Chapter 1

Real-Time Quantitative PCR, Pathogen Detection and MIQE

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Abstract

Keywords

1. Introduction

The last few years have witnessed a prodigious proliferation in the use of the real-time quantitative polymerase chain reaction (qPCR) for diagnostic applications designed to detect and quantify microbial pathogens (1). Key reasons are the characteristic sensitivity, specificity, and wide linear dynamic range of qPCR assays; this development is being accelerated by qPCR integration with nanotechnology, which has resulted in its emergence in novel areas such as high throughput, nanoliter qPCR (2), and microfluidic digital PCR (3). These innovations have extended the scope of qPCR technology by holding out additional advantages such as short assay times, low reagent usage, and exceptionally rapid heating/cooling rates. Importantly, integration of multiple processing modules allows further size reduction and power consumption, making qPCR technology viable for point-of-care diagnostics. Clearly, the combination of nanotechnology and qPCR has huge potential in clinical diagnostics, assuming the availability of adequate and appropriate sample material, the need for the technology to accommodate fairly crude biological samples as analytical targets, optimal sampling timing regarding the course of disease and standardization of pre-assay and assay protocols (4, 5). The contribution qPCR can make to improve the diagnosis of life-threatening diseases is clearly illustrated by its application to the detection of invasive

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aspergillosis (IA), caused by pathogenic *Aspergillus* fungi, where early diagnosis and treatment are essential for adequate therapeutic management. There is an increasing incidence of systemic fungal infections in patients immunocompromised as a result of HIV infection or organ transplantation, patients from intensive care units, and those with prolonged neutropenia after intensified chemotherapy. These include patients with acute leukemia during induction therapy or after transplantation of allogeneic hematologic stem cells. IA has a high mortality rate and, in surviving patients, leads to considerable complications often limiting further anti-neoplastic therapies. The reliability of current diagnostic tools in early detection of fungal infections in neutropenic patients is limited; hence the definitive diagnosis of IA remains a challenge.

2. qPCR and Pathogen Detection

The committee of the European Organization for Research and Treatment of Cancer/Mycosis (EORTC) and Mycoses Study Group of National Institute of Allergy and Infectious Diseases (NIAID) has thus far not recommended the routine use of PCR in the diagnosis of IA (6), mainly because of the absence of standardized protocols (7). However, the feasibility of using qPCR in these patients as a potentially more accurate alternative to conventional diagnostic procedures has been evaluated extensively (8–10). There are a number of compelling reasons for utilizing qPCR as a diagnostic assay for IA:

- A qPCR assay can be completed in minutes. This immediacy allows virtually instantaneous reporting of results.
- Judicious targeting of amplified genomic regions, e.g., the rRNA gene region, maximizes the reliability of the assay. It substitutes the uncertainty of having to identify a single copy of a gene on an infectious fungal particle or single genome with the consistency and reliability of being able to target tens of copies specified within that single genome.
- Primer and probe design is extremely flexible and assays can be tailored to be specific for most fungal genera or species.
- The quantitative potential of qPCR allows determination of fungal load, which can distinguish between colonization and infection.
- Multiplexing capacity allows qPCR assays to detect multiple targets and/or include appropriate controls in a single reaction.

In principle, the only requirements for obtaining quantitative data are a set of reagents (target-specific primers and optional probes, a source of DNA and an enzyme/buffer/dNTP mix), a qPCR instrument that detects the fluorescence emitted by the PCR reaction and software that calculates DNA copy numbers or relative abundance from the quantification cycles recorded by the instrument. In practice, the development and successful application of an optimal qPCR diagnostic assay depends on understanding and addressing several challenges and shortcomings. A systematic review and meta-analysis on the use of PCR tests for the diagnosis of IA highlights the lack of homogeneity of the PCR methods used (11) and another recent study shows that lack of rigorous experimental controls, together with a prevalence of false-positive and -negative results hinders the interpretation of diagnostic performance, thus impeding widespread acceptance of qPCR-based technology (12). False negatives can occur due to suboptimal DNA extraction, for example low recovery of DNA and the presence of PCR inhibitors. Since all fungi produce small, hydrophobic conidia that are difficult to disrupt, it is not surprising that sample preparation is a source of heterogeneity and a critical parameter for obtaining reliable qPCR results (10). Other causes are the presence of large amounts of human genomic DNA competing with the microbial target for amplification and of course suboptimal analytical sensitivity of the qPCR reaction itself. False positives can occur either due to contamination at any time during the pre-assay stage, i.e. during sample collection, DNA extraction, and qPCR set-up, or be the result of poor assay design, resulting from cross-reactivity of the target qPCR assay with other DNA. Hence any serious qPCR assay must incorporate controls to assess for these factors contributing to false-positive and false-negative results, something sadly lacking from many published studies.

3. Quality Control Issues

It is remarkably difficult to make a reaction fail completely but alarmingly simple to produce poor quality data (13). When properly executed, qPCR assays can be highly reproducible; however, since pathogen abundance is frequently low, assay reproducibility is influenced by parameters such as distribution statistics (14), with stochastic sampling effects affecting the reliability of the qPCR data (15). This emphasizes the importance of repetitive testing in clinical samples and one of the strengths of qPCR assays is the ease with which it is possible to obtain quantitative data for every sample, which encourages the use of biological replicates and permits the

application of powerful statistical analyses to the quantification procedure.

Like any clinical diagnostic assay, qPCR assays must be properly validated (16) and meet the criteria expected of any laboratory test applied in clinical medicine: (1) standardization of the test across different laboratories, (2) reproducibility of positive and negative predictive values, and (3) reliable sensitivity and specificity. This involves the establishment of a set of quality standards dealing with experimental protocols, the use of appropriate positive and negative control samples, and suitable analysis and reporting guidelines. Standardization, in particular, is all-important (11, 17, 18) as it provides the foundation for robust, reliable, and comparable results that are of critical importance for the consistent management of patients (19).

Specifically, a successful diagnostic qPCR assay requires careful consideration of these issues.

1. Optimal sample quality is a prerequisite for the generation of valid quantitative data (20). Hence sample collection, preparation, and transport and nucleic acid extraction methods are critical parameters in test performance and must be optimized and, ideally, standardized. In principle, extraction of fungal nucleic acids, especially if present in a cell-free state, from bronchoalveolar lavages, blood, and serum is relatively straightforward; however, it is easy to co-purify inhibitors of the PCR that will generate inconsistent and unreliable results. This must be assessed for every sample prior to carrying out any qPCR assay aimed at pathogen detection. Amongst the numerous methods for *Aspergillus* DNA extraction the bead-beating methods appear to be the most successful (21), although no single extraction method is optimal for all fungal species (22). However, extraction of DNA from conidia, combined with the presence of a complex, sturdy cell wall, can require extensive and harsh extraction methods that can damage DNA and RNA to the extent that amplification becomes unreliable.

2. Primer selection is critical since it affects the sensitivity of the qPCR assay. The structure of the nucleic acid target at the primer-binding site must be taken into account, as this affects the accessibility of the target to the primers and extensive secondary structure at a primer-binding site will result in a less efficient and sensitive assay.

3. Regular calibration of the real-time instrument is crucial for obtaining consistent and accurate results. The quantification cycle (C_q) is neither absolute nor invariant, but varies between assays carried out on different days with different reagents or on different instruments. This is because the C_q depends on the instrument's threshold setting, which in turn depends on background fluorescence, which varies with different probes,

chemistries, instruments, and assay protocols. Therefore, samples should not be compared by C_q values (23), but they should be converted to target copy numbers and reported as such.

4. Analytical sensitivity and specificity are critical parameters of any diagnostic qPCR assay. Analytical sensitivity refers to the smallest number of nucleic acid molecules that can be detected and distinguished from a zero result and is best calculated using a standard curve, which defines the range of the assay. It is inappropriate to report results that lie significantly outside the upper and lower concentration of target defined by the standard curve. Analytical specificity is determined by identifying the percentage of samples without the target sequence that generate a positive result. If a well-designed assay is used, this will be zero.

5. There must be agreement between technical replicates (to within ±0.5 C_qs), as this provides important information about the reliability of the assay and its operator. Repeatability is measured as the amount of agreement between replicates tested in different runs on the same instrument in the same laboratory. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents, and controls). It is important to maintain the internal quality control by monitoring the assay for both parameters. If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate it under the new conditions as mutations within the primer sites, especially at their 3′ ends, or the prevalence of other subtypes, will affect the performance of the assay and render the established validation no longer valid.

6. False-positive results may arise from product carryover from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments (24). A recent report suggests that nutritional supplements can harbor fungal DNA that can pass into the serum from the intestinal tract and cause false-positive results (25). It is critically important to include negative controls, i.e., samples that are as similar to the test samples as possible but exclude the target. Since false-negative results in an optimized assay are mostly due to inhibitory effects and/or pipetting errors, it is important to always include a positive control with any qPCR assay (26), ideally in the form of a dilution curve. In addition, all samples should be tested for inhibition using a simple “alien” assay (27) and any nucleic acid preparations showing inhibition must be repurified. Dilution curves are useful, as the highest dilutions provide information about the variability of the assay at very low target copy numbers and qPCR results are questionable if they are not supported by data demonstrating the overall sensitivity of

the assay applied (16). Furthermore, running a dilution curve with each assay immediately reveals any problem with that particular run and increases confidence when reporting negative results. The same argument applies to running samples in duplicate or, preferably, in triplicate and in this qPCR is no different from any clinical diagnostic assay.

Robust and precise qPCR usually correlates with high PCR efficiency; consequently the primary aim of assay optimization is to achieve the most efficient qPCR assay optimized at the sensitivity and specificity appropriate for pathogen detection. Detailed optimization requires the consideration of a range of parameters that include the concentration of reaction components, such as salts and oligonucleotides, as well as reaction conditions such as primer annealing temperatures, incubation periods, and even ramp rates. In addition, qPCR results are affected by the fluorescent signal, an important concern when designing multiplex assays. Since variations in experimental protocols lead to highly variable data, it is essential that all relevant experimental conditions and assay characteristics are reported when publishing qPCR results (28).

4. Optimization Parameters

4.1. Dilution Curves

A dilution curve, generated by performing qPCR with a serial dilution of template, is a convenient tool to test assay efficiency. A well-optimized assay should be linear over a range of at least nine logs of template concentration, with efficiency close to 100% and high reproducibility between technical replicates. Amplification efficiency can be determined from the slope of the linear regression of a plot of C_q (y-axis) vs. log [quantity]. The template may be any suitable material such as cDNA, genomic DNA, PCR product, or synthetic DNA oligonucleotides that match the sequence of the target amplicons. The advantages of using synthetic oligonucleotides are that (1) one synthesis is sufficient for several million reactions, thus providing a consistent positive control and (2) they can be accurately quantified, thus making it possible to obtain a fairly accurate pathogen copy number value, making comparison of different assays more reliable. However, artificial templates must be handled with extreme caution since they constitute highly concentrated targets that could potentially contaminate all oligonucleotides and reagents if handled carelessly.

4.2. Amplification Efficiency

Amplification efficiency, E, is calculated from the slope of the standard curve using the formula: $E = 10^{(-1/\text{slope})}$, which is usually converted into a percentage efficiency (% Efficiency $= (E-1)\times 100\%$). A combination of a good assay and accurate pipetting will generate

dilution curves that demonstrate near-perfect doubling (or consistent amplification) with each amplification cycle. Thus, the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves. For example, with a tenfold serial dilution of DNA, $2^n = 10$. Therefore, $n = 3.32$ and the C_q values should be separated by approximately 3.32 cycles. If doubling occurs at each cycle, $E = 10^{-(1/-3.32)} = 2$ and the % Efficiency $= (2-1) \times 100\% = 100\%$. In practice, amplification efficiency will be around 90–105% and result in gradients of between −3.2 and −3.5. Note that the presence of inhibitors can result in an apparent increase in efficiency. This is because samples with the highest concentration of template also have the highest level of inhibitors, and therefore display a greater lag between C_q values than samples with lower template concentrations and lower levels of inhibitors. As a result, the absolute value of the slope decreases and the calculated efficiency appears to increase. Similarly inaccurate pipetting can lead to data suggestive of higher or lower efficiency and so it is important to assess the correlation coefficient of the line to determine the linearity and reproducibility of the assay. It provides a measure of how well the data fit on a straight line and is influenced by pipetting accuracy and by the range of the assay. During assay evaluation and optimization, three technical replicates of each template dilution should be processed in parallel in order to establish that the assay is reproducible. A stable assay will demonstrate an $R^2 > 0.98$ over at least six logs and with three replicates.

Finally, DNA templates themselves can affect the efficiency and quality of a qPCR assay. A qPCR template may be present at any concentration from a single copy to approximately 10^{11} copies. High concentration of template will inhibit the reaction resulting in reduced yield and inaccurate C_q differences between amplification plots, so inaccurate quantification. Low initial concentration can result in lack of detection of amplified product if the final yield is extremely low.

4.3. Assay Specificity

Reliable pathogen detection requires absolute assay specificity. In a qPCR experiment all detectable products, be they specific or nonspecific, contribute to the final amplification plot and hence any qualitative or quantitative result. This can be a problem when using a generic detection system such as SYBR Green I dye, but can also affect the quality of a probe-based assay designed to detect only a single target sequence. Although nonspecific amplification may not affect the shape of the amplification plot and is not detected by a probe-based assay, it nonetheless affects amplification efficiency and assay sensitivity. Performing a post-reaction melt analysis using SYBR Green I dye during the assay optimization stage can validate the specificity of amplification. An assay with high specificity will produce a single peak at a high temperature with nothing in the

no-template controls. If the melting curve has more than one major peak, agarose gel electrophoresis and DNA sequencing should determine the identities of the products. Lowering the primer concentrations and increasing the annealing temperatures will often reduce the amount of nonspecific products.

4.4. Buffer and Sample Considerations

The composition of the reaction buffer has a significant influence on assay specificity, as it influences binding of primer to template. Reaction buffers contain variations on a basic composition consisting of ammonium sulfate, Tris, EDTA, BSA, β-mercaptoethanol, dNTPs, $MgCl_2$, KCl, NaCl, and DNA polymerase. Optimum buffer composition is dependent upon the DNA polymerase used: different enzymes can influence PCR efficiency and therefore product yield. It is generally accepted that *Taq* DNA polymerase performs optimally in a basic buffer of 50 mM KCl and 10 mM Tris–HCl, pH 8.3 (measured at room temperature). Some enzymes have a requirement for added protein (BSA is usually added, when required). Although dNTPs are the standard substrate for DNA polymerases, dUTP may be incorporated into qPCR reactions to provide target for subsequence contamination control steps using Uracil–N-Glycosylase to remove UTP containing templates from reaction mixes prior to amplification. In a standard reaction, the concentration of dNTPs is included in equimolar ratios, usually 200 μM (or up to 500 μM) of each dNTP. Many commercially available buffers may also contain PCR enhancers such as single-stranded binding protein (SSBP), betaine, formamide, or DMSO. The presence of detergent improves the activity of some enzymes, presumably by reducing aggregation.

The salt concentration within the buffer affects the T_m of the primer–template duplex and is required for primer annealing. Concentrations of KCl or NaCl above 50 mM can be inhibitory, while $MgCl_2$ is required as a cofactor for DNA polymerase. The most influential factor effecting free magnesium ions is the concentration of dNTPs in the reaction and so the magnesium ion concentration must exceed the dNTP concentration. Typical reactions contain 1.5 mM $MgCl_2$ in the presence of 0.8 mM dNTPs resulting in approximately 0.7 mM free magnesium. Optimal $MgCl_2$ concentrations can differ for every primer/template combination and this leads to problems if the reactions are to be multiplexed, as there will be significant differences in the efficiency of the individual reaction and, hence, in the yield of the different PCR products. Hence primer optimization is an essential step in multiplex assay development.

4.5. Primer Optimization

Primer optimization serves to drive the kinetics of binding of the primers to the specific template sequence. Annealing is a kinetic result of the annealing temperature of the reaction and also the concentration of the primers. Optimization is a two stage process and should proceed as follows.

1. Optimal annealing temperature T_a is approximately 5°C lower than the T_m (melting temperature) of the primer with the lowest T_m. The T_m for a short oligonucleotide can easily be calculated by the approximation: $T_m = 4(\text{number G} + \text{number C residues}) + 2(\text{number A} + \text{number T residues})$. Taking this temperature as a starting point, different temperatures (±5°C) should be tested in steps of 0.5°C; alternatively, a simpler option is to use a temperature gradient PCR block (as for example is standard on the BioRad CFX). Whilst unexciting, applying the optimal T_a will result in higher specificity and yield, since using too low a T_a results in nonspecific priming, whereas too high a T_a results in inefficient priming and elongation.
2. Primer concentration should be optimized using a primer concentration matrix in which all primer concentrations from 50 to 300 nM are tested against each other and the conditions producing the highest concentration of specific template are selected. When optimizing a multiplex reaction it is essential that each single reaction is of high quality (as defined above) before attempting to combine them. The single most important factor for multiplex success is the primer design and it is recommended that a program such as Beacon Designer is used since this will compare the compatibility of assays in silico. A serial dilution containing each of the targets is then interrogated with the assays in pairs. Any deviation from the original single assay indicates that the oligonucleotides are interfering. Optimization may then include reassessment of oligonucleotide concentration, change in $MgCl_2$ concentration and potentially in the amount of DNA polymerase in the reaction.

5. The MIQE Guidelines

The Minimum Information for the Publication of Quantitative PCR Experiments (MIQE; (http://www.rdml.org/miqe) guidelines have two aims: first, to provide a standardized template for good assay design and second, to encourage transparency of protocols, data analysis, and conclusions. MIQE is part of a drive that promotes minimum guidelines for the reporting of biological experiments (29) and is modeled on similar guidelines such as MIAME (Minimal Information about a Microarray Experiment), developed several years ago (30) and MIAPE (Minimal Information about a Proteomics Experiment) (31). All of these are initiatives developed under the umbrella of the MIBBI (Minimum Information for Biological and Biomedical Investigations) standardization body, which has the goal of unifying all of the standardization guidelines for biological and biomedical research. In addition, the Real-Time PCR Data Markup Language (RDML; http://www.rdml.org) has

been developed by a consortium, upon the request of MIBBI, to enable the straightforward exchange of qPCR data and related information between qPCR instruments and third party data analysis software, between colleagues, and with journals or public data repositories (32).

The four key areas of standardization that define any qPCR experiment are (1) study design, (2) technical detail, (3) analysis methods, and (4) statistics. MIQE defines the minimum information required for evaluation of qPCR results and addresses these under a set of nine captions that describe a large number of individual elements. There is a clear hierarchy with some parameters, labeled E (essential) in the published guidelines, indispensable for attaining the ambition of the main aims, whereas other components, labeled D (desirable) more peripheral, yet constituting an effective foundation for the realization of best practice protocols. It is essential to report as much information about sample acquisition, storage, and handling as possible; it is also important to provide details of sample processing procedures, since any sample has to pass through a number of preparative steps prior to the qPCR assay, every one of which can introduce additional variability (33, 34). For pathogen detection, it is critical that the sequences of primers and probes are reported, since an experiment cannot be reproduced if information on one of the principal reagents is lacking. The most commonly used models for the analysis of qPCR data use either the $\Delta\Delta C_q$ (35) or the more generalized efficiency calibrated model (36) and updates or variations continue to be introduced (37, 38). However, confidence interval and statistical significance considerations are still not accorded a high enough priority (39) and there is a tremendous reluctance to use dilution curves to test the amplification efficiencies of individual assays, even though, as discussed above, this method remains by far the easiest, most transparent and informative method for determining amplification efficiency. Furthermore, dilution curves also provide convenient positive controls, can act as inhibition controls, and help define the dynamic range and the limits of detection all at the same time. Ideally such a dilution curve should be run with each sample, as all these parameters could (and probably do) vary between samples.

5.1. Brief Troubleshooting Guide

Below are some basic recommendations for troubleshooting qPCR experiments.

5.1.1. No/Poor/Late Amplification

No amplification is characterized by no significant increase in fluorescence above background. Poor amplification is typified by a very low ΔRn value (<0.05) or by a slope that varies significantly between samples amplifying the same target. Late amplification is anything that generates a $C_q > 35$.

First check qPCR reaction conditions.

- Check amplification products on a gel. If no product is present, repeat the assay checking all reagents are added and that the thermal cycling conditions are correct. If a product is present, the instrument detection settings may be incorrect; for example the wrong filter may have been used to detect the light emitted from the reporter fluorophore.
- Make sure all required reagents, i.e. enzyme(s), reaction buffer, primers, probe, and template were added.
- Check annealing temperature (<60°C), elongation times and cycle number.
- Check that primers or other reactants have been diluted correctly.
- Keep pipettes well calibrated.
- Is the thermal cycler programmed to detect fluorescence during the wrong PCR step?
- Is there too little starting material?
- Is there too much starting material?
- Is the amplicon too long?
- If using homemade buffers ensure that the salt and buffer concentrations are correct.
- Incomplete thawing of frozen buffers will change the salt concentration in the remaining buffer. Increases in $MgCl_2$ concentrations will affect the efficiency of primer binding and may cause the appearance of primer dimers and reduce the efficiency of the PCR reaction.
- If there have been changes in the reaction volumes or number of reactions for which master mix has been prepared, errors may have been made during pipetting. Changing the volumes of the reaction mixtures can cause different amounts of error in the volumes being dispensed. This is partially due to differences in the tolerances between large and small volume pipettes. This error may not be propagated linearly during scale up. Highly sensitive methods such as PCR can significantly magnify these problems. It is best to scale up in stages.
- Increase the number of amplification cycles, especially if the PCR efficiency is low.

Then check the reagents/instrument

- If the assay is probe-based and has previously worked well, the probe may have gone off. For example, it may have been photobleached if it has been left in the light. Always store fluorophore-labeled oligonucleotides in aliquots in the dark at –20°C.
- If the background level of the probe is very high, it is possible that it may have become hydrolyzed, e.g., if subjected to repeated freeze/thaw cycles.

- If the assay is a new assay, it may well be that the probe manufacturer is to blame. Test the quality of the probe using a DNAse I digestion assay; following digestion the fluorescence should greatly increased due to reporter and quencher becoming separated.
- Has the SYBR Green "gone off"? Once SYBR Green has been diluted, it goes off very quickly and can only be kept at 4°C for approximately 2 weeks. It must also be kept in the dark.
- If using a new batch of polymerase, note that different lots of polymerase, even from the same supplier, can have different amounts of specific and exonuclease activity, but still be within the manufacturer's specifications.
- Different thermal cyclers, particularly if they using heating blocks, and thermal cyclers from different manufacturers have different ramping kinetics and heating efficiencies, hence can affect the efficiency of the PCR reaction. Variability in heating efficiency may also occur in different wells within the same block, again particularly so in 96 or 384 well instruments.

5.1.2. Poorly Designed Primers and/or Probe

This results in poor PCR efficiency

- Optimize primer concentrations; this is essential for optimal assay efficiency.
- Confirm annealing temperature is appropriate for primer.
- Are primers binding in a region with secondary structure? If this has not already been checked using Beacon Designer or a similar program. Use alternative primers.
- Is the probe too long?
- Is there probe secondary structure?

5.1.3. Reaction Conditions Not Optimized

This results in poor PCR efficiency

- Is the annealing temperature too high?
- Are the annealing/extension times too short?
- $MgCl_2$ concentration too high/low.

5.1.4. Positive NTC

The master mix may be contaminated with DNA template or PCR product.

- Use fresh aliquots of all reagents including sterile water.
- Only use pipettes, tips, solutions (especially water), and racks dedicated to setting up reactions. Do not use pipettes and accessories that have been exposed to amplicon.
- Decontaminate surfaces, pipets, and racks.
- Change gloves and tips frequently.
- Change location of PCR set-up.

- Check melt curves. If the NTC melt curve is different from the amplicon melt curve, you may still be able to use data.
- An excess of probe can generate an artifactual positive result. This can be determined by leaving out the *Taq* polymerase from the NTC.

5.1.5. Primer Dimers

Primer dimers are more likely to occur in no target controls, or when there is very little target nucleic acid. Even small amounts of target can suppress primer dimer.

- If dimers occur in the presence of normal amounts of target, primers need to be redesigned. Check for absence of complementary sequences at the 3′ ends.

5.1.6. Multiple Peaks in Melt Curve

Improve the stringency by raising the annealing temperature or lowering magnesium concentration.

- Run reaction on gel to check for specificity and consistent presence of additional bands.
- Shoulders in the melt curves do not necessarily mean that the assay is nonspecific. The amplicon may contain AT-rich subdomains. Run reaction on gel to check for specificity.

5.1.7. Standard Curve Is Unreliable ($R^2 < 0.99$)

The low (or high) concentration point(s) of the dilution series can sometimes be removed to improve the R^2 value. However, if the unknowns fall in the low range, the experiment will need to be repeated.

5.1.8. Inhibitor Present

Reagents such as SDS, EDTA, glycerol, sodium pyrophosphate, spermidine, formamide, guanidinium salts, and DMSO can inhibit *Taq* DNA polymerase.

- Dilute DNA sample by 1/10 and 1/100 and repeat the assay, as this may dilute out the inhibitor.
- If this fails, remove inhibitor by ethanol precipitation of the DNA. Include a 70% (v/v) ethanol wash of the DNA pellet. Glycogen (0.25 μg to 0.4 μg/μl) can be included to aid in DNA recovery for small samples.

5.1.9. Erratic Amplification Plots/High Well-to-Well Variation

Use extra care when pipetting solutions containing low amounts of target.

- Is the baseline set using wrong cycle range?
- Is there sample evaporation due to loose lid or poor sealing?
- Was there incomplete mixing of reagents?
- Are there air bubbles at the bottom of the reaction tubes?

- Were the frozen stocks of target, primers or buffer not completely thawed or mixed when used?
- Spikes in the signal can be caused by problems with the lamp, misaligned optics or other mechanical and/or electronic issues.

6. Conclusions

qPCR is a powerful enabling technology that has started to play a central role in clinical diagnostics. However, the combination of ease of use and lack of rigorous standards of practice has resulted in widespread publication of poor data, resulting in inappropriate conclusions. In the context of reliable detection of pathogens in general, and *Aspergillus* in particular, the major limitation of qPCR is the lack of standardization at every stage of the molecular process from sample type and processing to interpretation of results. Nonetheless, when optimized, it is extremely sensitive and highly specific (40).

Any solution to the challenge of how to make PCR-based assays more reliable requires both an appreciation and an understanding of numerous attributes that include statistics, mathematical modeling, technical know-how and a willingness to share this intelligence. MIQE constitutes a reference framework for communication within the research community, instrument, and reagent manufacturers and publishers that promises to deliver guidelines that promote transparency of experiments and confidence in results and conclusions that advance, rather than impede our knowledge.

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