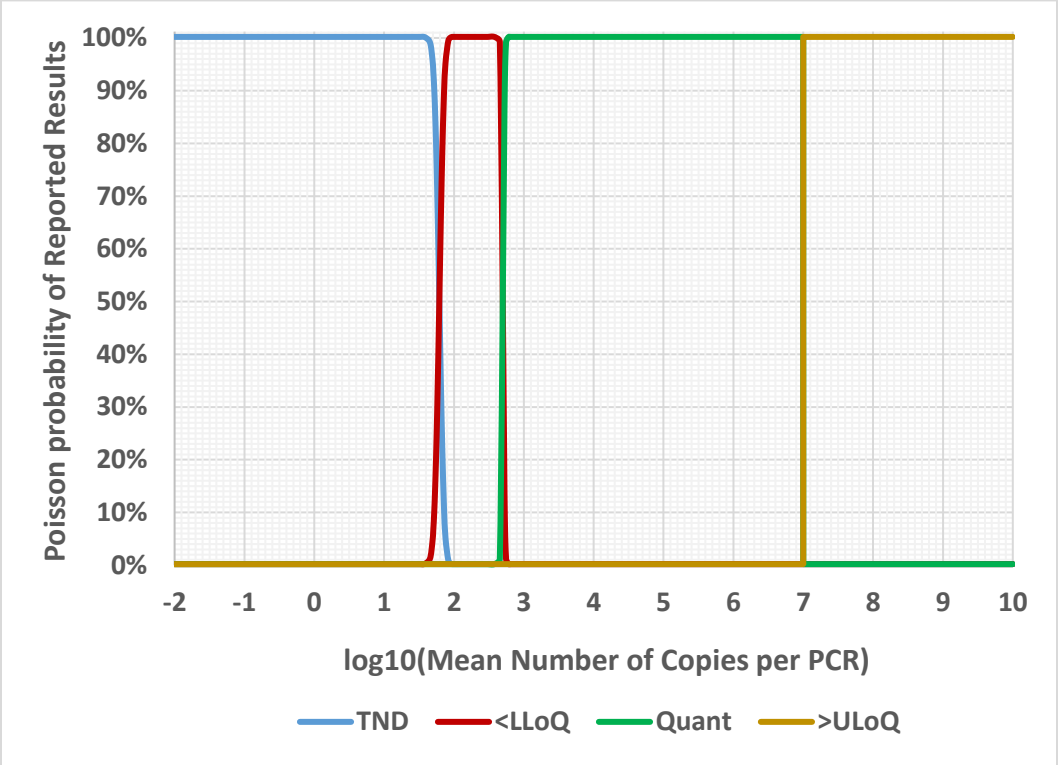

Figure 3. Probabilities of four types of reported test results vs. target concentration, $v = 62$

As noted before, in the graphs, the sums of the probabilities (expected proportions) of four types of reported test results are equal to 1 for any sample concentration. In the examples presented in Figures 2 and 3, under the Poisson model:

- Any test sample randomly drawn from a large pool can produce any one of the four types of the reported results with the probabilities that are functions of the target concentrations described by equations (1) to (7).
- Ignoring possible small effects of cross-contamination or cross-reactivity, the proportion of *TND* result is 1, while the proportions of three other types of reported results are zeros, when the target nucleic acid concentration in the pool is zero.
- The proportion of Quant results approaches 1, while the proportions of three other types of reported results approach zeros as the pool concentration exceeds about 2 x *LoD* and is below *ULoQ*.
- The proportions of Quant and >*ULoQ* results are both ~0.5 at *ULoQ* concentration, while the proportions of *TND* and <*LLoQ* results are practically 0. At concentrations exceeding the *ULoQ* by less than 1%, the proportion of >*ULoQ* results is practically 100% and the proportions of all other types of results are practically 0.
- The probability curves in Figure 3 with v = 62, are steeper than those in Figure 1 with v = 1 and are shifted to the right since more target copies are required for detection.
- With single copy detectable,
    - quantitative tests on specimens having *LLoQ* = *LoD* concentration, 57.6% results are quantitative, 37.4% results are < *LLoQ*, 5% results are *TND*, and ~0% of results are >ULoQ, summing up to 100%.
    - The proportion of < *LLoQ* results attains maximum of about 58.5% at the concentration of about 0.46 *LoD*.

4. Cumulative Poisson approximation of cumulative Binomial probabilities

The expected proportions of the four types of the reported test results with finite volumes of the pools from which test samples are randomly drawn are somewhat different. They are described by the Binomial probability distribution and get closer to the results obtained under Poisson model as the number of test samples that can be drawn from the pool - pool volume - increases. Tables 2 and 3 show extreme values of the Poisson – Binomial probability difference along with the target concentration and the smaller Binomial probability. The largest difference with a small pool of $k = 10$ sample volumes is below 0.02, and it is below 0.002 with a pool size of $k = 100$.

Table 2. Extreme values of the Poisson – Binomial (k = 10) probability differences of types of reported results along with corresponding target concentrations (cp/PCR) and Binomial probabilities of reported results

	TND	<LLoQ	Quant	>ULoQ
min diff	0	-0.0192	-0.0136	-0.0127
cp/PCR	>17	1	24	~ULoQ
Binom Prob	0	0.651	0.833	0.857
max diff	0.0192	0.0136	0.0127	0.0127
cp/PCR	1	24	~ULoQ	~ULoQ
Binom Prob	0.349	0.167	0.143	0.159

Table 3. Extreme values of the Poisson – Binomial (k = 100) probability differences of types of reported results along with corresponding target concentrations (cp/PCR) and Binomial probabilities of reported results

	TND	<LLoQ	Quant	>ULoQ
min diff	0	-0.0018	-0.0013	-0.0012
cp/PCR	>15	1	24	~ULoQ
Binom Prob	~0	0.632	0.821	0.830
max diff	0.0018	0.0013	0.0012	0.0012
cp/PCR	1	24	~ULoQ	~ULoQ
Binom Prob	0.368	0.179	0.155	0.159

The graphs of differences of cumulative Poisson – Binomial probabilities – cumulative Poisson approximation errors of cumulative Binomial probabilities are shown in Figures 4 and 5.

The difference with a real 'pool size' of $k > 1000$ - the volume of blood in human body, the largest difference between the cumulative Binomial and Poisson probabilities is less than 0.0002. This shows that simplified equations based on Poisson cumulative probability can be used in practice for quantification of the uncertainty of the types of reported results in molecular diagnostics. The cumulative Binomial probability based equations are given in Appendix.
Figure 6 shows expanded area around the ULoQ (10^7 cp/PCR) of graph in Figure 5. It has similar look, but about 10-fold higher approximation errors for the case of $k = 10$ in Figure 4.

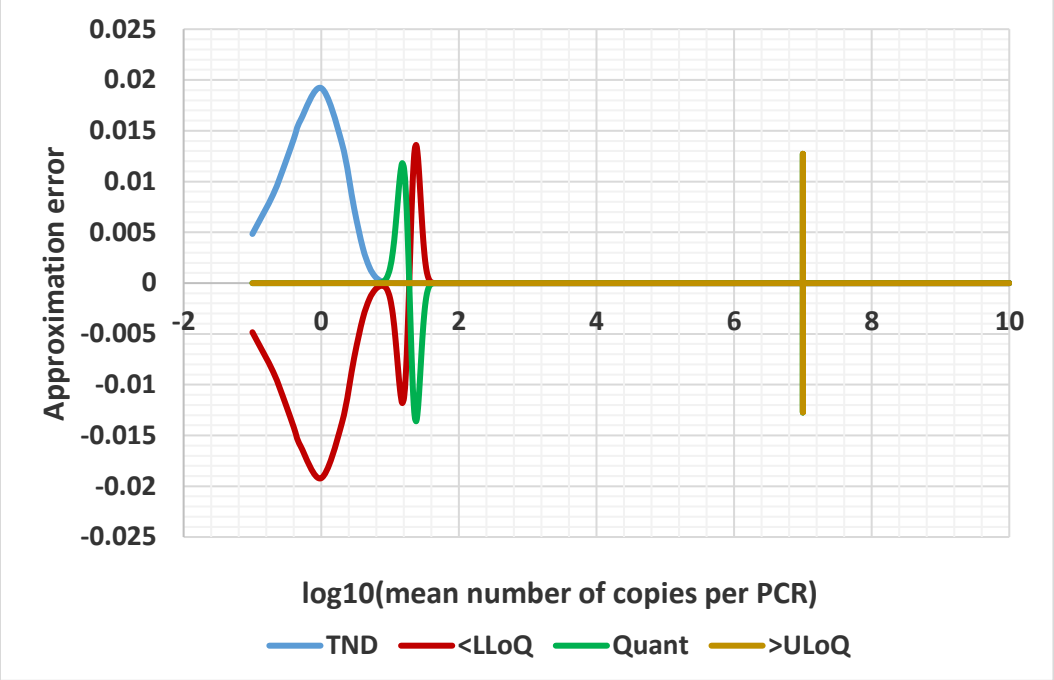

Figure 4. Approximation errors of cumulative Binomial probabilities of types of reported results with cumulative Poisson vs target concentration with pool of k = 10 sample volumes

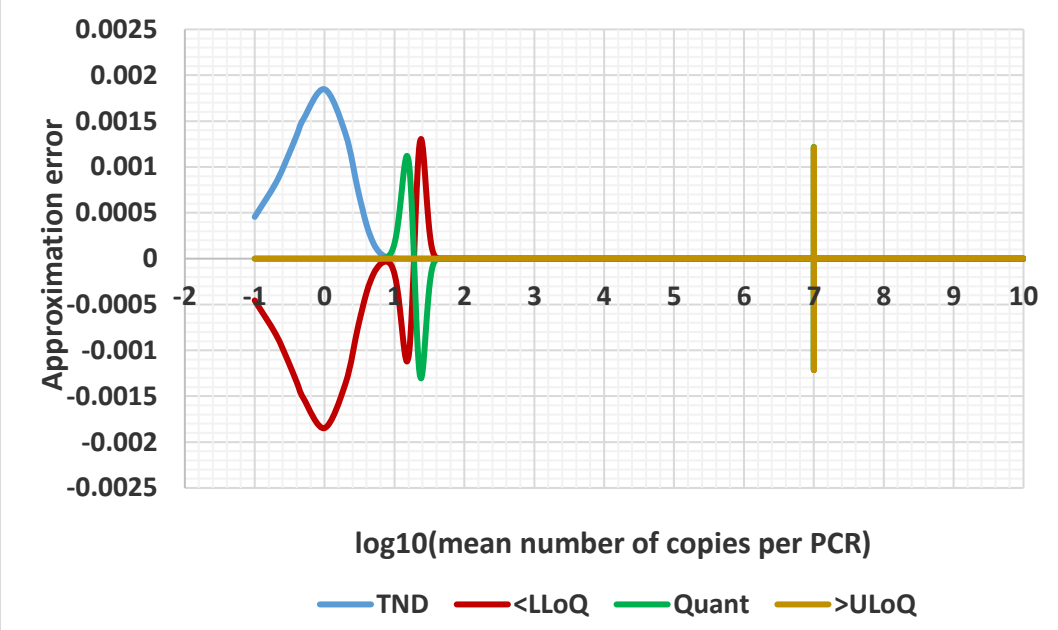

Figure 5. Approximation errors of cumulative Binomial probabilities of types of reported results with cumulative Poisson vs target concentration with pool of k=100 sample volumes

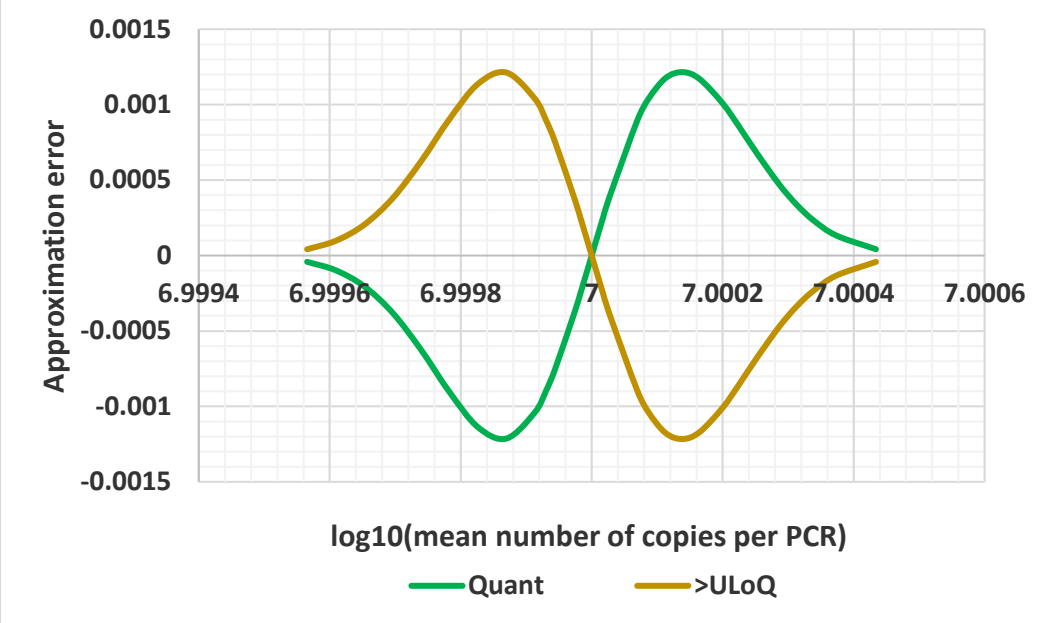

Figure 6. Approximation errors of cumulative Binomial probabilities of types of reported results with cumulative Poisson vs. target concentration around ULoQ = 10^7 with pool of k=100 sample volumes

5. Uncertainty of quantitative results

A quantitative result obtained with a randomly drawn sample are subject to random variation. Clinical sample with expected target concentration μ can be tested in some lab (site, S_i), using sample preparation and amplification / detection kit-lot L_j, on instrument I_k, by operator O_l, on day D_m, with random error ε_{ijklmr} primarily caused by random variation of the number of copies of target nucleic acid in randomly drawn samples. Quantitative result, R, (normally distributed log(concentration) or number of amplification cycles used for detection), subject to the above sources of variation, can be modeled as:

$$R_{ijklmr} = \mu + S_i + L_j + I_k + O_l + D_m + \varepsilon_{ijklmr} \qquad (9)$$

In accordance with (9), quantitative result can be characterized by the estimates of the mean, $\hat{\mu}$, and total standard deviation, $\hat{\sigma}_T$, along with the number of its degrees of freedom, f. With the stochastically independent random variation due to the sources in (9), the reproducibility total variance is sum of the variance components:

$$\sigma_T^2 = \sigma_S^2 + \sigma_L^2 + \sigma_I^2 + \sigma_O^2 + \sigma_D^2 + \sigma_\varepsilon^2 \qquad (10)$$

The number of degrees of freedom of the total variance estimate is[6]:

$$f \simeq \frac{2\left(\hat{\sigma}_T^2\right)^2}{\hat{\sigma}^2\left(\hat{\sigma}_T^2\right)} \tag{11}$$

The variance of the estimate of the total variance in the denominator of (11) is calculated as a sum of elements of asymptotic covariance matrix of the estimates of the variance components in (10). The latter is reported by the variance components analysis routines available in statistics software such as SAS® VARCOMP and MIXED procedures and JMP® by SAS Institute. The MIXED procedure also reports the population mean, $\hat{\mu}$, estimated with data from model (9), along with the standard error, SE, and its number of degrees of freedom, f_μ. The random variance component σ_ε^2 is the estimate of the repeatability variance.

6. Conclusions

With qualitative and quantitative PCR tests in molecular diagnostics there is uncertainty of the types of reported results and of the quantitative results. There are four types of reported results with quantitative methods: TND, <LLoQ, Quant and >ULoQ. There are two types of reported results with qualitative method: TND and TD. These results are reported when the number of copies per PCR is within respective interval. With binomial variation of the number of copies per PCR between test samples randomly drawn from a pool, there are probabilities of obtaining any type of the reported result. Those probabilities quantify the uncertainty of the types of reported results. Repeatability and reproducibility standard deviations with their degrees of freedom characterize the uncertainty of the quantitative results.

Formulas for calculating the probabilities of reported results based on Binomial and Poisson distributions have been derived, and graphs of probabilities produced for the cases of 1 copy and 62 copies of target nucleic acid required for detection. The cumulative Poisson approximation errors of cumulative Binomial probabilities of the reported results have been analyzed and found small enough for the simpler Poisson formulas to be recommended for the calculations.

The methods developed in this paper help in evaluation and clear understanding of the uncertainty of the types of reported results and of the quantitative results in molecular diagnostics, that in-turn helps laboratorians interpreting and QC the test results, clinicians diagnosing and monitoring treatment of disease, blood banks minimizing the risk of transmission of infectious agents from donors to recipients, pharma evaluating efficacy of new medications.

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Appendix. Formulas for calculating cumulative Binomial probabilities characterizing the uncertainty of various types of reported results in molecular diagnostics

Probability of a type of reported result in molecular diagnostics is the probability of having x copies of target nucleic acid within the interval for the respective type of reported result. This probability depends on the number of copies, n, (after extraction / reverse transcription) in the pool from which the test samples are randomly drawn and on the size of the pool expressed as the number of test sample volumes, k, contained in it. Then the probability to draw any copy into a random sample is $1/k$, and the probability to draw x copies into a random sample is binomial:

$$P(x) = \binom{n}{x}\left(\frac{1}{k}\right)^x\left(1-\frac{1}{k}\right)^{n-x} \tag{A1}$$

The formulas for the probabilities of various types of reported results shown below are derived using the above $P(x)$ for the respective intervals of x corresponding to the types of reported results and intervals of n.

$$P(TND) = \binom{n}{x}\left(\frac{1}{k}\right)^x\left(1-\frac{1}{k}\right)^{n-x}\Bigg|_{x=0} = \left(1-\frac{1}{k}\right)^n, \quad 0 \le n < \infty \tag{A2}$$

$$P(<LLoQ)=\begin{cases}0, & n=0\\ \sum_{x=1}^{n}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x}, & 0<n<LLoQ\\ \sum_{x=1}^{LLoQ-1}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x}, & LLoQ\le n<\infty\end{cases} \quad \text{(A3)}$$

$$P(Quant)=\begin{cases}0, & n<LLoQ\\ \sum_{x=LLoQ}^{n}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x}, & LLoQ\le n<ULoQ\\ \sum_{x=LLoQ}^{ULoQ}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x}, & ULoQ\le n<\infty\end{cases} \quad \text{(A4)}$$

$$P(>ULoQ)=\begin{cases}0, & n\le ULoQ\\ \sum_{x=ULoQ+1}^{\infty}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x}=1-\sum_{x=0}^{ULoQ}\binom{n}{x}\left(\frac{1}{k}\right)^{x}\left(1-\frac{1}{k}\right)^{n-x} & \\ ULoQ<n<\infty\end{cases} \quad \text{(A5)}$$

Calculations with the formulas for $P(<LLoQ)$ and $P(Quant)$ are easier when the probabilities are re-expressed as differences between cumulative probabilities using, e.g., Microsoft Excel function for cumulative Binomial probability.

The number of copies in a pool, n, can be expressed as:

$$n=k\mu\theta=\frac{k\mu\ln(20)}{LoD} \quad \text{(A6)}$$

μ = mean number of copies in test samples
θ = extraction / reverse transcription efficiency
LoD = limit of detection, concentration corresponding to 95% probability of detection

Since n is integer, μ can have values $\upsilon\cdot LoD/\left(k\cdot\ln(20)\right)$, with $0\le\upsilon$ being a natural number.