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Checklist for Optimization and Validation of Real-Time PCR Assays

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Real-time polymerase chain reaction (PCR) is a frequently used technique in molecular diagnostics. To date, practical guidelines for the complete process of optimization and validation of commercial and in-house developed molecular diagnostic methods are scare. Therefore, we propose a practical guiding principle for the optimization and validation of real-time PCR assays. Based on literature, existing guidelines, and personal experience, we created a checklist that can be used in different steps of the development and validation process of commercial and in-house developed real-time PCR assays. Furthermore, determination of target values and reproducibility of internal quality controls are included, which allows a statistical follow-up of the performance of the assay. Recently, we used this checklist for the development of various qualitative and quantitative assays for microbiological and hematological applications, for which accreditation according to ISO 15189:2007 was obtained. In our experience, the use of the proposed guidelines leads to a more efficient and standardized optimization and validation. Ultimately, this results in reliable and robust molecular diagnostics. The proposed checklist is independent of environment, equipment, and specific applications and can be used in other laboratories. A worldwide consensus on this kind of checklist should be aimed at. J. Clin. Lab. Anal. 23:145–151, 2009.

Key words: real-time PCR; checklist; molecular diagnostics; optimization; validation

INTRODUCTION

After the description of the polymerase chain reaction (PCR) by Saiki et al. (1) in 1985 and Mullis et al. (2) in 1987, PCR kinetics could be analyzed by Higuchi et al. (3,4) through the construction of a system that detects PCR products as they accumulate, using an intercalating dye (''real-time PCR''). In 1991, the cleavage of a target-specific probe during PCR, using the 5′ nuclease activity of Taq DNA polymerase, was demonstrated by Holland et al. (5).

The development of fluorogenic probes (6,7) further improved the real-time PCR assay by enabling the monitoring of a fluorescent signal, which is generated only in case of a specific hybridization between probe and target.

In contrast to conventional PCR, this real-time PCR methodology allows a nonlaborious, reliable detection and quantification of most nucleic acid target sequences. Thanks to these characteristics, real-time PCR has revolutionized molecular biology and an extensive number of applications have been developed, both in research and clinical diagnostics (8). The majority of these applications are noncommercial in-house developed assays. As for all clinical laboratory testing, standardization and quality assurance are required for molecular diagnostics as well. This is described in the international quality standard for medical laboratories ISO 15189:2007, which demands for verification and validation procedures for each assay. In Belgium, ISO 15189:2007 certification is required for reimbursement of molecular tests. However, ISO 15189:2007 guidelines are general and do not specify requirements for molecular assays. Practical guidelines for the optimization and validation of commercial and in-house developed molecular diagnostic methods found in literature often focus on either good laboratory practice or on subdivisions of the validation process (9–15).

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Therefore, we propose a practical guiding principle that allows standardization of the complete process of optimization and validation of commercial and in-house developed real-time PCR assays. In our laboratory, the use of the proposed checklist has allowed a consistent and standardized validation process for microbiological and hematological, qualitative and quantitative assays. Because it is independent of environment, equipment, and specific applications, it can be exchanged between laboratories.

CHECKLIST FOR OPTIMIZATION AND VALIDATION OF REAL-TIME PCR ASSAYS

Choice of Method

This article focuses on the technical and diagnostic performance of molecular diagnostic assays. Our checklist is based on literature, existing guidelines, and personal experience. It describes the different steps for the selection and the validation of the chosen assay. Besides general recommendations, that can be used for all assays and that are included in the checklist, test-specific criteria should be defined for each individual test validation. The complete list is depicted in the table and is clarified below.

In-house assay or commercial assay

As real-time PCR has only been used commonly for 10 years, hardly any commercial assays are yet available for many clinically important parameters. Consequently, it is often necessary to develop in-house assays, which need adequate optimization and validation before they are introduced in routine diagnostics.

For commercially available FDA or IVD-CE labeled assays, the manufacturer is responsible for the performance as stated in the kit insert. Nevertheless, the user should verify that the indicated performance characteristics are achieved in the local laboratory (9).

Choice of target gene

The first step in the development process of an in-house assay is the choice of a nucleic acid target. A literature review often reveals which target is most suitable for each particular assay. For viruses and bacteria, a specific and conserved nucleic acid target sequence is selected.

For real-time PCR assays that are used for the detection of somatic mutations, rearrangements, breakpoint fusion regions of chromosome aberrations, fusion-gene transcripts, aberrant genes, and aberrantly expressed genes, the region of aberration should be targeted. Additionally, for reverse transcription (RT) hematological assays, primers and probes should span an exon–exon splice junction, enabling amplification and detection of RNA sequences only. This prevents co-amplification of genomic DNA, which can compromise assay sensitivity and efficiency by competition between the desired PCR product and the product derived from genomic DNA. Screening the genome databases with the amplicon sequence also helps to ensure that an assay does not detect pseudogenes (16).

Choice of detection method

The detection method used at present in real-time PCR is monitoring of fluorescence. Nonspecific labels and sequence-specific probes are available as reporters.

Melt curve analysis, using intercalating dyes, allows the detection of double-stranded DNA, generated during PCR. The use of an intercalating dye gives the opportunity to detect nonsequence-specific amplified products. On the other hand, mis-priming events can generate a false-positive signal.

The use of fluorogenic probes leads to a specific hybridization between probe and target. Probes can be labeled with two kinds of dyes: (i) fluorophores with intrinsically strong fluorescence, which are brought in contact with a quencher molecule through structural design and (ii) fluorophores that can change their fluorescence capacities upon binding the target DNA. Examples of the former kind of probes are hydrolysis probes (based on oligonucleotides (6,7) or on locked nucleic acids (17,18)), minor groove binding probes (19), molecular beacons (20,21), and hybridization probes (22). More recently, fluorescent-labeled primers were also developed (23). The second kind of probes includes Light Up probes (24) and displacement probes (25). The advantages and disadvantages of each chemistry are discussed by Gunson et al. (10), Kubista et al. (26), Arya et al. (27), Aslanzadeh (28), Bustin and Nolan (29), Mackay (30), Tan et al. (31), Muller et al. (32), and Silvy et al. (33).

Choice of oligonucleotides

The use of optimal primer and probe sequences is one of the critical steps for a successful PCR. For the design of primers and probes, criteria are described in literature (34,35). In our experience these criteria are very effective, which was also proven by the extensive validation of several assays in our laboratory. At present, several software packages, such as Primer Express (35), Primer 3 (36), and Oligo (37), are available to design primers and probe sets. However, it should be checked that the suggested primers and probe set meets the criteria listed in the table.

G+C rich regions in the target sequence should be avoided because they are difficult to amplify (38). Any

stretch of polypurines or polypyrimidines within the expected amplicon should also be avoided (39). PCR amplification is difficult if mRNA is used as a starting template for RT-PCR because of the complicated structure of the 3′ untranslated region (40).

The melting temperature (T_m) of the oligonucleotides is used as an indicator for the hybridization strength of oligonucleotides. Although many attempts have been made to predict the T_m (41–43), the formula that calculates the T_m most accurately is based on the nearest-neighbor model where thermodynamic values for hybridization are assumed to depend on interactions between a particular base and its nearest neighbors (43–46).

Guidelines concerning the T_m of the primers are based on annealing and extension at 60°C (35). In most three-step PCR protocols the elongation is performed at 72°C, the optimal temperature for the Taq polymerase but hydrolysis probes elongate at 60°C, which was demonstrated to be equally efficient (5). The T_m of sense and antisense primers should be similar to avoid false hybridization (45).

Primer–template hybrids are stabilized when the Taq polymerase extends the primer. The fluorogenic probe is not extended and thus not stabilized. Consequently, the probe–template hybrid must be stabilized by using a probe with a higher T_m than the primer–template hybrids and than the actual annealing temperature. Both hydrolysis and hybridization probes should have a T_m that is 5–10°C higher than the T_m of the primers to ensure strong binding of the probe during the annealing phase (5,47).

Oligonucleotides with higher G+C content will also stabilize probe hybridization. However, a high G+C content at the 3′ end of a primer may prevent the complete annealing of the remainder of the primer sequence and reduce the specificity of the reaction (48).

Short amplicons (less then 400 bp) are more easily amplified due to a more efficient PCR reaction and to a minimum of potential secondary structure within the amplicon (49). It is presumed that elevated elongation temperatures are important to melt any secondary structures that may form in the template and may block extension (26). Hence, real time PCR amplicons should be short with limited capacity to fold.

Consecutive presence of guanines may fold the template into a tetraplex structure, which is very stable and cannot be transcribed by the polymerase (50). Self-complementary regions in the template can fold into hairpin and other structures that interfere with the extension. Primers and probes should also have a low potential to form secondary structures, including self- and cross-hybridization with other oligonucleotides in the PCR (primer–dimer) (26,39,51).

There is no consensus on the size of primers, but generally primers ranging between 18 and 24 nucleotides are used. Shorter primers (<17 bases) may decrease specificity (45), longer primers (>30 bases) are not more specific (52) and the T_m calculations become less reliable.

The design of optimal probes should focus on their hybridization specificity instead of on the length of the probes. The use of longer probes allows more mismatches and does not improve the sensitivity. Shorter probes increase the chance of nonspecific appearance of these sequences in test material but exhibit a higher penalty on mismatches.

Probes should also contain more C than G because such probes produce a greater normalized change in fluorescence (ΔR_n). A larger ΔR_n allows easier interpretation of the results, as low positive signals can be more easily differentiated from background signal (10,35).

Choice of sample material and sample processing

The selection of a disease-specific specimen must be based on literature. The population and sample types should be fully described. The results obtained with a certain method for a given population may not be comparable for another population. Analysis of different sample types within the same population may also give a different result (53).

A sample material-specific validation approach is absolutely necessary because of possible matrix-induced effects (54). The performance of a diagnostic PCR may be limited by the presence of inhibitory substances within individual samples. Therefore, efficient sample processing procedures prior to PCR are needed to improve the performance of the test. Correct sample processing should remove PCR inhibitors, concentrate the target nucleic acids, and turn a heterogeneous biological sample into a homogeneous PCR-compatible sample (54–56).

Quantification strategies

Quantification of RNA and DNA with real-time PCR can be performed by the standard curve and the comparative method (57). The first method is based on the close relationship between the input copy number and the increase of fluorescence in the exponential phase. Quantification can be either absolute or relative. Absolute quantification requires the construction of a standard curve, plotting the C_t values against the logarithm of the initial copy numbers of standards with known concentration. Standard material must be stable, reliable, and precisely quantified. The copy numbers can be calculated after linear regression of the standard curve. Absolute quantification allows the

exact determination of copy number per cell, per total RNA/DNA concentration or per sample matrix.

Relative quantification determines the changes of steady-state transcription of a gene. A relative standard curve consists of a dilution series created with a calibrator with arbitrary units.

To circumvent the use of standard material and standard curves, relative changes in the expression of the target gene can also be determined by the use of the comparative $\Delta\Delta C_T$ (58), when PCR efficiencies are the same, or by the mathematical model proposed by Pfaffl (59,60), when PCR efficiencies are different.

To compensate for differences in the amount of biological material in the tested sample, normalization is necessary. Many normalization procedures have been suggested but the most popular strategy is normalization to internal reference genes (26). Finding appropriate reference genes for data normalization is a problem because evidence suggests that there is no universal reference gene with a constant expression in all tissues (57,61–63).

Validation

Verification of design of oligonucleotides

Once the method is chosen, the specificity of the amplicon is verified by using the BLAST algorithm (64,65). This program performs sequence-similarity searches against various databases, returning a set of gapped alignments with links to full database records. The query coverage and the maximum identity should be 100%. Each alignment returned by BLAST is scored and assigned a measure of statistical significance, called the "expectation value" (*E*-value), which is an indicator of the probability for finding the match by chance. The *E*-value is a widely accepted measure for assessing potential biological relationship. Smaller *E*-values represent more likelihood of having an underlying biological relationship. Sequences with *E*-values equal to or smaller than 0.01 are most often found to be homologous (66,67).

Verification of amplification

The absence of primer–dimer formation should be checked by analyzing a well-documented sample (reference material, e.g. proficiency testing sample) with melt-curve analysis, resulting in one single peak. The length of the amplicon, analyzed by gel electrophoresis, should be of the expected size. The amplification product must be analyzed by sequence analysis, followed by a comparison of the target sequence with sequences in Genbank (64).

Optimization of reaction conditions

Optimization of reaction conditions can reduce primer–dimer formation and increase the efficiency and specificity of the amplification process. An initial optimization of both primer and probe concentrations ensures the most sensitive and most efficient assay. An optimization matrix is performed using two tenfold dilutions of a positive control near the expected limit of detection. The optimal primer and probe concentration is that for which the lowest threshold cycle, the highest ΔR_n and a difference in C_t values between the two dilutions of approximately three is obtained (35).

Although the design of the primers and probes assumes that the annealing will be performed at 60°C, software programs do not account for the stabilizing effect of the Taq polymerase, making optimization of the annealing temperature necessary. The temperature for which the PCR characteristics meet the criteria listed below is the optimal temperature.

Further, the DNA/cDNA input must be optimized to ensure a maximal sensitivity with minimal inhibition.

PCR characteristics

The PCR characteristics can be defined from a standard curve based on tenfold serial dilutions of the DNA or cDNA (reference material), within the dynamic range of the method. Each dilution is analyzed in triplicate. In our experience, results are reliable if the standard curve is analyzed ten times on different days. C_t values of the diluted reference material are plotted vs. the logarithm of the samples' concentrations, number of template copies or dilution factor (59,68). The slope (m) must be calculated by linear regression. For the slope to be an indicator of real amplification (rather than signal drift), there has to be a breakpoint in the amplification plot. The slope of the linear regression line, ideally -3.3219, results in a real-time PCR efficiency (E) of 1. At a PCR efficiency of 1, the number of target molecules exactly doubles in one PCR cycle (29). Slopes between -3.1 and -3.6, with efficiency % between 90 and 110 are generally acceptable but there is no evidence supporting these limits. A number of variables, e.g. PCR inhibitors, PCR enhancers, DNA degradation, DNA concentration, length of the amplicon, secondary structure, and primer quality, can affect the efficiency of the PCR (29,69,70).

The efficiency calculated by the standard curve method assumes equal amplification efficiencies between quantification standards and unknown test samples. The sample-specific amplification efficiency can be calculated via "sigmoidal" (71–74) or "logistic" (75) curve fitting, (76) and is theoretically 2 (77).

The correlation coefficient (r^2) is a measure of the closeness of relationship between two variables, more specific, of their closeness of linear relationship (78).

Analytical and clinical verification

The several steps for the analytical and clinical verification of a molecular diagnostic assay are described in the Clinical and Laboratory Standards Institute guidelines (9,79), which are comparable to those described for other clinical diagnostic assays (Table 1) (11,80).

Internal quality control

Amplification of an internal control (IC) must be included in every assay to exclude false negative results due to interference of inhibitors and to ensure the performance of the nucleic acid extraction procedure. Amplification of a human gene as IC can be used for cell-rich specimens. On the other hand, for cell-free specimens, a synthetic IC can be added to the specimen in advance. The IC must be added at a suitable concentration to prevent competition for reagents with the target template. The real-time PCR for IC amplification should be optimized in a way that the target gene amplification is preferential to that of the IC.

Analysis of a negative control, simultaneously with the specimens, enables detection of possible contamination during the extraction or the amplification. Also, the specificity of the assay can be demonstrated. The use of a blank control (no template control) can be used to detect reagent contamination or increased back ground signal. Although the problem of contamination (11,28) is not part of this scoop, it is important to mention that each laboratory should validate its own decontamination procedure because there is no consensus.

A statistical follow-up of a positive control (reference material) is necessary. The concentration of the control should be near the limit of detection of the assay but high enough to obtain reliable results. For quantitative assays, at least two concentrations of reference material should be tested (9). The target values are determined by calculating the mean and the corresponding standard deviation based on 20 measurements on different days (81).

Proficiency testing

External quality assessment is necessary for each assay that is performed, if available (82). If an external proficiency program survey is not available, alternative testing can include blind sample testing, exchange of samples with other laboratories, or medical chart review and should be conducted yearly twice (83).

TABLE 1. Checklist for Optimization and Validation of Real-Time PCR Assays

Choice of method
- Commercial assay or in-house assay
- Choice of target gene
- Choice of detection method
- Choice of oligonucleotides
  - T_m of primers: 58–60°C
  - GC content of oligonucleotides: 30–70%
  - Not more than two C or G in last five positions at 3′ end of primer
  - Length of amplicon: max 400 bp
  - No more than four constitutive guanines
  - Avoid primer–dimer
  - Length of primer: 18–24 base pairs
  - T_m of probe: 68–70°C
  - More C than G in probe
- Choice of sample material and sample processing
- Quantification strategies
  - Standard curve method
  - Comparative method
- Normalization

Validation
- Verification of design of oligonucleotides: Expectation value ≤ 0.01
- Verification of amplification
  - Melt curve analysis
  - Gel electrophoresis
  - Sequencing+blast of amplicon
- Optimization of reaction conditions
  - Optimization of primers and probe concentration
  - Optimization of annealing temperature
  - Optimization of sample input
- PCR characteristics
  - Slope m: $C_t = \log \text{conc.} \times m + y$-intercept (criterium: $-3.6 \leq m \leq -3.1$)
  - Efficiency E: $E = 10^{-1/\text{slope}}-1$ (criterium: $0.9 < E < 1.1$)
  - Coefficient of correlation r^2 (criterium: $0.99 \leq r^2 \leq 0.999$)
- Analytical verification
  - Precision
  - Linearity, measuring range
  - Trueness
  - Limit of detection ($\geq$95%)/limit of quantification
  - Analytical specificity
- Clinical verification
  - Clinical question (CAT)
  - Clinical performance
  - Correlation to disease or disorder
    - Negative predictive value
    - Positive predictive value
  - Comparison to current methods/standards
- Internal quality control
  - Amplification and inhibition control
  - Negative control
  - Statistical follow-up of a positive control
- Proficiency testing

CONCLUSION

We hereby propose practical guidelines for the optimization and validation of commercial and in-house developed real-time PCR assays. In our experience, the use of the proposed guidelines leads to a more efficient

optimization and validation of commercial and in-house diagnostic assays. Since it is independent of environment, equipment, and specific applications, it can be exchanged between laboratories. Ultimately, a worldwide consensus on this kind of checklist should be aimed at.

The proposed checklist is a crucial step in harmonization of different methodologies. These recommendations also include the use of sample material and sample processing, appropriate standards, reference material, calibrators, and international scale of measurement (84,85).

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