Internal and External Controls for Reagent Validation

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Abstract

PCR applications that require a high confidence in the result should be designed to control for the occurrence of false negatives. False negatives can occur from inhibition of one or more of the reaction components by a range of factors. While an external, or batch control is often used, the ideal control is one that is included in the reaction cocktail in a multiplex format. Early approaches used different sized amplicons combined with end-point analysis. Fluorescent homogenous real-time PCR methods have a number of advantages for implementing internal controls. Here we discuss the application and development of molecular mimics for use as controls in real-time PCR, and explain a number of concepts and experimental considerations that will aid in the optimisation of the controlled multiplexed assay.

Introduction

The confidence in assays based on the polymerase chain reaction (PCR) (Saiki *et al.,* 1985; Mullis *et al.,* 1987) may be compromised by the sporadic occurrence of either false positive or false negative results. False positives are a problem that is common to the general application of PCR. False positives occur mainly as a result of cross contamination from either positive samples or reaction products. A combination of preventative methods including good laboratory practice, delineated preparation/analysis areas, PCR cabinets with UV treatment, UV air scrubbers, closed tube assays, and uracil glycosylase carry-over prevention chemistry (Longo *et al.,* 1990), has effectively eliminated the occurrence of false positives for the majority of applications. However, there are a number of PCR applications where the avoidance of false negative results is of equal importance. False negatives occur through failure of one or more of the reagents, the presence of inhibitors, or the failure of the PCR thermal cycling process (Rossen *et al.,* 1992; Wilson *et al.,* 1997). The applications requiring high confidence in the PCR include pathogen detection in clinical diagnosis, food quality control and environmental analysis.

For most molecular tests the use of reference material in a batch test is the only control that can be implemented. However, PCR and other nucleic acid amplification techniques provide not only extremely sensitive detection, but also have the added advantage that they can be readily multiplexed to include an internal control (IC). An IC is a second target molecule that can be amplified with, but distinguished from, other products in the same tube. In an ideal assay this should be able to control for all of the reagents in a reaction cocktail, and for variations in machine operation parameters. This can be achieved by the use of a molecular mimic, a synthetic molecule that may be co-amplified using the same set of amplimers as the target species.

Early approaches for ICs used amplicons with different molecular masses and subsequent analysis using a separation method. For example, the molecular mimic could contain both priming sites, but an internal sequence changed by insertions or deletions (Ursi *et al.,* 1992). The products of amplification could be easily analysed by the

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use of agarose gel electrophoresis and ethidium bromide staining. There are a large number of such examples reported in the literature. The main drawback of this approach is that the analysis method is time consuming, the smallest amplicon tends to be amplified more efficiently, and generation of specific and control product heteroduplexes (Henley *et al.,* 1996) can be unpredictable and therefore confusing for data interpretation.

Real-time PCR approaches using fluorescence are particularly useful for high confidence applications because they can be easily multiplexed. The assays are also generally carried out in a closed tube format which significantly reduces the risk of cross-contamination by amplification products. Most commercial fluorimeter instruments can co-amplify and detect at least two targets in a quantitative manner as discussed further in Chapter 3. However, increasing the level of multiplexing adds significantly to the complexity of the design strategy for the controls and the assay optimisation.

In this short chapter we will present various strategies for producing internal controls for different fluorescent chemistries, and discuss some experimental considerations for their optimisation and implementation. In the literature the term internal control is often used incorrectly and so we will first discuss some definitions. The experimental considerations described here are based largely on our own experiences and we make some useful suggestions for the development and implementation of controlled assay systems. Whilst users will find that for many applications the use of a homologous or competitive control is not necessary, the information will be of equal use to those developing heterologous controls and synthetic standards alike. Competitive homologous internal controls are state-of-the-art for PCR and the main focus of this chapter.

Nomenclature

In the literature multiplex PCR is used for a number of applications and the second internal (in the same reaction tube) amplicon usually serves to control, normalise or standardise the result. This chapter covers the

application of molecular mimics to check reaction outcomes. However, the strategies described are equally applicable in the development of other internal and external mimics. In particular the use of mimics as either external or internal standards has advantages and in Table 1 we summarise some nomenclature used to describe the types of second amplicon. It is easy to see how a homologous internal control will add more confidence to a result. However, it should be noted that for controls that utilise nucleic acid reporter probes it is almost impossible to control for their function. This is discussed in more detail in the next section.

Strategies for Development of Internal Controls and their Analysis

The choice of strategy for detecting an internal control is dependent upon the instrument's capabilities and the reporting chemistry. The simplest strategy is one that uses a strand-specific probe using a reporting dye with a different emission wavelength to that of the probe for the specific target. This requires that the emission wavelength of these dyes is either spectrally separated enough that they do not overlap, or that these emissions can be effectively deconvoluted using the instrument's calibration algorithm. For example on the ABI 7700, a 5' nuclease assay may use FAM as the specific reporter and either VIC, JOE, HEX etc as the reporter for the control probe. A cycle-by-cycle increase in the emission of either dye reports the amplification of the respective species. This strategy may be applied to virtually all fluorescent chemistries and instruments.

Since the internal control serves only to report the integrity of the reagents the signal does not necessarily need to be quantitative. The ability to detect the species at the end of thermal cycling should suffice most applications. Therefore, for those assays based on hybridisation, and when an instrument that can determine melting point (T_m) is used, one can design the control amplicon and/or probe to have a different T_m from that of the specific nucleic acid target. This allows discrimination between target and control species and may be achieved by using DNA binding dyes for amplicons with different melting points (Lee

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Table 1. Definitions and types of standard, control and reference nucleic acids.

Definition / Type	Second amplicon type			
	Standard	**Control**	**Reference**	
Definition / Type	A standard is a nucleic acid preparation that has a set or known concentration. Unknown samples may be compared to one or more standards and an absolute value for the unknown can be determined by numerical interpolation/extrapolation from experimentally determined values.	A control is an amplicon added to the PCR and allowed to amplify to verify the integrity of one or more reagent(s) in the cocktail	A nucleic acid target that is used to compare the sample for either a qualitative or relative quantitative analysis.	
External: Amplified in a different well.	The standards are amplified separately from the target species in different wells on the same instrument. *	"Batch control" This involves the use of a control in a separate well on the instrument. This approach cannot control for false negatives in different samples. It controls only for successful thermal cycling and the integrity of a common core reaction mix.*	A related nucleic acid that is used for a qualitative comparison of amplification.	
Internal: Amplified in the same well.	*Homologous*		**Exogenous**	This is an internal reference that is added to the sample and is co-amplified with the target nucleic acid using a second set of amplimers.
	A competitive standard that has the same amplimers as the specific target.	A competitive control that is included in the same well of the instrument that has the same amplimers as the specific assay and controls for the core reagents and primers, and the thermal cycling.		
	Heterologous		**Endogenous**	A "Housekeeping gene." This is usually a gene that is naturally present in the sample at constant levels such that the up or down regulation of the target species can be relatively quantified using a second set of amplimers.
	A non-competitive standard with different amplimers to those of the specific target	A control that is included in the same well of the instrument but has different amplimers to that of the specific assay that controls for both the thermal cycling and the core reagents.		

et al., 1999), or labelled probes for different sequences. When the melting point method is used with sequence specific probes each may be associated with a dye that has a different emission wavelength. The merits of strand-specific probes over DNA binding dyes have been discussed in chapter 3. Whilst DNA binding dyes have some disadvantages in terms of specificity, the same probe is used for detecting the control and the specific target. Therefore, their function may be somewhat better controlled than strand-specific probes. Using dual hybridisation probes in either quantification and/or melting point analysis it is possible to use one common probe, either the donor or the acceptor, this also provides a better approach for control of the probe. The methods for generating these mimics are now briefly described.

Synthesising and Optimising Molecular Mimics for Use as Internal Controls

To generate a homologous internal control mimic there are several different approaches using recombinant DNA techniques. In each case the objective is to create a synthetic target that will effectively co-amplify with the specific amplicon, and whose "motif" may be easily distinguished from that of the specific amplicon. This is achieved by the use of dyes with different emission wavelengths and/or melting point analysis as described above. Several methods have been reported. These include: insertion of the specific target/amplimers into a vector and insertion/deletion of a sequence between the amplimers (Ursi *et al.,* 1992; Zimmerman *et al.,* 1996; Brightwell *et al.,* 1998), the amplification of a generic target from a related sequence (deWit *et al.,* 1993), the use of overlapping and tailed PCRs (Müller *et al.,* 1998; Sachadyn *et al.,* 1998), site directed mutagenesis (Nash *et al.,* 1995), and polymerase extension of oligonucleotides and subsequent PCR (Rosenstraus *et al.,* 1998). In addition a complete synthesis of the gene is possible for smaller amplicons (Zimmerman *et al.,* 2000) and some commercial suppliers will now make the gene and supply it in a vector ready for use. Since the ability to discriminate amplicons by size is not a requirement, the amplicon may be similar in size, and thus achieve a similar amplification efficiency. The two main techniques that we use to generate mimics are:

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- Direct insertion of amplimer and probe sequences into cloning vectors using recombinant techniques. This can be done by amplifying amplimer/probe sites, or the use of oligonucleotide linkers. In either case the use of restriction overhangs will assist in the correct directional insertion of the fragments.

- Generation of targets using a PCR approach of amplimers tailed with specific amplimer sequences to amplify alternate probe regions. The products contain the specific amplimer sequences incorporated into the PCR product. This product may be subsequently cloned into the desired vector (See Figure 1). The use of the additional overhangs with unique restriction sites will assist in directional cloning that may be required for some applications (*e.g.* sense-specific RT-PCR).

For reverse transcriptase PCR (RT-PCR) a mimic may be made using the methods described and subsequent insertion of the product into a vector. *In-vitro* transcription using the T7/T3 promoters allows the RNA molecule to be then synthesised in quantity. The sequence could be cloned into an RNA virus such as the bacteriophage MS2. Armoured RNA™ is a product available from Ambion Inc (Pasloske, *et al.,* 1998). This is a method for packaging recombinant RNA into pseudoviral particles using a plasmid system whereby the MS2 bacteriophage coat protein is located downstream of the promoter and the target gene of interest. Under the control of the inducer, the RNA is transcribed encoding the coat protein and the target sequence. Coat protein is translated and subsequently binds to recombinant RNA to form the product. The packaged molecule is resistant to RNAase digestion yet easily extracted for amplification. The process has been shown to stabilise the RNA compared to *in vitro* transcripts made from plasmid and is an ideal method for RNA IC production.

Using any approach there are a number of experimental considerations that should be considered in the design and use of the control molecule and these will now be discussed.

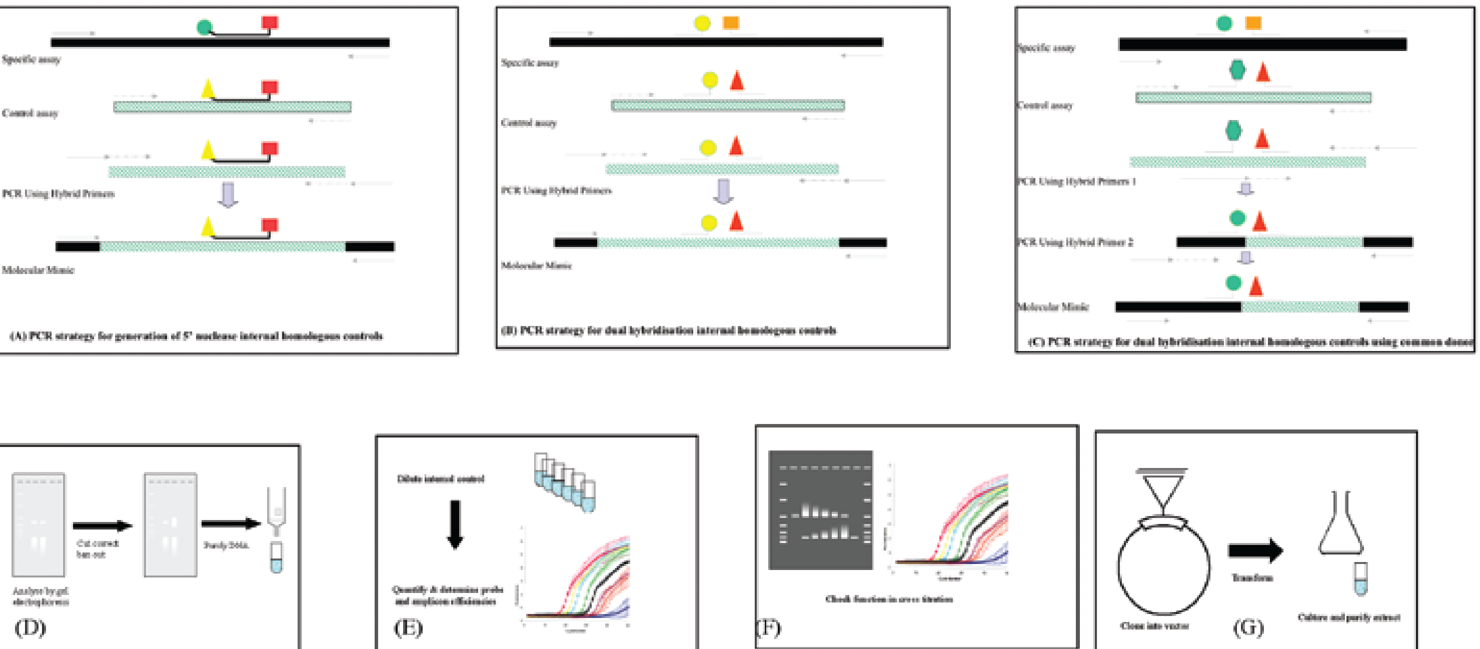

Figure 1. Schematic showing strategies for rapidly generating and evaluating competitive internal homologous controls by PCR. (A) For assays such as the 5' nuclease where there is only one probe, (B) For dual hybridisation assays using two additional probes, (C) dual hybridisation probes using one common probe for both the control and specific assay, (D) Amplify the IC using PCR and extract the correct band from the gel, (E) Serial dilute the product and determine the amplification and probe efficiencies (see Figure 2), (F) Cross titre the IC with specific target to assess the efficacy of the IC in multiplex PCR before cloning into a suitable vector and (G) transformation, culture, extraction and use.

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Experimental Considerations

In designing an experiment the sequence of the specific target and the assay are important considerations. Reference should be made to chapter 3 covering the utility, design and sequence dependency of different chemistries. The design of the assay is best facilitated by software tools that can automatically select amplimers and probes and predict (score) amplimer-amplimer and amplimer-probe interactions for the multiplexed assay. Here we highlight some of the considerations that are important in achieving a successful IC implementation.

Control Sequence

The choice of control sequence is the most important factor since the other considerations are mostly dependent on this. When using nucleic acid probes one useful approach is to use a sequence that has been previously reported/optimised, or that is commercially available for the same assay type. For example, the ABI Human β-actin sequence for a 5' nuclease assay is useful because the probe is efficient and a kit containing it is commercially available. Ideally the probe sequence should not be of the same origin (or endogenous to the sample) as the target for the specific test. For example, it would not be ideal to use the human β-actin probe for a human test. Providing the probe sequence has been shown to work, a sequence from almost any other source could be suitable. Ideally, the GC content for the sequence should be similar to that of the specific target.

Amplicon Efficiency

Whilst the differences in amplification efficiency may be compensated for by changing the concentration of the initial target they should ideally be similar and this can be empirically determined. This is most important if the mimic is to be used for competitive quantification since the amplification efficiency can vary significantly between amplicons (Zimmerman *et al.,* 1996). It is useful to include native sequence on either side of the amplimer when designing the mimic to ensure that

the effects of neighbouring sequence is maintained. The amplification efficiency is best determined using a DNA binding dye such as SYBR® Green I combined with an efficient hot-start to amplify a broad range or concentrations across the dynamic range of the assay. Whilst using a probe directly to do this is satisfactory, checking the amplification efficiency without the probe allows a direct measurement of efficiency and is useful in understanding the efficiency of the probe when it is later evaluated. It will avoid dismissing a satisfactory reaction when it may only be necessary to have a poor probe batch re-synthesised or to revise the probe design. The efficiency (E) can be calculated by using the slope value for the derived standard curve using (Figure 2):

$$E=10^{-1/slope}$$

The efficiency of different amplicons for the same (or similar) dilution series can then be directly determined.

Probe (Nucleic Acid) Efficiency

Probe efficiency is important if the signal of the control is to be commensurate with that of the specific probe. The probe efficiency can be affected by a number of factors that are discussed in chapter 3. The probe sequence to be chosen should be checked for complementarities

Figure 2. Determining and comparing amplification and probe efficiencies of different amplicons. A: Determining amplification efficiency (E). Ideally, using a generic method such as a DNA binding dye, *e.g.* SYBR®Green-1, the cycle threshold (CT) values are determined for a dilution series of target material over a range of concentrations and the plot of fluorescence intensity against cycle number generates the amplification curves. The derived CT values can be used to generate a standard curve. The slope of the curve can then be used to calculate E for the range tested. This value can be used to compare E with other amplicons using the same or similar dilution series. B: Determining probe efficiency. Using the 5' nuclease assay and other assays by comparing the total change in fluorescence (as a function of a reference probe/assay) when the reaction is allowed to go to completion. C: Using probes based on hybridisation by comparing (as a function of a reference probe/assay) the change in fluorescence when the probe is hybridised (at a specific temperature) to when it is un-hybridised (at high temperatures).

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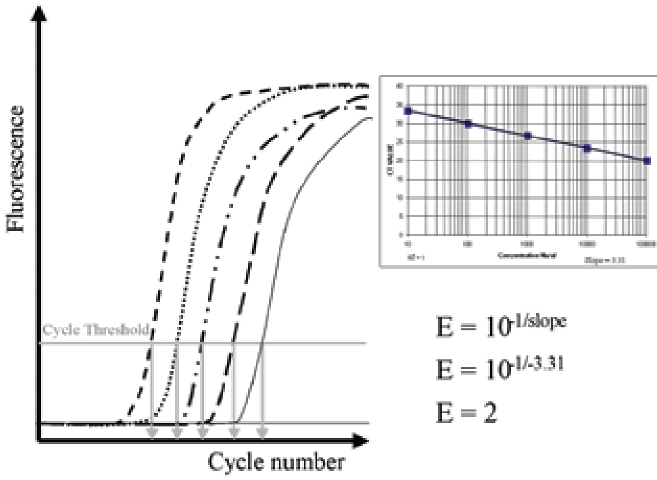
A



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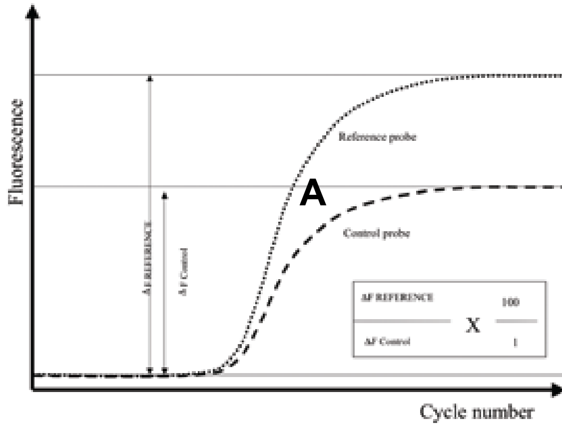

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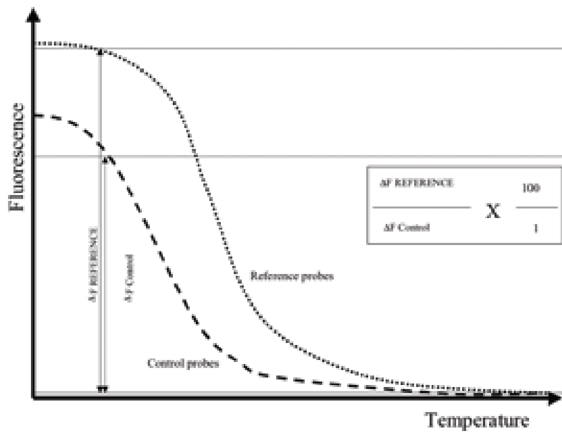

to other probes and amplimers in the reaction. This is most important when the probe melting point approach is used since the signal from a control probe that is too large may swamp a low signal obtained in positives. When assessing potential probes for the mimic sequence it is useful to score the probe efficiency relative to a reference assay. For example, the percentage of signal:noise for both 5' nuclease assay specific and control probes can be compared to that of another assay such as the ABI Human β-actin probe. For this to be done the reaction for either probe must be taken to completion (plateau). This data may be expressed as:

$$\frac{\text{Fluorescence signal value specific assay/control assay at (cycle 50- cycle 1)}}{\text{Fluorescence signal value } \beta\text{-actin (Cycle 50-cycle 1)}} \times \frac{100}{1}$$

This provides a comparative means for matching candidate control sequences to given assays. Likewise, the same may be done for comparing dual hybridisation probes using:

$$\frac{\text{Fluorescence signal value specific assay/control assay at (95°C- 60°C*)}}{\text{Fluorescence signal value } \beta\text{-globin (95°C- 60°C*)}} \times \frac{100}{1}$$

*or at the temperature where the fluorescence data are to be acquired.

This approach may be carried out using any reference probe and is equally applicable to other assay systems to estimate the relative probe efficiency (see Figure 2). The quotient used to compare should be based on the values derived from the maximum signal (when the target is in excess of the probe) subtracted from the minimum signal (the background) for each probe. The minimum signal is usually at the start of the amplification or when the probe is disassociated from the target at elevated temperatures.

Control Vector

The choice of control vector is important since the vector may affect the stability and amplification efficiency. Plasmid constructs are commonly used and the efficiency of amplification may drop dramatically at

lower target numbers (<100 copies per reaction). Linearising the plasmid by digestion at a unique restriction site in a vector can improve amplification efficiency. The use of other vectors such as bacteriophage λ vectors and M13 (ssDNA rescue by co-infection with helper phage) may also be useful approaches for generating large amounts of linear IC molecules. PCR product itself is fine for use as a control. We recommend the initial amplification of control DNA by PCR since the products may be used to evaluate potential mimics prior to the cloning of the molecule into a vector. However, the use of an *in vivo* amplification method for the final synthesis of DNA that is to be employed as the control is superior in that individual clones may be readily sequenced, to check their correct identity, and this process avoids the spurious artefacts generated in PCR. These artefacts can be minimised during the evaluation stages by cutting the predicted PCR band out of an agarose gel and using an appropriate method, such as column purification and/or dialysis, to clean up the product before it is diluted and evaluated as a suitable mimic.

Number of Molecules

When using a competitive control the number of molecules to be added into the reaction must be empirically determined so that the required sensitivity of the specific assay is not compromised. The control must be amplified effectively in the absence of specific target. With increasing numbers of specific target molecules in the reaction the control amplification may be out-competed. We have observed that it is possible to prevent this if the Tm of the specific amplicon is higher than that of the control. Reducing the denaturing temperature of the thermal cycle (which is possible on some thermal cyclers) to below that of the specific target, but higher than that of the control, suppresses specific amplification thus allowing the IC to amplify. However, this is not necessary since the amplification of the control only serves to validate the reagents in the absence of the specific target. A cross titration of specific target to control should be carried out to determine the correct amount of control molecules to include in a reaction. In practice, this amount is routinely determined to be 10-100 copies per reaction if the specific assay is to maintain a sensitivity of

10 or more copies of specific target. Accurate determination of the IC mimic concentration is therefore important. The use of fluorescent dyes and standards is inherently more accurate than ultra-violet optical density quantification at 260 nM (UV OD_{260}). An alternative method for statistically determining the correct concentration of a stock of internal controls using PCR is provided by Rosenstaus *et al.,* 1998. This method describes using a dilution series of the control preparation and performing multiple amplifications. At a given dilution the amplification will become stochastic due to the presence of low or no copies of the template in the reaction in accordance with Poisson's law. The relationship between the average number of internal control copies in a dilution (C) and the probability (P*n*) that no molecules exist in a sample of this dilution is given by:

$$C=-ln(Pn)$$

Where, P*n* is determined by counting the number of negative replicates used to calculate C.

Target Nucleic Acid

The control must be RNA if it is to be used for controlling the RT step. If a specific primer initiated RT step is used then utilising the same primers in the mimics will control for both the RT and the PCR, and negates the requirement of a second mimic for the PCR step in a one-step PCR. Depending on the application, controls at both the RNA and DNA levels may be required in a two-step PCR. The RT efficiency of the mimic and the specific target must be similar if the mimic is to be used as a standard in any application. When the RT-PCR is designed to be sense-specific, directional cloning and the choice of vector orientation is important.

Dye/Channel Assignment

The most efficient dye and/or detector should be used for the specific target assay. This is of importance where a single excitation source is

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used in a universal acceptor arrangement, in which case it is usually the shorter wavelength dye that will be most efficiently excited. When a universal donor arrangement is used, it will be the acceptor to which energy is most efficiently transferred from the donor. In either multiplex arrangement, the optimum channel will be dependent on the instruments specification and the dyes used.

Summary and Future Improvements

The further development of instruments and methods of detection will increase the ability to multiplex PCR to higher levels in a quantitative manner. This introduces new possibilities for controlling for/and or normalising sample extraction, and/or RT-PCR steps. This will be achieved through a combination of competitive (homologous) and non-competitive (heterologous) internal controls for each process. It is not always possible to control for these processes with one control since each may be subjected to different inhibitory factors. The ability to do this will improve the usefulness of the PCR for both research and diagnostic applications. The ability to control for all such processes will be of most use in emerging applications where remote testing in the non-laboratory environment necessitates the use of internal validation.

Acknowledgements

The authors would like to thank the editors for the invitation to contribute to this text. The underlying work leading to this publication was funded by the UK Ministry of Defence.

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