Probability of Detection of Target Nucleic Acid Sequence with PCR Assay in Molecular Diagnostics: Math Model Derivation, Validation and Applications

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Abstract
Probability of detection as function of target nucleic acid sequence concentration and minimum number of copies detectable determines important performance characteristic, analytical sensitivity, of polymerase chain reaction assay. Only math model for the case of single copy detectable was published elsewhere. General math model for probability of detection with any minimum number of copies detectable was derived and validated with χ^2 goodness-of-fit test using data collected on several types of instruments for limit of detection evaluations of several viral and microbial assays. The *p*-values of 84 χ^2 goodness of fit tests on such data ranged from 0.050 to 0.999, with χ^2 test on combined data having 157 degrees of freedom and *p*-value of ≈1 successfully validating the math model.

Examples of applications provided are: (1) estimation of sample concentration with confidence bounds from observed detection rate, (2) estimation of concentrations corresponding to 5%, 50% and 95% detection rates with confidence bounds, (3) probability of detection vs. concentration and minimum number of copies detectable curves corresponding to several values of limit of detection with single copy detectable, and (4) bounds for concentrations corresponding to 5% and 95% detection rates, recommended as precision characteristic of qualitative assays in CLSI guideline EP12-A2.

Key Words: Polymerase chain reaction, analytical sensitivity, concentrations at specified detection rates: C_5, C_{50} and C_{95}

1. Introduction

PCR assays are used for detection of infectious viruses and bacteria based on sequences in their DNA or RNA and of mutations in human genes. The viral and bacterial test results on human samples are used in diagnosis of disease and monitoring of the therapy. Donor blood screening prevents transmission of infections to transfusion recipients. Tests of mutations of human genes are used in companion diagnostics for identifying patients for treatments with proper medications. This brief description shows the importance of the PCR assays in health care. One of the most important PCR assay performance characteristics is its analytical sensitivity, which is determined by the probability of detection of the target nucleic sequence as a function of the concentration – the mean number of copies in the test sample volume – and the minimum number of copies required for detection. A brief description of the PCR process sufficient for derivation of the probability of detection math model is given in the following section.

Statistical methods based on empirical model describing probability of detection of target nucleic acid as a function of concentration are often used for evaluation of performance characteristics of PCR assays. An example is using cumulative normal probability curve in probit analysis to describe the relationship between the probability of detection and the concentration for estimating the limit of detection (LoD), along with a confidence interval (CI), as recommended in the CLSI (Clinical and Laboratory Standards Institute, Wayne, PA, USA) EP17-A2 guideline[1]. Other examples are using logistic regression model[2,3] in probit analysis.

A math model based on theory of the underlying process has many advantages over an empirical model[4]: "1. It contributes to our scientific understanding of the phenomenon under study. 2. It usually provides a better basis for extrapolation (at least to conditions worthy of further experimental investigation if not through the entire range of all input variables). 3. It tends to be parsimonious (i.e., frugal) in the use of parameters and to provide better estimates of the response."

In case of a single copy detectable, the probability of detection, assumed to be the probability of at least 1 copy of the nucleic acid target sequence derived from a test sample and present at PCR input, was modeled with Poisson distribution[5,6,7]. An evaluation of probabilities (proportions) of three types of reported results with quantitative PCR assays: (i) target not detected, (ii) target detected with the concentration below the limit of detection (LoD), and (iii) target detected with quantitative result reported, also was based on the Poisson model assuming single copy is detectable[8]. Some publications provide information that is derived from the math model for the case of a single copy detectable[9]: LoD cannot be smaller than mean number of 3 copies per test sample, and the probability distribution of the number of copies in randomly drawn samples is Poisson. A different math model for the probability of detection was described that incorporates the probabilities of false negatives and false positives estimated experimentally[10]. The latter math model is semi-empirical. We did not find publications describing statistically rigorous validation with data of the above math models.

More than one copy per PCR is required for detection when there is incomplete separation between the background fluorescence noise arising from testing negative samples and the fluorescence light signal arising from testing low positive samples, and the fluorescent light intensity threshold for detection is set to provide for high specificity with no false positives. There are situations, described in the following section, when it is desirable that more than one copy is required for detection. We did not find publications describing the probability of detection math model for the general case when more than one copy per PCR is required for detection.

The purpose of this work is to derive and validate with data a general math model describing the probability of detection as a function of concentration of target nucleic acid sequence and minimum number of copies required for detection, based on the PCR assay process description given in the following section, and provide examples of practical applications.

2. Probability of Detection

2.1 PCR Assay Process Description

The process description here includes details needed for derivation of a simplified math model for probability of detection vs. concentration. A sample of specified volume is randomly drawn from a bulk, e.g., a 1 mL blood sample is drawn from a subject whose

blood volume is about 5,000 mL. To simplify derivations, it is assumed that the bulk volume contains a whole number, k, of test sample volumes, with a mean number of copies per test sample volume, μ, and a total number of copies in the bulk, μk. The sample preparation usually includes centrifugation to remove blood cells, extraction of nucleic acid from plasma or serum, etc., and, in case of RNA target nucleic acid, reverse transcription to produce complementary DNA. With extraction / reverse transcription efficiency, $0 < \theta \leq 1$, which is the probability of extraction / reverse transcription of any copy of target nucleic acid present in the sample, the mean number of copies at PCR input is $\xi = \mu\theta$. In the course of PCR, the primers bind to the target sequences, the latter are copied in cycles, producing amplicons, and the probes bind to those amplicons. The dye molecules, initially attached to the probes and subsequently cleaved from the probes by the polymerase enzyme, are excited by laser light to emit fluorescent light at certain wavelength. The number of amplicons approximately doubles each cycle, and so does the fluorescent light intensity. Detection of target sequence takes place when the intensity of fluorescent light emitted by the above dye molecules attains a specified threshold. Modern PCR assays in molecular diagnostics provide for separation between the background fluorescence, produced by samples containing no target sequence, and the fluorescent light signal, produced by the PCR with samples containing a single copy of the target sequence. The fluorescent light intensity threshold is set between the observed maximum of randomly varying noise and minimum of randomly varying signal. In an ideal case, this assures detection of a single copy of target sequence at PCR input and non-detection of zero copies at PCR input, so maximizing both analytical sensitivity and analytical specificity.

In some cases, the analytical sensitivity needs to be reduced, while high analytical specificity is always required. That is achieved by setting a higher fluorescent light intensity threshold and/or using smaller number of amplification cycles than those needed for detection of a single copy. Some examples of the cases when the analytical sensitivity needs to be reduced include matching PCR microbiology assay performance that detects genetic material of both live and dead or non-growing bacteria to the performance of bacterial culture growth tests[11], and reducing the rate of false positives due to cross-contamination of negative samples with high positive samples or due to non-specific signal generation. Also, more than one copy may be required for detection in case of PCR inhibition by interferents in the sample matrix, e.g., hemoglobin[12].

Possible random variation of the number of amplification cycles required for detection of various numbers of copies may lead to random variation of the minimum number of copies required for detection. Such random variation of the minimum number of copies detectable is ignored in derivation of a simplified math model for probability of detection vs. concentration.

2.2 Math Model Derivation

As described in the previous section, samples of a specified volume are randomly drawn for testing with PCR assay from a bulk of a volume containing k test samples. The mean number of copies of target nucleic acid sequence at PCR input is ξ. The total expected number of copies in k samples, after extraction/reverse transcription, is ξk. The probability of any copy to be included into any randomly drawn sample is $1/k$. Based on the above description, the probability, $P(x)$, of having x copies at PCR input is binomial:

$$P(x) = \binom{\xi k}{x} \left(\frac{1}{k}\right)^x \left(1 - \frac{1}{k}\right)^{\xi k - x}, \quad 0 \leq x \leq \xi k \qquad (1)$$

A PCR assay requires at least v ($v \geq 1$) copies for detection. Then the probability of detection is the probability of having $x \geq v$ copies at PCR input, which is complementary to the cumulative probability of having $x \leq v - 1$ copies:

$$P(x \geq v) = 1 - \sum_{x=0}^{v-1} \binom{\xi k}{x} \left(\frac{1}{k}\right)^x \left(1 - \frac{1}{k}\right)^{\xi k - x} \quad (2)$$

The probability (2) is a function of the mean number of copies per PCR, ξ, the number, k, of sample volumes contained in the bulk volume, and the minimum number of copies required for detection, v. The asymptotic probability found as the limit of $P(x \geq v)$ when k tends to infinity is Poisson:

$$\lim_{k \to \infty} \{P(x \geq v)\} = 1 - \sum_{x=0}^{v-1} \frac{\xi^x e^{-\xi}}{x!} \quad (3)$$

The relative error of Poisson approximation (3) of the binomial probability (2) is determined as the difference between the probabilities calculated using formulas (3) and (2) divided by the latter, and it is a function of k, ξ and v. An example for v = 1, 10, 60, k in the range from 10 to 200, and two concentration levels, ξ , corresponding to $P(x \geq v)$ around 0.26 and 0.95 for each v, is presented graphically in Fig 1. In the figure, the curve for v = 10, μ = 15.8 is masked by the curves for v = 1, μ = 13 and v = 60, μ = 73.5.

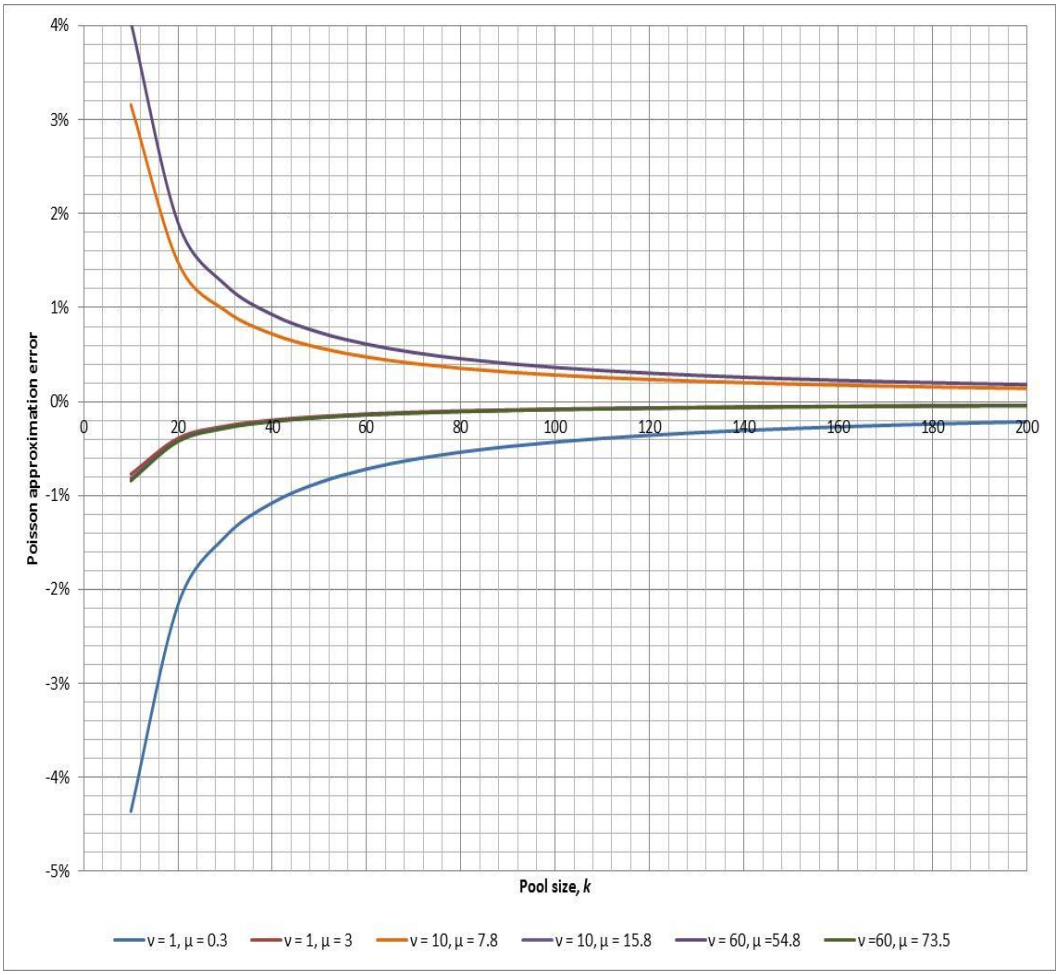

Figure 1: Error of Poisson approximation (3) of the binomial probability (2)

While for $v = 1$ the approximation error is negative for both lower and higher concentrations, with $v = 10$ and 60 the approximation error is positive for the lower concentrations and negative for the higher concentrations. The error curves are almost the same for $v = 1$, 10 and 60 at the higher concentration corresponding to $P(x \geq v)$ around 0.95, while they are different for the lower concentration corresponding to $P(x \geq v)$ around 0.26. In the above cases shown in Fig. 1 the approximation error is within ±5% with a small bulk size $k = 10$, and it is utmost ±1% with a bulk size $k \geq 45$. Such Poisson approximation errors being much smaller than the quantification errors of the bulk concentrations can be ignored. Therefore, being simpler than the binomial probability (2) and having one parameter less, the Poisson approximation (3) is useful in practical applications. It also should be noted that the random variation of the sample volume is small compared to the variation of the number of copies between randomly drawn samples of constant volume, and, for that reason, can be ignored. In addition, based on successful validation with data of the simplified model describing the probability of detection vs. the target concentration, discussed in the following section, using it in practice is justified.

From the previous section, $\xi = \mu\theta$, and the probability, p_v, of having at least v copies of target nucleic acid at PCR input, from (3), can be expressed as:

$$p_v = 1 - \sum_{x=0}^{v-1} \frac{(\mu\theta)^x e^{-\mu\theta}}{x!} \tag{4}$$

Formula (4) simplifies for $v = 1$, the probability of detection of a single copy per PCR:

$$p_1 = 1 - e^{-\mu\theta} \tag{5}$$

With the limit of blank being zero with PCR assays, by definition[1], the limit of detection, LoD, is the concentration corresponding to 0.95 probability of detection. Setting the probability of detection, p_1, at 0.95 in (5), replacing the concentration, μ, with the concentration corresponding to 0.95 probability of detection, which is the LoD_1, and solving equation (5) for θ yields:

$$\theta = \frac{\ln(20)}{LoD_1} \tag{6}$$

Substituting θ from (6) into (5) yields for the probability of detection with single copy per PCR detectable[7]:

$$p_1 = 1 - e^{-\frac{\mu \ln(20)}{LoD_1}} \tag{7}$$

Formula (7) allows for calculation of the probability of detection as a function of the target concentration, expressed as a multiple of LoD_1, while concentration, μ, and LoD_1 may not be known separately. This is convenient for planning various studies before the limit of detection has been evaluated.

Obviously, the extraction/reverse transcription efficiency, θ, is independent of the minimum number, v, of copies at PCR input detectable. Replacing θ in (4) with its expression (6) and setting $p_v = 0.95$ and μ to its respective value LoD_v yields:

$$1 - \sum_{x=0}^{v-1} \frac{(r_v \ln(20))^x e^{-r_v \ln(20)}}{x!} = 0.95 \;\Rightarrow\; \frac{e^{-r_v}}{\ln(20)} \sum_{x=0}^{v-1} \frac{r_v^x}{x!} = 0.05 \tag{8}$$

In (8), $r_v = LoD_v / LoD_1$ is a function of v only.

Replacing LoD_1 with LoD_v / r_v in (6) yields:

$$\theta = \frac{\ln(20) r_v}{LoD_v} \qquad (6,a)$$

Replacing θ in (4) with (6,a) yields for the probability of detection, p_v , as a function of the concentration, μ, and the minimum number of copies detectable, v:

$$p_v = 1 - \sum_{x=0}^{v-1} \frac{\left(\frac{\mu \ln(20) r_v}{LoD_v} \right)^x e^{\frac{-\mu \ln(20) r_v}{LoD_v}}}{x!}, \; 1 \le v < \infty \qquad (9)$$

This completes derivation of the general math model describing the probability of detection vs. target sequence concentration and the minimum number of copies detectable. From (9) it is seen that the target concentration, μ, and the limit of detection, LoD_v, do not have to be known separately if the concentration is expressed as multiple of the limit of detection, μ / LoD_v. Formula (9) is easily simplified for the case v = 1 and r_1= 1 to obtain (7). As expected, both equations (7) and (9) provide for expected probabilities of detection 0, 0.95 and 1 for μ = 0, $\mu = LoD_v$ and $\mu \to \infty$, respectively. The r_v ratios for v = 1 to 100, calculated as numerical solutions of (8), are summarized in Table 1.

Table 1: The limits of detection ratio, $r_v = LoD_v / LoD_1$, vs. v.

v	r_v	v	r_v	v	r_v	v	r_v	v	r_v
1	1	21	9.701	41	17.381	61	24.832	81	32.162
2	1.584	22	10.095	42	17.758	62	25.201	82	32.527
3	2.102	23	10.487	43	18.134	63	25.569	83	32.891
4	2.588	24	10.877	44	18.509	64	25.938	84	33.255
5	3.056	25	11.267	45	18.884	65	26.306	85	33.618
6	3.509	26	11.655	46	19.259	66	26.674	86	33.982
7	3.953	27	12.043	47	19.633	67	27.041	87	34.345
8	4.389	28	12.429	48	20.007	68	27.408	88	34.708
9	4.818	29	12.815	49	20.380	69	27.775	89	35.072
10	5.243	30	13.199	50	20.753	70	28.142	90	35.434
11	5.662	31	13.583	51	21.126	71	28.509	91	35.797
12	6.078	32	13.966	52	21.498	72	28.875	92	36.160
13	6.490	33	14.348	53	21.870	73	29.241	93	36.522
14	6.899	34	14.729	54	22.241	74	29.607	94	36.884
15	7.306	35	15.110	55	22.612	75	29.973	95	37.246
16	7.710	36	15.490	56	22.983	76	30.338	96	37.608
17	8.112	37	15.869	57	23.353	77	30.703	97	37.970
18	8.512	38	16.248	58	23.723	78	31.068	98	38.332
19	8.910	39	16.626	59	24.093	79	31.433	99	38.693
20	9.306	40	17.004	60	24.463	80	31.798	100	39.055

The table shows that r_v increases with increase of v. Therefore, LoD_v increases, and the analytical sensitivity decreases r_v - fold with v copies required for detection compared to the case of a single copy per PCR detectable. E.g., doubling the number of target copies

per PCR required for detection from 1 to 2 increases the LoD and reduces the analytical sensitivity 1.584 - fold, and so on. Values of $\boldsymbol{r_v}$ in Table 1 have been calculated using commercial mathematical software[13] Maple® 2016.

2.3 Math Model Validation with Data

2.3.1 Statistical method of math model validation

For using the asymptotic math models (7) and (9) in practical applications they have to be validated with data. Since the models describe the relationship between the probability of detection and the target sequence concentration expressed as a multiple of the limit of detection, μ / LoD_v, it is natural to use LoD evaluations data for the model validation. The concentration levels tested in LoD study are obtained by dilutions of a stock solution. Small random errors of the dilution factors and random variation of the numbers of copies in the respective amounts of the stock solution used for preparation of the concentration levels used for LoD evaluations can be ignored. Then the value assignment error of the stock solution affects the concentration levels of the panel members and the LoD_v estimate proportionally to their respective nominal values. Therefore, the value assignment error of the stock solution does not affect the results of validation of math models (7) and (9) when expressing the concentrations as multiples of the limit of detection estimate, μ / LoD_v.

In a LoD study, n_i valid results are obtained at the i-th concentration level, μ_i , $i = 1..N$, with x_i positive and u_i negative, where $x_i + u_i = n_i$. With the probabilities of positive results, p_i, calculated with (9) for proper v, and of negative results, q_i, summing up to 1 ($p_i + q_i = 1$), the probability of x_i positive results and u_i negative results is binomial - a special case of a multinomial distribution:

$$P(x_i, u_i) = \frac{n_i!}{x_i! u_i!} p_i^{x_i} q_i^{u_i} \qquad (10)$$

In such case, the Pearson χ^2 goodness of fit test statistic[14], calculated with data obtained in LoD study for the i-th concentration level is:

$$\chi_i^2 = \frac{(x_i - n_i p_i)^2}{n_i p_i} \qquad (11)$$

The probability of positive results, which is the probability of detection, p_i, at concentration μ_i is calculated using the math model (9) or its special case (7), as applicable, and the expected number of positive results is $n_i p_i$. For estimate of LoD_v available from the LoD study, the probability distribution of goodness of fit test statistic (11) is well approximated by χ^2 distribution when the following requirements are satisfied[14]:

$$n_i p_i \geq 5, \; n_i (1 - p_i) \geq 5 \qquad (12)$$

The χ^2 distribution of the statistic (11) has 1 degree of freedom, the latter calculated as the number of the random variables (x, u) equal 2, minus the number of restrictions, $x + u = n$, equal 1, for each concentration level satisfying (12). The random numbers of positive and negative results x_i, u_i across the concentration levels tested in LoD study vary independently. The goodness of fit test statistic (11) describes a variance, and a sum of variances of independent random variables is a variance that has χ^2 distribution. The number of degrees of freedom for such sum is equal to the sum of the numbers of degrees of freedom of the variances summed up. This allows summing up m terms calculated with (11) for the subset of m out of N contiguous concentration levels with $i = j, \ldots, j + m - 1$, satisfying the requirements (12):

$$\chi^2 = \sum_{i=j}^{j+m-1} \frac{\left(x_i - n_i p_i\right)^2}{n_i p_i} \tag{13}$$

As discussed before, each concentration level included in calculation of the goodness of fit statistic (13) contributes one degree of freedom. A subset of m concentration levels, satisfying (12) and used for calculations of the probabilities of detection, p_i, in (13), provides a total of m degrees of freedom. One degree of freedom used for estimation of two functionally related parameters, v and LoD_v, has to be subtracted. Therefore, the number of degrees of freedom of the χ^2 distribution of goodness of fit statistic (13) is m – 1. To have at least 1 degree of freedom, the number of concentration levels, m, satisfying (12) has to be at least 2, and only LoD study data having at least $m = 2$ concentration levels satisfying (12) can be used for the model validation. So, the test of goodness of fit of the models (7), (9) to data obtained in a LoD study consists of calculating the goodness of fit statistic (13) and the corresponding p-value from the χ^2 distribution with m – 1degrees of freedom. The p-value is the probability of a larger divergence between the experimental and theoretical numbers of positive results than the observed divergence. The hypothesis of goodness of fit is not rejected if $p \geq 0.05$.

2.3.2 Data analysis and results of testing goodness of fit hypothesis

The χ^2 statistics (13) of goodness of fit of the models (7), (9) to data were calculated using eight LoD studies data sets from a range of mostly qualitative PCR tests. These studies included 104 LoD evaluations, with 84 having at least 2 concentration levels satisfying requirements (12), with a few exceptions explained below. The LoD studies were conducted with Roche **cobas s** 201 system and **cobas®** 4800, 6800 systems for MPX v2.0 and v3.0 (HIV-1M, HIV-1O, HIV-2, HBV, HCV) and Zika virus Blood Screening Tests; HBV, HCV, HPV, HSV-1, HSV-2, MRSA virology and microbiology tests, and Roche **cobas®** Liat® Influenza A, Influenza B, and RSV tests. The types of instruments and assays used for LoD studies are listed in Table 2 below. The data were collected with multiple lots of reagents, different sample matrices (whole blood, plasma and serum, vaginal and nasal fluids) and different media (swab diluents). In 79 of the mentioned above 84 LoD evaluations, 6 out of 8 studies conducted with 21 to 105 replicates per concentration level, from 2 to 4 concentration levels satisfied requirements (12) and were used for calculations of the χ^2 goodness of fit statistics summarized in Table 2. In 2 studies that included 6 LoD evaluations (Zika, Flu A, B and RSV) conducted with smaller number of tests per concentration level, the requirement (12) was loosened, as shown in respective column of Table 2 below, to allow for including these studies in validation of the math model. The respective 84 p-values ranged from 0.0502 to 0.999, and there were only three cases with p-values below 0.1. The goodness-of-fit hypothesis was not rejected in any one of the 84 goodness of fit tests.

It should be noted that cobas HIV-1M and HCV tests, to increase detection capability of mutating viruses, each use two probes complementary to the respective target sequences. This allows for detection of the viruses with any one or both of the target sequences non-mutated increasing reliability of the tests. The probability of detection in such case is calculated with (7) or (9) for the mean number of non-mutated target sequences in test sample. The lowest probability of detection corresponds to the case when each virion in the sample has a single non-mutated sequence, and the highest when each virion has both sequences non-mutated.

For the goodness of fit χ^2 test statistics, combined within each of the eight studies that included from 2 to 54 LoD evaluations, the p-values summarized in Table 2 ranged from

0.637 to 0.999, and the *p*-value with the combined test statistic having 157 degrees of freedom for all 84 LoD evaluations was practically 1. All goodness of fit tests, except for MRSA and quantitative HBV, were done for $v = 1$, while 4 goodness of fit tests for MRSA were done for $v = 10$, 11, 15 and 62, and 6 tests for HBV were done for v from 1 (in single case) to 4. Two groups of the minimum number of copies, v, required for detection in MRSA LoD studies, $v = 10$, 11, 15 and $v = 62$, were obtained with two different cutoffs of the numbers of amplification cycles. The variation of the estimates of v is caused by using different lots of extraction and PCR reagents and random variation of the observed detection rates. The method of estimation of the minimum number of copies required for detection, v, will be published separately as a part of a new method of estimation of LoD based on the math models (7) and (9).

Table 2: Goodness of fit χ^2 test for the math models (7) and (9)

System cobas®	Assay	LoD eval's	Replicates per conc.	$n(1-p)$, $np \geq$	χ^2	Total DF	*p*		
							Min	Max	All
s 201	**MPX v2.0**	4	24	5	1.9	4	0.30	0.62	0.75
Liat®	**Flu A, B; RSV**	3	10 to 24	3	1.3	3	0.33	0.84	0.72
4800	**HPV genotypes**	54	30	5	72	107	0.09	0.97	0.99
6800	**MPX v3.0**	10	66	5	15	24	0.05	0.92	0.92
4800	**HSV-1, 2**	2	105	5	0.5	2	0.54	0.75	0.79
6800	**Zika Virus**	3	10 to 12	2	2.5	4	0.19	0.75	0.64
4800	**MRSA**	4	21; 63	5	3.1	6	0.21	0.92	0.79
6800	**HBV**	4	63	5	0.0	7	0.96	1.00	1.00
All	**All**	84	10 to 105	2-5	96	157	0.05	1.00	1.00

The interpretation of the results of goodness of fit tests with data combined across systems and assays is: the divergence between the experimental and theoretical numbers of positive results calculated with math models (7) and (9) can be attributed to random variation, with probability close to 1 of observing by chance a greater divergence. This means that the observed divergence is very small, and models (7), (9), nearly perfectly fit the data.

Based on the above results, the models (7), (9), describing the relationship between the probability of detection in PCR assays vs. the target nucleic acid concentration and minimum number of target copies required for detection, have been successfully validated with substantial amount of data collected on several types of instruments for multiple molecular diagnostics PCR assays. Therefore, models (7), (9) can be used with a reasonable confidence in practical applications.

3. Examples of Applications of the Math Model for Probability of Detection

3.1 Estimation of the Mean Concentration from Observed Detection Rate

Theoretical model (7) describing the relationship between the probability of detection and the target sequence concentration in the ubiquitous special case $v = 1$ can be inverted for calculating concentration from the observed proportion, $p_1 = x/n$, of positive results, yielding a closed form expression for calculation of the concentration from the observed proportion of positives:

$$\hat{\mu} = -\frac{\ln(1 - x/n) \cdot LoD_1}{\ln(20)} \tag{14}$$

Using the lower and upper limits of 95% confidence interval for the proportion of positives in (14) yields 95% confidence limits for the concentration. E.g., LoD = 10 cp/mL, and the observed proportion of positives is x / n = 25 / 30. The Clopper-Pearson[15] confidence limits for the proportion of positives are [0.65, 0.94]. From (14), the estimate for the mean target concentration is $\hat{\mu}$ = 5.98 cp/mL with the 95% confidence interval [3.53, 9.60]. Larger number of tests, n, tightens the confidence interval for the same observed proportion of positives.

3.2 Estimation of LoD with a Single Concentration Level Tested

Using empirical probit model[1] or model-free method, several concentration levels have to be tested for estimation of LoD. The PCR process based math model (7) allows for estimation of LoD from the observed detection rate at a single concentration level. From (14), the LoD_1 can be estimated as:

$$LoD_1 = -\frac{\mu \ln(20)}{\ln(1 - x/n)} \tag{15}$$

E.g., sample with target sequence concentration μ = 5 cp/mL is tested n = 48 times, and the observed number of positive results is x = 39. The LoD_1 estimate from (15) is 8.95 cp/mL. The Clopper-Pearson 95% confidence interval for the observed proportion 39 / 48 is [0.67, 0.91]. The corresponding 95% CI for the LoD_1 is [6.21, 13.37], the lower confidence limit for the LoD_1 being calculated with (15) using the upper confidence limit for the proportion, and the upper confidence limit for the LoD_1 being calculated with (15) using the lower confidence limit for the proportion. Uncertainty of μ is ignored in calculations of the 95% CI for the LoD_1 and if taken into account would make the confidence interval somewhat wider. Method of estimation of LoD and the minimum number of copies required for detection, v, exceeding 1 will be subject of a future publication.

3.3 Concentration Corresponding to a Particular Probability of Detection

In some cases, target concentration, C_p , corresponding to a particular probability of detection, p, is of interest. In case v = 1, C_p is calculated using the following formula obtained by inverting (7) and substituting C_p for μ :

$$C_p = -\frac{\ln(1 - p_1) \cdot LoD_1}{\ln(20)} \tag{16}$$

Often used in practice C_5 and C_{50} concentrations corresponding to 5% and 50% detection rates[16], with p_1 = 0.05 and p_1 = 0.5, respectively, from (16), are: C_5 = 0.017 LoD_1, C_{50} = 0.231 LoD_1. C_{95}, the concentration corresponding to 95% detection rate, in case of PCR assays having zero limit of blank, equals to the limit of detection[1, 16].

For LoD_1 = 8.95 cp/mL with the 95% confidence interval [6.21, 13.37] of the previous example, C_5 = 0.152 cp/mL with a 95% CI [0.106, 0.227], and C_{50} = 2.06 cp/mL with a 95% CI [1.43, 3.08].

For v > 1, C_5 and C_{50} can be calculated as fractions of LoD_v, R_5 and $R_{50,}$ by solving equation (9) numerically for μ / LoD_v with p_v = 0.05 and p_v = 0.5, respectively. Values of those fractions calculated using specially written script with commercial mathematical software platform Maple® 2016[13], are given for v from 1 to 100 in Tables 3 and 4.

Table 3: Concentrations at 5% detection rate expressed as fractions of the limit of detection, $R_5 = C_5 / LoD_v$, for v from 1 to 100

v	R_5	v	R_5	v	R_5	v	R_5	v	R_5
1	0.0171	21	0.4842	41	0.5966	61	0.6553	81	0.6932
2	0.0749	22	0.4925	42	0.6004	62	0.6575	82	0.6947
3	0.1299	23	0.5004	43	0.6040	63	0.6598	83	0.6963
4	0.1762	24	0.5079	44	0.6075	64	0.6619	84	0.6978
5	0.2152	25	0.5150	45	0.6109	65	0.6641	85	0.6993
6	0.2485	26	0.5218	46	0.6143	66	0.6661	86	0.7007
7	0.2774	27	0.5283	47	0.6175	67	0.6682	87	0.7022
8	0.3028	28	0.5345	48	0.6207	68	0.6702	88	0.7036
9	0.3253	29	0.5404	49	0.6237	69	0.6722	89	0.7050
10	0.3455	30	0.5461	50	0.6267	70	0.6741	90	0.7064
11	0.3637	31	0.5516	51	0.6297	71	0.6760	91	0.7077
12	0.3803	32	0.5569	52	0.6325	72	0.6778	92	0.7091
13	0.3955	33	0.5619	53	0.6353	73	0.6797	93	0.7104
14	0.4095	34	0.5668	54	0.6380	74	0.6815	94	0.7117
15	0.4225	35	0.5715	55	0.6406	75	0.6832	95	0.713
16	0.4345	36	0.5761	56	0.6432	76	0.6849	96	0.7143
17	0.4457	37	0.5804	57	0.6457	77	0.6866	97	0.7155
18	0.4563	38	0.5847	58	0.6482	78	0.6883	98	0.7167
19	0.4661	39	0.5888	59	0.6506	79	0.6900	99	0.7180
20	0.4754	40	0.5928	60	0.6530	80	0.6916	100	0.7192

Table 4: Concentrations at 50% detection rate expressed as fractions of the limit of detection, $R_{50} = C_{50} / LoD_v$, for v from 1 to 100

v	R_{50}	v	R_{50}	v	R_{50}	v	R_{50}	v	R_{50}
1	0.2314	21	0.7112	41	0.7810	61	0.8155	81	0.8372
2	0.3538	22	0.7165	42	0.7833	62	0.8168	82	0.8381
3	0.4247	23	0.7216	43	0.7854	63	0.8181	83	0.839
4	0.4736	24	0.7263	44	0.7875	64	0.8194	84	0.8398
5	0.5103	25	0.7308	45	0.7896	65	0.8206	85	0.8407
6	0.5393	26	0.7351	46	0.7915	66	0.8218	86	0.8415
7	0.5632	27	0.7392	47	0.7934	67	0.8230	87	0.8423
8	0.5833	28	0.7431	48	0.7953	68	0.8241	88	0.8431
9	0.6006	29	0.7468	49	0.7971	69	0.8252	89	0.8439
10	0.6156	30	0.7503	50	0.7989	70	0.8264	90	0.8447
11	0.6290	31	0.7537	51	0.8006	71	0.8274	91	0.8455
12	0.6409	32	0.7569	52	0.8023	72	0.8285	92	0.8462
13	0.6516	33	0.7600	53	0.8039	73	0.8295	93	0.8470
14	0.6613	34	0.7630	54	0.8055	74	0.8306	94	0.8477
15	0.6702	35	0.7659	55	0.8070	75	0.8316	95	0.8484
16	0.6783	36	0.7686	56	0.8085	76	0.8326	96	0.8491
17	0.6859	37	0.7713	57	0.8100	77	0.8335	97	0.8498
18	0.6929	38	0.7738	58	0.8114	78	0.8345	98	0.8505
19	0.6994	39	0.7763	59	0.8128	79	0.8354	99	0.8512
20	0.7055	40	0.7787	60	0.8142	80	0.8363	100	0.8519

Tables 3 and 4 show that both R_5 and R_{50} get closer to 1, which means C_5 and C_{50} get closer to LoD_v and to each other with increase of v.

3.4 'Imprecision' of qualitative assay

Section 8.3 of CLSI EP12-A2 guideline[16] recommends a method of evaluation of 'imprecision' of qualitative methods: "Ideally, a laboratory likes to know entire imprecision curve for a candidate method under its stipulated conditions. However, the experiment to estimate this curve is beyond the scope of this document. Instead, this section describes an experiment that will allow a laboratory to determine whether or not a particular concentration range, for example ±20% of C_{50}, … bounds … the $C_5 - C_{95}$ interval. If the -20%, +20% concentration range bounds $C_5 - C_{95}$ interval, then samples 20% or more away from C_{50} can be expected to yield consistent results; ie, results from samples outside $C_5 - C_{95}$ can be considered precise because they will consistently yield a positive result if greater than C_{95} and a negative result if less than C_5."

The EP12-A2 term 'imprecision' in application to PCR assays, in our opinion, should be replaced with 'uncertainty', nevertheless, it has inspired the following example of application of the probability of detection math model (9). The 'entire imprecision curve' in application to PCR assays is the probability of detection vs. target sequence concentration and the number of copies required for detection. Such curves can be generated using cumulative Poisson distribution function in Microsoft Excel® for calculating the probabilities of detection using math model (9). Such curves are shown in Figure 2 for the numbers of copies required for detection 1, 2, 5, 20 and 100.

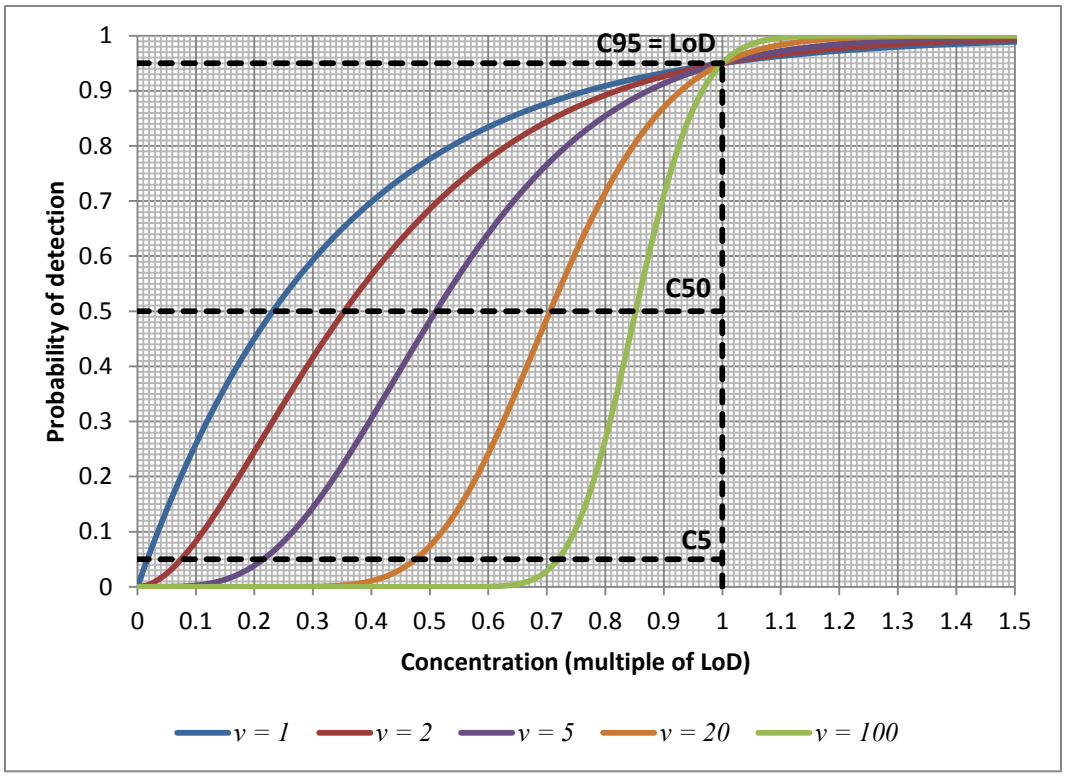

Figure 2: Probability of detection vs. concentration as multiple of LoD curves

So, the probability of detection math model (9) allowed to produce the 'entire curves' of interest to the clinical laboratories as identified in the above quote from the CLSI EP12-A2 guideline. Horizontal lines at probabilities 0.05, 0.50 and 0.95 cross the curves at concentrations C_5, C_{50} expressed as fractions of LoD_v and $C_{95} = LoD_v$. The expected $C_5 - C_{95}$ intervals as percent of C_{50} vs. the number of copies required for detection, v, can be easily calculated using the values of C_5 and C_{50} from Tables 3 and 4 and $C_{95} = 1$, all expressed as fractions of LoD_v, with the following formula:

$$[(C_5 - C_{50}) / C_{50},\ (C_{95} - C_{50}) / C_{50}] \qquad (17)$$

The intervals are shown in Figure 3 for $v = 1$ to 100. The intervals are asymmetrical, with upper bounds removed further from C_{50} in positive direction. They get within ±20% of C_{50} from C_{50} at $v \geq 77$. The $[C_5, C_{95}]$ interval of [-92.6%, 332%] in ubiquitous case of $v = 1$ is quite wide as percent of C_{50}. It gets tighter with increase of v, and for $v = 100$ it is [-15.6%, 17.4%].

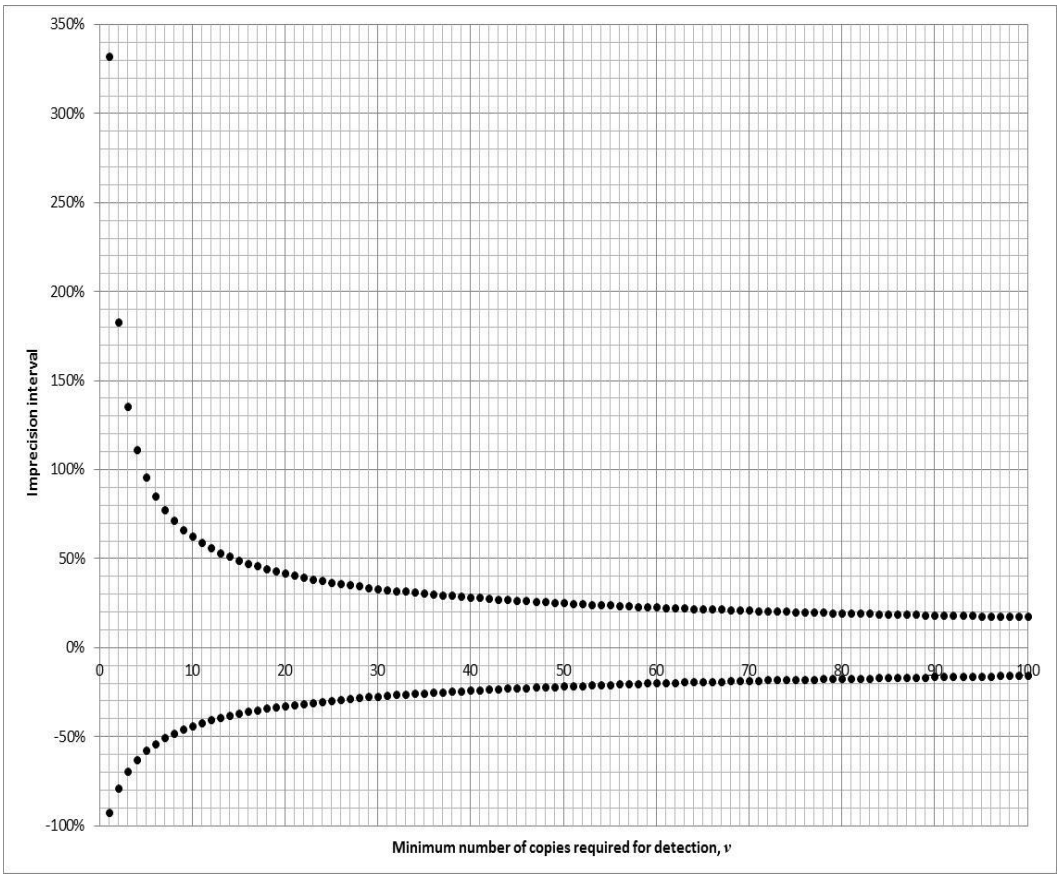

Figure 3: $[C_5, C_{95}]$ 'imprecision' interval as percent of C_{50} deviation from C_{50} for PCR assays

With $v < 15$, the upper bound of the interval exceeds 50%. In important case of single copy detectable, the interval is [-92.6%, 332%], which is not a meaningful estimate of imprecision. So, $[C_5, C_{95}]$ interval with bounds measured as above can be tightened by increasing the minimum number of copies detectable and sacrificing the assay analytical sensitivity. The analytical sensitivity of PCR assay is of utmost importance in donor blood screening and evaluating the ability of new medications completely healing patients of chronic diseases such as AIDS. For PCR assay, the bounds of $[C_5, C_{95}]$

interval, expressed as percent of C_{50} deviation from C_{50} following EP12-A2 guideline, are determined by the shape of the probability of detection vs. concentration curve corresponding to respective minimum number of copies detectable, v. The curves for some v values are shown in Fig. 2. Such [C_5, C_{95}] intervals do not depend on the PCR assay analytical sensitivity making them not very informative.

It can be also noted that in the above citation from EP12-A2 guideline, the statement "…results from samples outside $C_5 - C_{95}$ can be considered precise because they will consistently yield a positive result if greater than C_{95} and a negative result if less than C_5" is not accurate. Actually, sample with concentration > C_{95} is expected to have at least 95% positive results, while sample with concentration < C_5 is expected to have at least 95% of negative results. The actual proportions of observed positive and negative results will vary randomly.

The bounds of [C_5, C_{95}] interval, expressed as mean numbers of copies per sample volume, depend on the analytical sensitivity of PCR assay. For LoD_1 = 3, 5, 10 and 15 corresponding to extraction/reverse transcription efficiencies of 1, 0.6, 0.3 and 0.2, respectively, and minimum number of copies detectable from 1 to 100, the bounds of [C_5, C_{95}] interval are summarized in Table 5.

Table 5: [C_5, C_{95}] interval vs. minimum number of copies detectable for several values of LoD_1

	***LoD*$_1$**							
	3		**5**		**10**		**15**	
v	**C5**	**C95**	**C5**	**C95**	**C5**	**C95**	**C5**	**C95**
1	0.05	3.00	0.09	4.99	0.17	9.99	0.26	14.98
2	0.36	4.75	0.59	7.91	1.18	15.82	1.78	23.73
3	0.82	6.30	1.36	10.50	2.73	20.99	4.09	31.49
4	1.37	7.75	2.28	12.92	4.55	25.84	6.83	38.76
5	1.97	9.15	3.28	15.26	6.57	30.52	9.85	45.77
6	2.61	10.51	4.35	17.52	8.71	35.04	13.06	52.56
7	3.29	11.84	5.48	19.74	10.95	39.47	16.43	59.21
8	3.98	13.15	6.64	21.91	13.27	43.83	19.91	65.74
9	4.70	14.43	7.83	24.06	15.65	48.11	23.48	72.17
10	5.43	15.71	9.04	26.18	18.09	52.36	27.13	78.53
20	13.25	27.88	22.09	46.46	44.18	92.93	66.27	139.39
40	30.20	50.94	50.33	84.90	100.66	169.80	150.98	254.70
60	47.85	73.28	79.76	122.14	159.52	244.28	239.27	366.42
80	65.88	95.26	109.80	158.76	219.60	317.53	329.40	476.29
100	84.15	117.00	140.24	195.00	280.48	389.99	420.73	584.99

The graph based on Table 5 is shown in Figure 4 below. To produce Table 5 and Figure 4, C_5 values were calculated multiplying R_5 values in Table 3 with r_v from Table 1 and LoD_1. C_{95} values were calculated multiplying LoD_1 with r_v. In the graph, the pairs of curves of the same color are: for C_5 the lower curve, and for C_{95} the upper curve. With the curves in Fig. 4, the [C_5, C_{95}] intervals are visualized as vertical spaces between the pairs

of curves corresponding to the respective minimum numbers of copies detectable and LoD_1. Table 5 and Fig. 4 show that so measured $[C_5, C_{95}]$ interval is the tightest with single copy detectable and the lowest possible LoD of 3 copies per test sample volume.

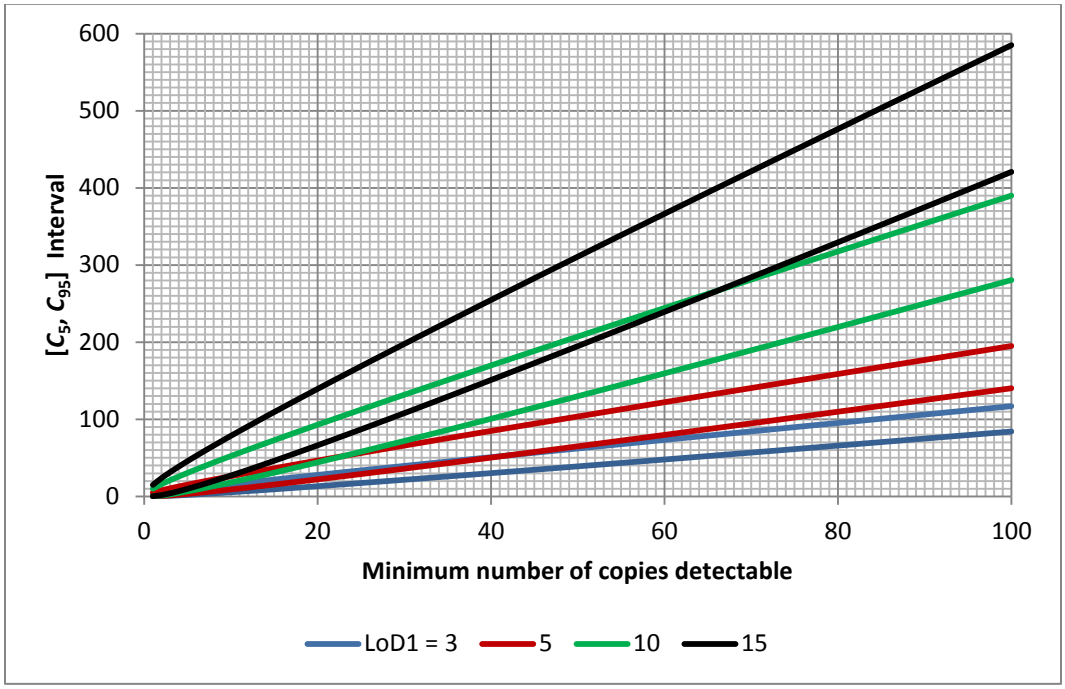

Figure 4: $[C_5, C_{95}]$ interval vs. minimum number of copies detectable for several values of LoD_1

With the curves in Fig. 4, the $[C_5, C_{95}]$ intervals are visualized as vertical spaces between the pairs of curves corresponding to the minimum numbers of copies detectable. Table 5 and Fig. 4 show that so measured $[C_5, C_{95}]$ interval is the tightest with single copy detectable and the lowest possible LoD of 3 copies per test sample volume. The bounds of $[C_5, C_{95}]$ interval expressed as mean numbers of copies per test sample volume are defined by the limit of detection characterizing the analytical sensitivity of the assay and the minimum number of copies required for detection. For this reason, such bounds are more meaningful than the bounds expressed as percent of C_{50} deviation from C_{50}.

In the above citations from CLSI EP12-A2 "Ideally, a laboratory likes to know entire imprecision curve …", which in case of PCR assays is the curve for the probability of detection as a function of concentration expressed as a fraction or multiple of LoD and the minimum number of copies detectable. Such curves calculated using math model (9) are shown in Fig. 2 above for several minimum numbers of copies detectable. Method of estimation of the minimum number of copies detectable, v, and of the limit of detection, LoD_v, using math model (9) with data collected for a PCR assay is a large subject, it is out of scope of this paper, and it will be published separately.

4. Conclusions

A general math model describing the probability of detection of target nucleic acid sequence vs. concentration and minimum number of copies required for detection has been derived based on description of the molecular diagnostics PCR assay process and

certain assumptions. The math model has been validated with data collected in 8 LoD studies using four types of Roche instruments for 12 blood screening, virology and microbiology DNA/RNA targets that included multiple genotypes. None of 84 χ^2 goodness-of-fit hypothesis tests was rejected so validating the math model. The p-value for the χ^2 test with 157 degrees of freedom on the combined across the studies and nucleic acid targets was practically 1, testifying of near perfect fit of the math model to data.

Examples of useful applications of the general math model for probability of detection provided are: (1) estimation of concentration, along with confidence bounds, as a multiple of LoD from observed proportion of positives; (2) estimation of LoD from sample concentration and observed proportion of positives along with confidence bounds; (3) estimation of concentration corresponding to the probability of detection of interest as multiple of LoD along with confidence bounds; (4) probability of detection vs. concentration curves for several minimum numbers of copies detectable and bounds of $[C_5, C_{95}]$ interval recommended in CLSI EP12-A2 guideline as characteristic of imprecision of qualitative assay. Other useful applications of the probability of detection math model will be subjects of future publications.

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References

1. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures, Approved Guideline, Second Edition. CLSI document EP17-A2, Wayne, PA, USA (2012).
2. Martin, B., Carriga, M, Aymerich, T. (2012) Pre-PCR treatments as a key factor on the probability of detection of *Listeria monocytogenes* and *Salmonella* in ready-to-eat meat products by real-time PCR, Food Control (Elsevier) , Vol. 27, Iss. 1, 163-169
3. Burns, M, Valdivia, H., Modelling the limit of detection in real-time quantitative PCR, European Food Research Technology (2008) 226:1513–1524
4. Box GEP, Draper NR, (1987), Empirical Model Building and Response Surfaces, John Wiley & Sons, New York, NY.
5. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. J. Immunol. 126**:**1614-1619.
6. Bustin, S., Benes, V., Garson, J., et al, The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clinical Chemistry 55:4, 611–622 (2009)
7. Vaks, J.E., Hemyari, P., Rullkoetter, M., Santulli, M.J., Schoenbrunner, N., Verification of claimed limit of detection in Molecular diagnostics, Journal of Applied Laboratory Medicine, Nov. 2016, pp. 260-270.

8. Cobb, B.R., Vaks, J.E., Do, T., Vilchez, R. A., Evolution in the sensitivity of quantitative HIV-1 viral load tests, Journal of Clinical Virology, 52S (2011) S77– S82.
9. Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., Erlich, H. (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase". *Science*. 239 (4839): 487–491.
10. Rodrigo, A. G., P. C. Goracke, K. Rowhanian, and J. I. Mullins. 1997. Quantitation of target molecules from PCR-based limiting dilution assays. AIDS Res.Human Retrovir. 13**:**737-742.
11. Van der Zee, A., Roorda, L., Bosman, G., Ossewaarde, J.M., Molecular Diagnosis of Urinary Tract Infections by Semi-Quantitative Detection of Uropathogens in a Routine Clinical Hospital Setting. PLoS One, 2016; 11(3): e0150755.
12. Al-Soud, W.A., Radstrom, P., Purification and characterization of PCR-inhibitory components in blood cells. Journal of Clinical Microbiology, 2001 Feb;39(2):485-93.
13. Maple® 2016, Mathematical software by Maplesoft, Inc., Waterloo, Canada.
14. Guttman I., Wilks S.S., Hunter J.S., Introductory Engineering Statistics, Wiley, 1982, pp. 287-291.
15. Clopper, C.; Pearson, E.S. (1934). The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika* 26: 404–413.
16. User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline—Second Edition. CLSI document EP12-A2, Wayne, PA, USA (2007).