Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Guidelines for the validation of qualitative real-time PCR methods by means of a collaborative study

Foreword

These guidelines for conducting a collaborative validation study of qualitative real-time PCR methods has been developed by a subgroup of the § 64 LFGB working group "Development of methods for identification of foodstuffs produced by means of genetic engineering techniques".

The present guidelines are intended to ensure that real-time PCR methods developed by the § 64 LFGB working group can meet the performance criteria defined at the European (ENGL, CEN) and international level (ISO, Codex) [1, 2].

In addition, the aim of this document is to propose novel method validation approaches to other relevant bodies (ENGL, CEN / ISO) in order to actively contribute to the process of defining minimum required performance criteria in this area.

The procedure described in this document is a **recommendation** that is underpinned by practical experience. It is possible to apply alternative approaches for which it can be shown that the performance criteria mentioned in the present document are achieved.

The sub-working group "validation of real-time PCR methods" was chaired by Dr. Lutz Grohmann. The members of the group were: Hermann Broll, Emilie Dagand, Dr. Sabine Hildebrandt, Dr. Peter Hübert, Dr. Heiko Kiesecker, Dr. Kathrin Lieske, Dr. Dietrich Mäde, Dr. Joachim Mankertz, Dr. Ralf Reiting, Dr. Manuela Schulze, Brigitte Speck, Dr. Steffen-Uhlig, Dr. Daniela Wahler, Hans-Ulrich Waiblinger, Dr. Karl Woll and Katrin Zur.

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1 Scope

These guidelines describe the performance characteristics which should be determined when conducting a collaborative study for the validation of qualitative real-time PCR methods

The validation data thus obtained will then inform the decision whether to include the method under consideration in the BVL's Official Collection of Methods.

2 Performance characteristics

The main criterion in the validation of a qualitative real-time PCR method by means of a collaborative study concerns the determination of the **false-positive rate** and **false-negative rate**.

Due to the use of different real-time PCR equipment from one laboratory to the next, information on the robustness of the method can also be derived.

Moreover, on the basis of a mathematical-statistical model for the validation of the probability of detection (POD) of qualitative PCR methods [3], the following performance characteristics can be computed if the design of the collaborative study is appropriate:

- **Laboratory standard deviation σ**
- **Mean amplification probability λ**
- **Slope parameter *b* of the POD curve (across laboratories)**
- **$LOD_{95\%}$ (across laboratories)**

Note: The limit of detection ($LOD_{95\%}$) should be determined in the course of single-laboratory validation.

2.1 False-positive rate / false-negative rate

Definitions:

The false-positive rate is a measure of the probability that a method will classify a known negative test sample as positive. The false-negative rate is a measure of the probability that a method will classify a known positive test sample as negative.

Procedure:

The following materials can be used as test samples for the determination of the false-positive and false-negative rates of a qualitative real-time PCR method:

- DNA solutions (in general extracted from reference material)
- Sample materials (reference material or samples from which the DNA must be extracted)

In collaborative studies, the test material consists generally of DNA solutions. However, if the target sequence is to be detected mainly for one specific matrix and if, for this matrix, no validated extraction procedure is available, it may make sense to include the DNA extraction in the collaborative study.

In the collaborative study, each participant receives encoded positive and negative samples. The positive samples contain definite quantities of the target sequence. The negative samples only contain non-genetically modified taxon-specific plant DNA or matrix-specific negative material.

a) Collaborative study with DNA solutions

A laboratory involved in the preparation of the collaborative study extracts DNA from the sample material and pre-tests it with respect to amplificability and inhibition. A detailed description of this procedure can be found in other documents [1, 4]. The positive samples should contain at least twice the number of copies corresponding to the limit of detection as determined in the course of the in-house validation (but no fewer than 20 copies of the target sequence) per reaction. The samples are to be prepared with up to 100 ng per 25 µl PCR reaction of suitable background DNA.

b) Collaborative study with sample materials

If the collaborative study uses sample materials from which DNA must be extracted, the participants must be given the opportunity to check the performance of the DNA extraction method. For this purpose, the participants may be sent an additional sample for positive extraction control (P). This sample will allow each participant to test the extraction method and its own reagents.

To this end, a PCR analysis is performed both for the DNA from P and for a positive control DNA provided by the organizer. The analysis is carried out in duplicate and the mean Ct values are then compared. The mean Ct value for the DNA from P should not exceed the mean Ct value for the positive control by more than 1 (for example: Ct value of positive control = 23 → Ct value of DNA from P should not exceed 24). This applies to the target-sequence PCR as well as to the taxon-specific PCR. Moreover, it is recommended to carry out an inhibition control test [cf. 1, 4].

For screening methods, samples from more than one relevant species (e.g. corn and soya) may be included in the pool of test samples.

The reagents for the PCR (oligonucleotides, PCR master mix) are supplied to the participants in order to ensure that different PCR reagents, which have not been checked for suitability, do not influence the results.

Experience shows that, in order to be able to carry out the procedure described in Table 1, the number of participants should be ≥ 12.

Each participant receives at least 6 positive and 6 negative samples, which have been encoded beforehand. The participants perform the PCR measurements in single determination. Thus, for each laboratory, at least 6 results for positive and 6 results for negative DNA samples are available for the evaluation.

Table 1: Example of a collaborative study design with sample materials containing the target sequence in different species

Type of sample	Quantity of target sequence in PCR	Examples for the number of copies of the target sequence	Number of samples per participant	Required number of laboratories (with evaluable results)
DNA species 1	2 x $LOD_{95\%}$	20	6	10
DNA species 2	2 x $LOD_{95\%}$	20	6	10
DNA species 1	0	0	6	10
DNA species 2	0	0	6	10

Note: It may make sense to test another concentration level of the target sequence in the collaborative study (e.g. 3 x $LOD_{95\%}$).

Evaluation:
On the basis of the available results, the false-positive and false-negative rates are calculated as follows [2]:

$$\%\ \boldsymbol{false-positive\ results} = \mathbf{100} \times \frac{\boldsymbol{number\ of\ misclassified\ known\ \ negative\ samples}}{\boldsymbol{total\ number\ of\ known\ negative\ samples}}$$

$$\%\ \boldsymbol{false-negative\ results} = \mathbf{100} \times \frac{\boldsymbol{number\ of\ misclassified\ \ known\ positive\ samples}}{\boldsymbol{total\ number\ of\ known\ positive\ samples}}$$

Performance criteria:
Neither the false negative rate nor the false positive rate should exceed 5 %.

False positive rates above 5 % should be investigated on a case-by-case basis. The method documentation should then provide relevant instructions.

Note: Apparently false positive results could occur, for example, in screening tests for the detection of genetic elements which occur naturally.

Annex 1 provides further information regarding the conduct of the collaborative study, the preparation of test samples and the evaluation of the results and pertaining documentation.

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2.2 Robustness

Definition:

The robustness of a method is a measure of its capacity to remain unaffected by small, accidental changes in the environment or in the conditions in which the measurement was performed. In order to determine robustness, measurement results corresponding to small, deliberate changes in the measurement conditions are collected.

Procedure:

In the collaborative study, different types of real-time PCR equipment are used.

Performance criteria:

The method must produce the expected results despite these changes. There must not be any noticeable difference between the results obtained using different equipment.

Note: Robustness is primarily tested in the framework of single-laboratory validation.

2.3 Probability of Detection (POD)

On the basis of a mathematical-statistical model for the validation of the probability of detection (POD) of qualitative PCR methods [3], the following performance characteristics can also be computed if the design of the collaborative study is appropriate:

- **Laboratory standard deviation σ**
- **Mean amplification probability λ**
- **Slope parameter *b* of the POD curve (across laboratories)**
- **$LOD_{95\%}$ (across laboratories)**

Definitions:

The probability of detection (POD) describes the probability that, for a given number of copies of the target sequence, PCR amplification will take place. The mean number of copies of the target sequence yielding a probability of detection of 0.95 is called limit of detection or $LOD_{95\%}$.

The laboratory standard deviation σ characterizes the dispersion of the log-transformed laboratory-specific values for $LOD_{95\%}$.

The mean amplification probability λ describes the probability that, for a randomly selected copy of the target sequence, PCR amplification will occur.

The slope parameter ***b*** of the POD curve (across laboratories) describes the deviation from the ideal POD curve ($b = 1$). The ideal POD curve is based on the assumption that the mean amplification probability is independent of the number of copies of the target sequence.

The across-laboratory $LOD_{95\%}$ corresponds to the $LOD_{95\%}$ of a theoretical median laboratory, i.e. of a laboratory whose sensitivity corresponds to the median of the theoretical distribution of the laboratory-specific sensitivities.

Procedure:

In order to obtain suitable data for the computation of the different performance characteristics of the POD, the laboratories receive standard DNA with a calculated number of copies of the target sequence (cf. “Guidelines for single-laboratory validation”, Annex 1).

On the basis of the standard DNA, a dilution series for the target sequence is prepared. The dilutions are prepared in a buffer solution having a uniform non-target DNA concentration. For this purpose, standard DNA (genomic DNA, plasmid DNA or amplicon DNA) is added to the corresponding amount of background DNA and thereby stabilized for the PCR (**Table 2**).

PCR measurements are carried out in triplicate determination for dilution levels 1 to 4, and 6 replicate determinations are performed for dilution level 5 and onwards.

The specified replicate numbers and dilution levels represent the minimum number necessary to obtain a sufficient statistical reliability for $LOD_{95\%}$ and for the corresponding precision data [3].

Annex 1 provides further instructions concerning the conduct of the collaborative study and the preparation of the standard DNA.

Table 2: Example of a scheme for the preparation of a dilution series

Dilution level	Preparation (example)	Number of copies of target sequence (in 5µl)	Number of PCR replicates
1	dilute DNA stock solution with 0.2xTE*	2 500	3
2	10 µl (500 copies/µl) + 40 µl 0.2xTE*	500	3
3	20 µl (100 copies/µl) + 80 µl 0.2xTE*	100	3
4	50 µl (20 copies/µl) + 50 µl 0.2xTE*	50	3
5	40 µl (10 copies/µl) + 60 µl 0.2xTE*	20	6
6	50 µl (4 copies/µl) + 50 µl 0.2xTE*	10	6
7	50 µl (2 copies/µl) + 50 µl 0.2xTE*	5	6
8	40 µl (1 copy/µl) + 60 µl 0.2xTE*	2	6
9	50 µl (0.4 copies/µl) + 50 µl 0.2xTE*	1	6
10	10 µl (0.02 copies/µl) + 90 µl 0.2xTE*	0.1	6

*contains background DNA (e.g. 20 ng/µl of salmon semen DNA in 0.2xTE-buffer)

Evaluation:

On the basis of the standard curve (2500 to 50 copies), the values for the slope and the coefficient of determination of the calibration function are computed for each laboratory and are presented in a table (**Table 3**). The results submitted by the laboratories are to be checked for deviations and outliers (plausibility check and outlier tests, e.g. Grubbs test for the laboratory-specific values for the calibration function slope and the coefficient of determination).

A calibration function slope of approx. -3.1 to -3.6 is considered to be an indication of good PCR efficiency. The coefficient of determination should be at least 0.98.

DNA dilutions in the range from 20 to 0.1 copies per PCR reaction are used for the validation of the POD performance characteristics. The corresponding qualitative PCR results (positive and negative results) for the 6 dilution levels are presented (**Table 4**).

Table 3 Example showing the evaluation of the standard curves in a collaborative study [5]

Laboratory code	PCR equipment	Pubi-cry PCR method		
		Slope [s]	Coefficient of determination [R^2]	PCR efficiency [%]
A	ABI 7500	-3.60	1.00	89.6
B	Bio-rad CFX96	-3.29	0.99	101.1
C	MX3000p	-3.31	0.94	100.4
D	ABI 7500Fast	-3.54	0.99	91.8
E	ABI 7500	-3.40	1.00	96.8
F	ABI 7500	-3.44	1.00	95.4
G	Roche LC480	-3.41	0.99	96.6
H	ABI 7500	-3.32	0.99	100.0
I	Rotorgene 6000	-3.34	0.99	99.1
J	ABI 7500	-3.43	0.99	95.5
K	ABI 7900HT	-3.44	0.99	95.5
L	ABI 7500	-3.47	1.00	94.0
M	ABI 7500	-3.55	0.99	91.3
N	ABI 7500	-3.41	0.99	96.5
O	ABI 7500Fast	-3.36	1.00	98.3
P	ABI 7900HT	-3.38	0.99	97.6
Q	ABI 7500	-3.66	0.99	87.6

Table 4 Example showing the qualitative evaluation of the results from 17 laboratories [3]

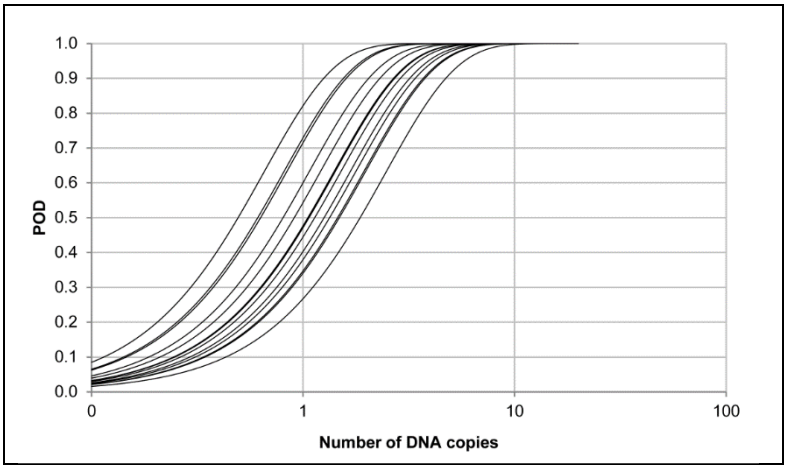
Laboratory No.	Nominal number of copies of target sequence in PCR					
	0.1	1	2	5	10	20
01	0	3	5	5	6	6
02	0	4	6	6	6	6
03	1	0	5	6	6	6
04	0	4	3	6	6	6
05	0	0	5	6	6	6
06	0	4	6	6	6	6
07	0	5	6	6	6	6
08	0	5	6	6	6	6
09	0	6	4	6	6	6
10	0	2	5	6	6	6
11	0	1	6	6	6	6
12	0	4	6	6	6	6
13	0	4	4	6	6	6
14	0	3	3	5	6	6
15	0	6	5	6	6	6
16	0	2	6	6	6	6
17	1	4	6	5	6	6

On the basis of the qualitative data, the laboratory-specific POD curves can be computed, and then the slope parameter *b* (across laboratories). Plausibility checks and outlier tests (e.g. according to Grubbs) are carried out for the laboratory-specific POD curves, the amplification probability λ and the slope parameter *b*.

Note: The value calculated for b should be in the range 0.65 < b ≤ 2 in order to ensure sensible POD curves. This range is based on the condition that $LOD_{95\%}$ is not higher than 20 copies and that the amplification probability in the range from 0.1 to 100 copies is not higher than 1.

Figure 1 shows the laboratory-specific POD curves which were calculated on the basis of the slope parameter *b* (across laboratories) in the framework of a collaborative study [3].

Figure 1 Example of laboratory-specific POD curves with slope parameter *b* = 1.29 [1]



Once the lab-specific POD curves have been computed, the values for the other performance characteristics can be calculated [1]. An example is presented in **Table 5**.

Table 5 Example of the statistical evaluation of the POD performance characteristics of the real-time PCR method for the detection of Pubi-cry [5]. Values for the following performance characteristics are provided: mean amplification probability (λ_0) and corresponding 95 % confidence interval, slope parameter *b* of the POD curve across laboratories, laboratory standard deviation (σ_L) as well as the $LOD_{95\%}$ (in copies of the target sequence) of the theoretical median laboratory at POD = 0.95.

Parameters		**Value [Pubi-cry PCR]**
Number of participating laboratories		17
Number of PCR replicates per concentration level		6
POD curve	mean amplification probability λ_0	0.77
	95 % confidence interval for λ_0	0.60 – 0.98
	slope parameter b (across laboratories)	1.19
	standard deviation σ_L (across laboratories)	0.31
$LOD_{95\%}$ [in copies]	theoretical median laboratory	3.1

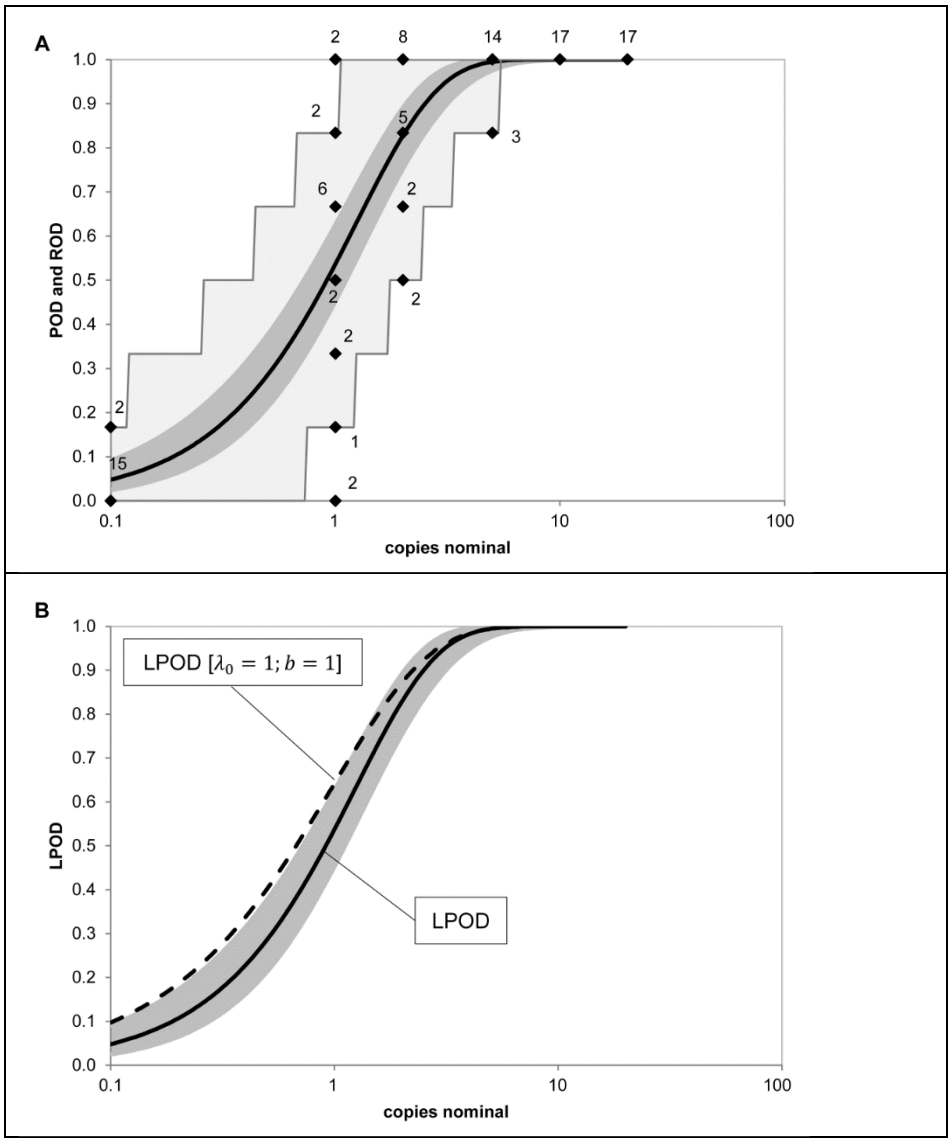
If no statistically significant deviation from the value 1 is observed for the slope parameter *b*, the computation of the other performance characteristics is based on $b = 1$.

A value for $b \leq 1$ is an indication that the amplification is inhibited already for low numbers of copies.

A value of $\lambda \geq 1$ is an indication that the actual number of copies of the dilution series is higher than the nominal value (the value calculated beforehand).

The computed values for the POD curve across laboratories along with the corresponding prediction intervals as well as the rates of detection for the different concentration levels of the target sequence can be presented graphically (**Figure 2)**.

Figure 2 **(A)** POD curve across laboratories (LPOD) with corresponding 95% prediction interval (dark grey) and 90% prediction interval (light grey) for the rate of detection (ROD). The diamonds with the numbers show the distribution of the qualitative PCR results of 17 laboratories (number of laboratories and corresponding rate of positive PCR results for the 6 replicates, cf. **Table 4**).
(B) LPOD curve under ideal conditions ($\lambda = 1$ and $b = 1$; dotted line) in comparison to the calculated LPOD curve (the corresponding 95% prediction interval is depicted in dark grey).



Performance criteria:

The upper 95 % prediction limit of the laboratory-specific $LOD_{95\%}$ values should not exceed 20 copies of the target sequence.

As a general rule, the $LOD_{95\%}$ is subject to considerable variability across laboratories. For this reason, a test is carried out on the basis of the ratio of the 5 % and 95 % quantiles. This ratio should not exceed a value of 5.

Example: If $LOD_{95\%}$ is less than 4 copies for 5 % of all laboratories, lies in the range of 4 to 20 copies for 90 % of all laboratories and exceeds 20 copies for the remaining 5 % of all laboratories, this ratio has a value of 5.

Note: With a value of 5 for the ratio of the upper and lower prediction limits of the laboratory-specific $LOD_{95\%}$ values, the mean amplification probability λ_0 is approx. 15 % (an estimate based on first computing the mean $LOD_{95\%}$ value corresponding to a distribution of $LOD_{95\%}$ values with 3 copies as 5 % quantile and 15 copies as 95 % quantile, and then on the relationship derived from the Poisson distribution: $\lambda \times LOD_{95\%} = 2.996$).

The laboratory standard deviation σ_L describes, in the log domain, how much the POD curves of the individual laboratories differ from one another. A value of $\sigma_L = 1$ should not be exceeded.

Note: In the case of a value for σ_L exceeding 1, the $LOD_{95\%}$ (corresponding to a "theoretical median laboratory") exceeds the limit of 20 copies, and the ratio of the upper and lower prediction limits of the laboratory-specific $LOD_{95\%}$ values exceeds the value of 50. In other words, a value of $\sigma_L \geq 1$ means that the method has a very poor mean sensitivity and, moreover, that sensitivity can be expected to be subject to considerable variability from one laboratory to the other.

2.4 Precision data for samples (optional)

The standard curves make it possible to compute copy numbers for the test samples. Moreover, precision data for the statistical parameters (mean, repeatability and reproducibility standard deviation, etc.) according to DIN ISO 5725-2 can be calculated (**Table 6**).

In connection with the computation of these precision data, it is necessary to identify and eliminate possible outlier data sets.

Table 6 Example of statistical precision data (according to ISO 5725-2) from the collaborative validation study of the cry1Ab/Ac method [5]. The Poisson standard deviation *s* and the expanded measurement uncertainty* U (coverage factor 2) are provided.

Materials for sample DNA	Number of laboratories	Ratio pos. tests/ all tests	Computed copies/µl				
			Mean* ± *U*	SD_r	SD_R	*s*	RSD_R [%]
0.05 % Bt63 rice	17	34/34	0.8 ± 0.2	0.3	0.4	0.9	46.9
KeFeng6 rice	17	34/34	5.3 ± 0.6	0.9	1.4	2.3	26.3
4.89 % Bt11 maize	17	34/34	11.0 ± 0.9	1.7	2.2	3.3	20.2
KeFeng6-positive rice noodles (RASFF 2009.0717)	17	32/34	0.4 ± 0.1	0.2	0.2	0.6	58.0
GM-positive Basmati rice (RASFF 2011.1646)	17	34/34	1.6 ± 0.2	0.5	0.6	1.3	34.7

*The expanded measurement uncertainty corresponds to twice the standard error of the mean.

Annex 1 - Instructions for the conduct of the collaborative study

A. Collaborative study samples and standard DNA

DNA solutions containing the target sequence

Reference DNA or reference material with a certified percentage of GMOs, which is characterized as well as possible with regard to the target sequence, e.g. regarding the number of copies and zygosity, is especially well suited.

DNA solutions not containing the target sequence

Reference DNA or reference material which does not contain the respective GMO and which, for example, corresponds to the isogenic lines, can be used.

It must be kept in mind that the certification of such materials only refers to the indicated GMO content. This means that contaminations with other GMOs at trace level (< 0.1 %) are still possible. For this reason it can be an advantage for the production of negative DNA samples to use vegetative plant material from individual plants of the same species, which have been proven not to contain the target sequence.

Usually, for each species analyzed in the collaborative study, one non-genetically modified material is analyzed.

If background DNA is used, it must be checked for inhibition.

Standard DNA

Standard DNA can be produced from genomic DNA, plasmid DNA or amplicon DNA.

Sufficient standard DNA must be provided to allow for the preparation of the dilution series with at least 4 levels presented in **Table 2**. Ideally, enough volume is produced to enable each participating laboratory to repeat the preparation of the dilution series if necessary.

For the preparation of the dilution series, a buffer solution with background DNA (at least 20 ng/µl of a non-target DNA, e.g. salmon sperm DNA) is to be provided.

B. Reagents

The quantity of reagents shipped by the organizer must be sufficient to allow a repetition of the PCR measurements (e.g. in cases of equipment failure or apparent mistakes in the execution).

If commercial kits are used for DNA extraction or clean-up, they are to be purchased by the organizer and shipped to the study participants. Further reagents for DNA extraction (e.g. buffers, RNases, proteinases, amylases, etc.) can be purchased by the study participants themselves, if required.

Primers, probes and master mixes are purchased by the organizer and shipped directly to the participants.

Before being used in the study, the reagents must be checked along with an original set of test samples by a laboratory which will not participate in the study. Only after the successful completion of this check may the collaborative study start officially.

C. Documentation and evaluation

The following data and results are documented:

DNA extraction (if included)

- Initial weight and possible deviations from the protocol of the collaborative study
- Number of extractions carried out per encoded sample (standard = 1).
- Concentrations of the extracted sample DNA (along with the method used to determine the concentrations)

Real-time PCR

- Equipment and equipment settings (threshold, baseline)
- Number of PCR replicates per DNA extract for the encoded samples (standard = 1).
- Possible deviations from the instructions provided for the collaborative study
- Ct values of the DNA dilution series and of the encoded samples
- Results of the amplification reagent control (NTC) and, if applicable, of the negative extraction control

The organizer of the collaborative study provides the participants with a spreadsheet for the computation of the slope and the coefficient of determination by means of linear regression applied to the data of the standard series. For this purpose the Ct values of the dilution levels are plotted versus the log-transformed quantities as number of copies per reaction mixture.

After decoding, the results of each laboratory are assigned to the individual collaborative study samples (or DNA extracts) and concentration levels, and are evaluated separately.

The submitted laboratory results are to be checked for deviations.

The following data are calculated:

By laboratory (columns):

- Number/percentage of positive reactions per 6-fold blank sample (e.g. 6 out of 6)
- Number/percentage of positive reactions for all dilution levels of the DNA standard solution below 50 copies
-

Summary of all laboratories:

Table C-1: Example of a table for the evaluation with regard to false-positive / false-negative rates

Number of participating laboratories	10
Number of laboratories having submitted results	10
Number of samples per laboratory	12
Number of accepted results	120
Number of samples containing the target sequence	60
Number of samples not containing any target sequence	60
False positive results (rate in %)	0 (0 %)
False negative results (rate in %)	0 (0 %)

Further evaluation (optional)

Further data for the description of the performance of the PCR method can be determined from the following results (cf. **Annex D** of the “Guidelines for in-house validation”):

6-fold blinded (encoded) samples

- Mean Ct and calculated number of copies (if applicable, per DNA extract)
- Mean Ct and number of copies of all identical DNA extracts, or identical DNA samples
- Standard deviation for the number of copies of all identical DNA extracts carried out under repeatability conditions, or for the identical DNA samples, absolute and with regard to the mean value (repeatability standard deviation, s_r)

Dilution series

- Mean Ct and mean value of the number of copies per dilution level of the dilution series (if all reactions of the dilution level are positive)
- Standard deviation for the number of copies for a dilution level analyzed under repeatability conditions, absolute and with regard to the mean value (repeatability standard deviation, s_r) (only if all reactions of the dilution level are positive).

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ViSdP:
Frau N. Banspach (BVL, Pressestelle)

Picture:
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