Performing Real-Time PCR

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Abstract

Optimisation of the reagents used to perform PCR is critical for reliable and reproducible results. As with any PCR initial time spent on optimisation of a real-time assay will be beneficial in the long run. Specificity, sensitivity, efficiency and reproducibility are the important criteria to consider when optimising an assay and these can be altered by changes in the primer concentration, probe concentration, cycling conditions and buffer composition. An optimised real-time PCR assay will display no test-to-test variation in the crossing threshold or crossing point and only minimal variation in the amount of fluorescence. The analysis of the real-time PCR results is also an important consideration and this differs from the analysis of conventional block-based thermal cycling. Real-time PCR provides information on the cycle at which amplification occurs and on some platforms the melting temperature of the amplicon or probe can be determined.

Optimisation of Real-Time PCR Assays

Real-time PCR assays require optimisation in order that robust assays are developed which are not affected by normal variations in the target

DNA, primer or probe compositions. A robust assay is defined as an assay in which these 'normal' variations cause no effect on the crossing threshold (CT) also known as crossing point (CP) and have only a minimal effect on the observed amount of fluorescence. The important criteria for optimisation are specificity, sensitivity, efficiency and reproducibility. It is important to decide before commencing optimisation which type of assay is required for a particular application. For example, there is little point in developing a quantitative assay if a simple qualitative assay will be just as informative. Melting curve analysis may also be required for product differentiation and in this case should be considered at the planning stage. If the real-time assay is based on conversion of an existing block-based assay it is important to note that the cycling conditions used for conventional block-based thermal cycling may not always translate easily to a real-time format and so it is important to consider re-optimisation of the assay (Teo *et al*., 2001).

The same principles of optimisation apply to assays run on all real-time platforms. The following criteria should be optimised: buffer composition, cycle conditions, magnesium chloride ($MgCl_2$) concentration, primer concentration, probe concentration and template concentration. Commercial master mixes, which are widely available, simplify the optimisation and are convenient. In this chapter all aspects of optimisation and analysis of real-time PCR results will be discussed.

PCR Master Mix

Commercial master mixes are available for most of the real-time platforms and although some are marketed for specific instruments and probe formats, they often work equally well on other instruments. Mixes are provided in easy-to-use formats and often contain additional features such as use of dUTP allowing the enzyme uracil-DNA glycosylase to be used to prevent cross-over contamination. Some commercial master mixes contain the uracil-DNA glycosylase as well as the dUTP and carry-over contamination never needs to be considered. However, it is important to note that the use of dUTP

has been shown to decrease PCR sensitivity. There is little doubt that the use of commercial master mixes can simplify optimisation and promotes uniformity of assay performance over time. However, for some laboratories the cost cannot be justified.

Preparation of in-house master mixes can be effective and assays can perform well over time if the reaction components are well optimised. The master mix requires thermostable polymerase, buffer, dNTPs and $MgCl_2$ though the latter will be discussed later in the chapter. The correct amount of polymerase is essential and there can often be a narrow optimal concentration range. Not enough polymerase leads to inefficient amplification, low fluorescence and loss of sensitivity and leads to a high CT value. Too much enzyme also leads to low fluorescence and can contribute to the production of primer dimers and production of other non-specific amplicons. The composition of the core buffer can be essential for some real-time platforms and can affect the Tm of the primers/probes and the performance of the enzyme. For example, when using glass capillaries it is essential that a protein such as BSA is included in the buffer to prevent the DNA from binding to the glass. The optimal concentration of dNTPs is usually wide and the CT values are not affected by 2-4 x increases or decreases, however, the amount of fluorescent signal can be affected. An important consideration is the use of dUTP in the master mix. *Taq* polymerase preferentially incorporates dATP, dTTP, dGTP or dCTP and although it can incorporate dUTP this is usually less efficient. However, the use of dUTP may be considered essential to prevent carry over contamination and a balance has to be found between sensitivity and the consequences of contamination. Differences of up to two cycles can be observed between master mixes containing dUTP and dTTP.

When the assay produces a large quantity of primer dimer or if the sensitivity of the assay is low, a hot-start technique can be applied to increase the stringency of the reaction (D' Aquila *et al*., 1991; Chou *et al*., 1992). Hot-start PCR can be achieved by using a commercially available master mix with an in-built hot-start or by adding commercially available anti-*Taq* DNA polymerase antibodies. When the PCR is performed in glass capillaries, *i.e*. using the LightCycler, the use of hot-start has been shown to improve performance as it is thought

that the binding of *Taq* polymerase and magnesium ions to glass is reduced due to the hot-start mechanism (Teo *et al*., 2002). Antibody based hot-start methods can be advantageous as the recommended activation time is only 1-3 minutes compared to 10 minutes for chemically modified *Taq* polymerase. This time difference can be important where fast PCR results are required.

Regardless of whether commercial master mix or an in-house preparation is used the following components (primers, probes, $MgCl_2$ and template concentration) require optimisation.

Primers

Primers should be designed with the aid of appropriate software and attention should be paid to the optimal size of the amplicon produced in the real-time application. Real-time PCR products are usually <500 bp, this may be much smaller than the amplicon size generated on a block-based thermal cycler. The following guidelines should be observed for optimal and accurate primer design:

- The primer 3' ends should be free from secondary structure, repetitive sequences, palindromes and highly degenerate sequences.
- Forward and reverse primers should not have significant complementary sequences.
- The forward and reverse primers should have equal GC contents, ideally between 40-70%.
- The binding sites should not have extensive secondary structure.

The annealing temperature as determined using primer design software should be used as an initial guide, however, it is important to note that this can often vary greatly from the experimentally determined annealing temperature. One approach, which can be used to determine the true annealing temperature is to synthesize the complementary primer sequence, hybridise the two primers and perform a melting analysis that allows the Tm to be observed under the conditions that

will be used in the PCR reaction. The cost of synthesising another oligonucleotide is often justified by the reduction in time spent repeating the experiment at different annealing temperatures (Teo *et al*., 2002).

Time should also be spent determining the optimal primer concentration and it is best to try a range of final concentrations from 0.1 μM to 0.5 μM for both primers. For some assays the optimal concentration for the two primers will not be the same, for example, to achieve good melting curves it is sometimes necessary to use asymmetric PCR, where a lower concentration of one primer is used, to increase the amount of the strand complementary to the probe (Lyon *et al*., 1998; Phillips *et al*., 2000). This improves the melting curve because increased amplification of the target strand can reduce the competition between the template strands and template-probe hybridisations. Some manufacturers recommend performing a primer optimisation matrix as outlined in Table 1. The optimal combination is the one that gives the lowest CT value (Figure 1).

Fluorescent Probes

As with the primers, real-time PCR probes are best designed using dedicated software such as Primer Express (Applied Biosystems) for hydrolysis probes, hybridisation probe design software from Roche Applied Science or Beacon Designer from Bio-Rad. For any of the probe formats the annealing temperature should be higher than that of the primers in order to ensure that the primers are binding and

Table 1. Example of a primer optimisation matrix.

Reverse/Forward	50nM	300nM	500nM	900nM
50nM	*50/50*	*300/50*	*500/50*	*900/50*
300nM	*50/300*	*300/300*	*500/300*	*900/300*
500nM	*50/500*	*300/500*	*500/500*	*900/500*
900nM	*50/900*	*300/900*	*500/900*	*900/900*

synthesising the product before the probe is able to participate in the reaction. It is important that the probes are not able to act in the PCR as primers and to prevent this either a fluorescent molecule or a blocker, for example phosphate, should be placed at the 3' end of the sequence. Probes complementary to the 3' termini of the PCR primers should be avoided since they may hybridise to the primers. Primer elongation may then occur leading to primer-probe dimers that affect amplification efficiencies (Roche Molecular Biochemicals). As with the primers it is important to try a range of probe concentrations to determine which is optimal. A wide range of probe concentrations is possible but if the concentration is too low no fluorescent signal will be observed and if the concentration is too high it can lead to a high fluorescent background. The probe concentration should be optimised after optimising the primer concentration and as with the primer concentration, a range of probe concentrations should be tried between 0.1 uM and 0.5 uM and the concentration which gives the lowest CT values and the highest fluorescent signal should be selected (Figure 2).

SYBR Green I

If the DNA binding dye SYBR Green I is used in the fluorescent detection system it should be optimised for each set of PCR primers. As the amount of SYBR Green I is increased an increase in the amount of fluorescence can be observed. The recommended concentration is a 1:10,000 dilution of the neat stock as supplied by the manufacturer. High concentrations of SYBR Green I have been shown to adversely affect the PCR amplification as they inhibit *Taq* polymerase (Ririe *et al*., 1997; Wittwer *et al*., 1997). DNA binding dyes also influence melting temperatures and consequently it is important to use only the optimised level. Some researchers have suggested that SYBR Gold is preferable to SYBR Green I due to its stability during long-term storage (*Lee et al*., 1999).

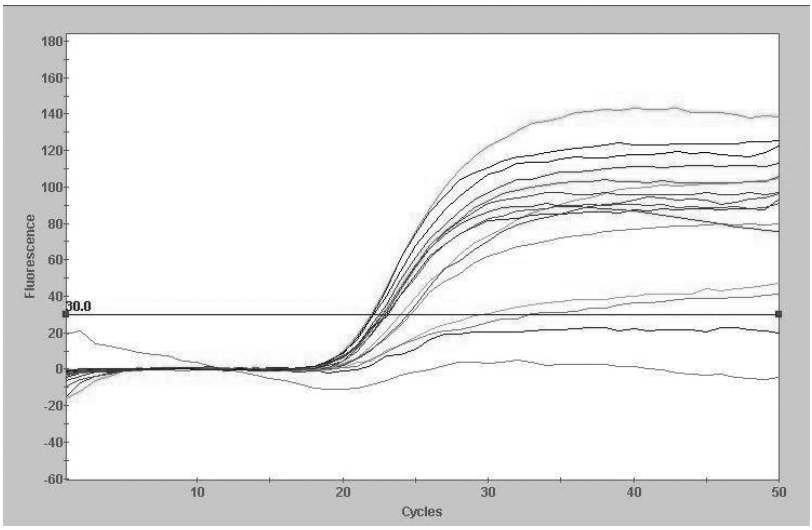

Figure 1. Primer optimisation experiment. The same sample was analysed at 12 different forward and reverse primer concentrations as detailed in Table 1. This experiment was run on the Smart Cycler using the same concentration of hydrolysis probe in each tube. The optimal primer concentration is the one that gives the lowest CT value.

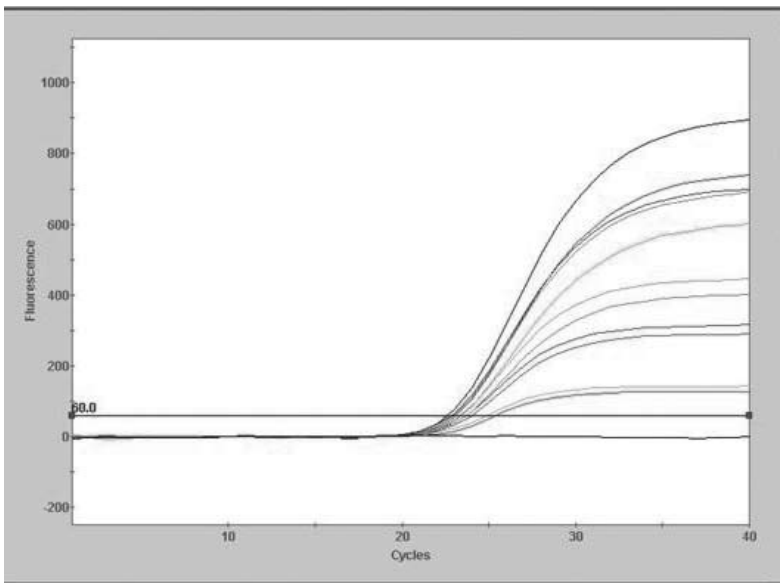

Figure 2. Probe optimisation experiment. Using two different primer concentrations, five different hydrolysis probe concentrations (250 nM, 200 nM, 150 nM, 100 nM and 50 nM) were run on the Smart Cycler. The optimal probe concentration is the one that gives the lowest CT value and the highest fluorescent signal.

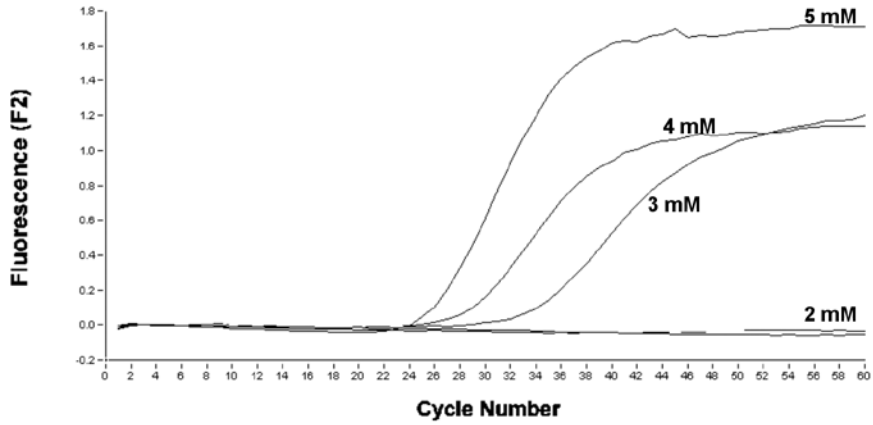

Figure 3. $MgCl_2$ optimisation experiment. The same sample was analysed at four different $MgCl_2$ concentrations (5 mM, 4 mM, 3 mM, and 2 mM) on the LightCycler. Using 5 mM $MgCl_2$ gave the lowest CP value and the highest fluorescent signal and was therefore the optimal concentration.

Magnesium Chloride

A key variable is the magnesium chloride ($MgCl_2$) concentration since Mg^{2+} ions are known to affect both the specificity and the yield of PCR (Oste, 1988). Concentrations that are too low or too high can have deleterious effects on PCR, high concentrations may lead to incomplete denaturation and low yields, whereas levels that are too low reduce the ability of polymerase to extend the primers. High $MgCl_2$ levels also lead to increased production of non-specific products and primer artifacts including primer dimers. This should always be avoided but especially in sensitive quantitative assays. It is recommended that a $MgCl_2$ titration is performed for each primer/probe set. This work is usually best performed after completing optimisation of primer and probe concentration. The optimal $MgCl_2$ concentration will generally be the lowest amount that gives the minimum CT value, the highest fluorescent intensity and the steepest slope of the fluorescence/cycle plot (Figure 3). The optimal $MgCl_2$ concentration for DNA assays is usually between 2-5 mM and for RNA is usually between 4-8 mM. Commercially available real-time PCR buffers are now available where

$MgCl_2$ optimisation is not required for example, QuanTitech (Qiagen, Crawley, UK) or FastStart Plus (Roche Diagnostics, Lewes, UK).

Template Purity and Concentration for Optimisation

When optimising assays it is advisable to use a minimum of a high and a low template concentration as too much DNA can inhibit the reaction and too little may be undetectable. For some assays it may not be necessary to use purified DNA or RNA however, if the primers are not amplifying it is worth repeating the PCR with pure template in the event that PCR inhibitors are present. If the CT is <10 cycles then a higher dilution should be prepared or if the CT is >30 then higher concentrations should be used. If a serial dilution series of template is prepared and amplified then an optimised assay should display an equal number of cycles between amplification of each dilution.

Cycling Conditions

The use of optimal cycling conditions are essential for real-time as with any PCR reactions (Rasmussen, 1992). The initial cycling conditions recommended for both hybridization probes and hydrolysis probes are given in Table 2. The optimal annealing temperature should be determined as described in the primer section and the optimal extension time should be calculated using the following formula: extension time (s) = amplicon length (bp) ÷ 25. Note: when hot-start techniques are employed the initial denaturation time may need to be increased. If Primer Express is used to design hydrolysis probes then they should all work optimally under the same conditions (Table 2).

PCR Controls

No template (negative) controls should always be included in real-time runs and where possible appropriate positive controls should also be included. When the assay is quantitative standards of known concentration should be included, although on some platforms it

Table 2. Cycling conditions

	Hybridisation Probe (performed on Roche LightCycler)	Hydrolysis Probe (performed on ABI Sequence Detection System)
Initial Denaturation	2-10 min @ 95°C	10 min @ 95°C
Denaturation	0 s @ 95°C	15 s @ 95°C
Annealing	5 s @ annealing temperature	60 s @ 60°C
Extension	Optimal time dependent on amplicon length @72°C	
Ramp Rate	20°C/s or reduce to 2-5°C if annealing temperature <55°C	
Fluorescent Acquisition	At end of annealing step	At end of annealing step
Melting Curve	30 s @ 55°CRamp rate 0.1°C/s with acquisition mode on continuous temperature increased to 80°C	

is possible to perform quantitative analysis using a standard curve generated in a previous run thus maximising the number of samples which can be analysed. Where melting curve analysis is used for product differentiation then it is advisable to include samples that will melt at each of the possible melting temperatures (Logan *et al*., 2000; Edwards *et al*., 2001). This allows for any drift in melting temperature that may be observed over time or with different batches of fluorescent probe.

To control for PCR inhibitors an additional inhibition control template should be included in each sample. This inhibition control should be amplified by the same primers as the experimental target but should contain a different probe binding site. When the reaction is significantly affected by inhibitors, their presence is indicated by the failure of both control and experimental target amplification, or by low amplification

efficiency. Inhibition may be minimised by diluting the sample, by using an alternative purification technique or by obtaining a different sample. If an inhibition control is not available, negative samples should be analysed for a different target or spiked with a positive control and re-amplified. The use of inhibition controls is particularly important when real-time PCR is being used for clinical diagnostics or other applications where a false negative result could be critical.

ROX Passive Reference

Real-time platforms that use peltier blocks as the heating/cooling mechanism require the use of a reference signal to normalize the fluorescent signal across the block. By using the ROX passive reference all fluorescent signals are normalized leading to more accurate and reproducible results. When the ROX passive reference is used it is important to note that everything in the real-time PCR is relative, which means that if the ROX passive reference changes then the baseline, CT value and amount of fluorescence will also change. When optimising the passive ROX reference there is a narrow optimal range. If the amount is too high then background noise will be reduced but a low signal will be hard to distinguish from the background and this may lead to false negative results. If the ROX level is set too low the background noise is increased but a strong signal will be easy to distinguish from the background for high copy number samples, however, weak signals may be lost in the background.

Analysis of Real-Time PCR Results

Real-time PCR results can be analysed in a variety of ways depending on the application. Analyses of quantitative standards can be used to generate PCR curves and from these the cycle number at which the fluorescent signal increases above the background fluorescence can be determined. This cycle number can be used for comparison of results from run-to-run and can be used to generate qualitative positive/negative results. In quantitative assays the standard curve is used to determine the copy number of unknown samples and again

this can be compared with other samples. Real-time PCR reactions can also be analysed by melting curve determination when methods including hybridisation probes and SYBR Green I intercalation have been used. Melting curves are useful for differentiating primer dimers from specific PCR products (primer dimers usually melt at lower temperatures than specific PCR products) or for differentiating different PCR products in mutation detection. During the initial optimization of real-time PCR assays it is useful to analyse the results by agarose gel electrophoresis in order to correlate product length with melting peaks and to identify any primer artifacts.

Conclusions

Real-time PCR results are dependent on the optimal concentration of each of the reaction constituents as well as on the interrelations of each of these components. The use of commercially available master mixes, which have already been optimised, provides a solid basis for a robust assay. Optimisation of $MgC1_2$, primer and probe concentration is still required to obtain maximum sensitivity and PCR efficiency but can be performed relatively easily. Depending on the platform and probe format employed some optimisation of the cycling conditions may be required. When using a well optimised assay the target will reliably amplify at the same cycle number although variations may be observed in the amount of fluorescence observed as this is more dependent on not only the PCR efficiency but also on variations in the availability of each of the different reaction components.

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