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LightCycler qPCR optimisation for low copy number target DNA

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Abstract

The LightCycler is a rapid air-heated thermal cycler which incorporates a fluorimeter for the detection and quantification of Polymerase Chain Reaction (PCR) amplified products. It provides real-time cycle-by-cycle analysis of product generation. Amplification occurs in glass capillary tubes. The products are detected using a fluorescent double stranded DNA binding dye or fluorescent probes. However, conditions that work well in conventional PCR reactions do not readily translate to the LightCycler. Whilst using this new technology to study an infectious pathogen in human tissue samples, several parameters were identified which can have an adverse effect on the reliable and reproducible quantification of low copy number target DNA. They included abstraction of PCR reagents on glass, primer–dimer formation, non-specific product generation, and a failure to amplify low copy number target when it is present in a high background of human chromosomal DNA. For each problem identified, several solutions are described. Novel approaches are also described to ensure that amplification of target DNA and of the quantification standards occurs with the same efficiency. With appropriate changes to the protocols currently in use, LightCycler quantitative Polymerase Chain Reaction (LC-qPCR) can be used to achieve a level of accuracy that exceeds that of an enzyme immunoassay. The LC-qPCR optimisation strategies described are of particular relevance when applying this technology to the study of pathogens in tissue samples. The technique offers the enormous potential for reliable and reproducible quantitative PCR of low copy number target DNA.

Keywords: LightCycler-quantitative PCR optimisation; Additives

1. Introduction

The Roche LightCycler (Wittwer et al., 1997a) is a rapid air-heated thermal cycler incorporating a fluorimeter for the detection and quantification of Polymerase Chain Reaction (PCR) amplified products. Unlike the ABI 7700 machine, the LightCycler offers real-time monitoring of PCR amplified products. Real-time PCR amplification requires no post-PCR analysis of the samples. This avoids the cumbersome quantifi-

Abbreviations: PCR, Polymerase Chain Reaction; LC-qPCR, LightCycler quantitative Polymerase Chain Reaction; HIV-1, Human Immunodeficiency Virus 1; 2-LTR, 2-Long Terminal Repeats; BSA, Bovine Serum Albumin; HDL, High Density Lipoprotein; PVP, polyvinylpyrrolidone; tRNA, transfer Ribonucleic Acid; TMAC, tetramethyl-ammonium chloride; LGT agarose, low gelling temperature agarose; HS, "hotstart".

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cation techniques normally associated with traditional PCR. Amplifications are performed using a modified PCR mix within glass capillaries, and the products detected with a fluorescent double-stranded DNA binding dye (Sybr Green or Sybr Gold), or using fluorescent probes. The use of glass capillaries enables rapid heating and cooling, and consequently very short cycle times. The LightCycler is now increasingly being used for the rapid detection of infectious organisms in clinical diagnostic laboratories.

Whilst evaluating the validity of LightCycler based quantitative Polymerase Chain Reaction amplification (LC-qPCR) and the Sybr Gold detection system, we have sought new solutions to several problems that we and others (Wittwer et al., 1997b; Morrison et al., 1998; Sturzenbaum, 1999) have identified that can severely affect the reliable and reproducible quantification of low copy number target DNA. They include: (1) abstraction of PCR reagents within the glass capillaries, (2) failure to effectively amplify low copy number target DNA when it is present in a high background of other DNA, and (3) competitive formation of primer–dimers.

We have previously encountered some of these problems (and devised solutions) whilst attempting to perform PCR in-situ on glass slides or within glass capillary microscope slide chambers (Teo and Shaunak, 1995a,b). In particular, abstraction of Taq polymerase, magnesium chloride and target DNA on glass surfaces was found to severely alter the efficiency of amplification. In both PCR in-situ and LC-qPCR, reagent abstraction has been reduced by the inclusion of Bovine Serum Albumin (BSA) in the PCR mix. However, the stability of this blocking agent during repeated cycles of denaturation at 95 °C has been a cause for concern. We have observed sporadic precipitation in some commercial PCR mixes with a concomitant failure of PCR amplification. We have now identified and validated several heat-stable blocking agents for use in LC-qPCR.

The presence of a large amount of non-target DNA severely restricts the use of LC-qPCR when Sybr Green is used as the detection method. This is because the high levels of basal fluorescence mask the increase in fluorescence generated by the amplified product. The fluorescence is due to high (>50 ng) levels of non-target DNA binding Sybr Green at the acquisition temperature. This effect is most likely due to the incomplete denaturation of complex DNA in the very short denaturation times used in the LightCycler. The problem is particularly problematical when quantifying low copy number target DNA in high DNA background. We have therefore developed alternative target enrichment strategies that remove non-target DNA or use cycle limited nested PCR to address this problem. Both methods have been applied to the quantification of episomal forms of Human Immunodeficiency Virus-1 (HIV-1) DNA in our model system.

Lastly, we have addressed the problem of primer–dimer formation and non-specific product generation. These artefacts compete with DNA target sequences for PCR reagents and lead to anomalous quantification of the true target. As accurate quantification requires simultaneous amplification of the target DNA and of the quantification standards with equal efficiency, novel approaches have been developed to achieve this.

2. Methods

Optimisation of the reagents used to perform the PCR reactions is critical for reliable and reproducible results. Conditions that work well in conventional PCR reactions do not readily translate to the LightCycler. This is not unique to the LightCycler and is seen with other new quantitative PCR platforms. Our model system relates to the detection of HIV-1 DNA in cells derived from blood. For the studies described here, LC-qPCR amplification was carried out for 2-Long Terminal Repeats (2-LTR) episomal (circular) forms of HIV-1 DNA (Farnet and Haseltine, 1990; Sharkey et al., 2000).

2.1. Primers

The PCR primers were selected to amplify across the junction of a 2-LTR circular form of HIV-1 DNA (Table 1a) A sequence comparison using the database at http://www.hiv-weblanl.gov showed that the primer nucleotide sequences selected were >99.2% identical within the 80 viral isolates in which this region was compared. None of the redundancies noted were in the distal 3′ ends of the primers.

The primers were synthesised by GenSet (http://www.helena-biosciences.com), GibcoBRL (euro-

Table 1a
Primers used for amplification of 2-LTR circles

	Sequence	Calculated melting temperature (°C)	Actual melting temperature (°C)	Comments
Forward primers				
M669	GGAACCCACTGCTTAAGCCTCAA	50–70	69	sense primer
R Cir3	TTGCCTGTACTGGGTCTCTCTG	52	not done	outer nested primer
Reverse primers				
M847	GTGTAGTTCTGCCAATCAGGGAA	52–72	67	antisense primer

prim@lifetech.com) or GenoSys (http://www.sigmagenosys.com), and are as detailed in Table 1a. Both standard desalted and HPLC purified primers were used. The complementary primer sequences were also synthesised (0.025 μM scale synthesis). The primers were 20–25 bp in length, had a GC content of ~50%, and had a calculated melting temperature of ~60–70 °C; (T_m (°C)=2(A+T)+4(G+C) °C). Primer melting temperatures were also calculated by the "nearest neighbour method" and "base stacking melting temperature" using software provided by Sigma-Genosys (http://www.sigmagenosys.com) and Promega (http://www.promega.com). To reduce potential primer–dimer artefacts, some primers incorporated an –AA sequence at their 3′ end (i.e., target dependant). Primer pairs were also checked for potential dimerisation on the Wheatstone Enterprise Primer analysis website (http://www.Wheatstone Enterprises.com).

2.2. Preparation of plasmid samples for standard curves

An HIV-1 2-LTR containing plasmid (gift from M. Stevenson, University of Massachusetts, USA) was purified using a Promega mini-prep kit (Promega UK). The concentration of DNA was determined spectrophotometrically (OD 260/280 nm) using a GeneQuant apparatus (Amersham-Pharmacia, UK) and the copy number calculated. Plasmid DNA was then linearised by *Eco*RI digestion and serially diluted in PCR grade water (Sigma UK) containing 5 ng/μl herring sperm DNA to make a working stock solution. All dilutions were made in siliconised microfuge tubes (Sigma UK). This is very important because we found that even short-term storage (i.e., up to 4 h) of low copy number target DNA in conventional tubes led to a significant binding of the target DNA to the walls of the tubes. Consequently, for each PCR amplification, standards were made daily from the *Eco*RI linearised plasmid working stock (100,000 copies/μl). Doubling dilutions of the standards from 200 copies/tube down to 13 copies/tube were then used to generate the standard curve. In this paper, 200 copies/capillary is denoted as "high" copy number and <25 copies/capillary as a "low" copy number. The 25 and 13 copy standards were prepared in duplicate because of the Poisson distribution of very low copy number target DNA in solution. Making dilution series every day adds to the length of time required to complete the PCR amplification. We have therefore also investigated the feasibility of "long-term" storage of pre-aliquotted, very low copy number target DNA standards in glass capillary tubes. Two microlitre aliquots of the plasmid standards were centrifuged to the bottom of glass capillary tubes and then stored for up to 2 months at 4 °C, prior to being run in parallel with freshly made standards.

2.3. Determining the optimum PCR cycling conditions

Quantification of the PCR amplified products can be performed with either Sybr Green or Sybr Gold (BioGene UK) or fluorescent probes. Whilst the probes confer high specificity detection of the amplified product due to their high signal to noise ratio, they have a major disadvantage in their considerable cost. Sybr Green or Sybr Gold detection is simpler, but does not confer absolute specificity of product detection. The level of background fluorescence present at DNA concentrations >100 ng/reaction can also limit its application. Having evaluated both approaches, we used the probe method to validate

Table 1b
LC-qPCR cycling parameter's for BioGene Hotstart and Qiagen HotStar mixes

Mix	Digestion	Activation	Denaturation	Annealing	Extension	Acquisition	Cycles	Melting point analysis
Biogene	(1) 37 °C, 5 min	95 °C, 3 min	(2) 95 °C, 10 s	62 °C, 5 s	72 °C, 10 s	83 °C, 5 s	5	
			(3) 89 °C, 10 s	60 °C, 5 s	72 °C, 10 s	83 °C, 5 s	35	(4) 65–95 °C
Qiagen	(1) 37 °C, 5 min	95 °C, 15 min	(2) 95 °C, 10 s	62 °C, 5 s	72 °C, 10 s	83 °C, 5 s	5	
			(3) 89 °C, 10 s	60 °C, 5 s	72 °C, 10 s	83 °C, 5 s	35	(4) 65–95 °C

the specificity of our amplified product, and the Sybr Gold method of detection for all subsequent day-to-day experiments. This approach resulted in a considerable cost saving when trying to develop an assay with real clinical utility. Although we used the Sybr Gold at the dilution recommended by the supplier (1/20,000 dilution of the stock), it is advisable to optimise the concentration of Sybr Gold. Using Sybr Green, Wittwer et al. (1997b) and Ririe et al. (1997) have reported that high concentrations of Sybr Green adversely affect the PCR amplification. Sybr Gold was chosen in preference to Sybr Green because it is more stable during long-term storage (Lee et al., 1999).

As with all PCR reactions, determination of the optimum PCR cycling conditions for each primer pair is crucial (Rasmussen, 1992). Importantly, conditions being used for conventional PCR machines such as the Perkin Elmer 9600 are inappropriate for LC-qPCR. The optimal primer annealing temperature was determined by hybridising each PCR primer to its complementary sequence, and then performing a melting curve analysis in each PCR buffer in the presence of Sybr Gold. It is important to note that melting temperatures vary considerably in different PCR buffers (Table 1a). The annealing temperature chosen for the LightCycler amplification was 2–5 °C lower than the lowest primer melting point temperature determined using this complementary primer hybridization method (Table 1b). The lowering of the annealing temperature allowed for potential redundancy in the target sequence.

It was also necessary to determine the optimal fluorescence acquisition temperature. PCR amplification of plasmid was first performed in a conventional Perkin Elmer 9600 PCR machine. After confirming the specificity of the amplified product by agarose gel analysis, a 10-μl aliquot of the amplified product was removed, added to 10 μl of 2× LC-PCR mastermix (BioGene, Roche, Qiagen or Plat Taq) in a glass capillary tube, and a melting curve analysis performed using the LightCycler. The fluorescence acquisition for each PCR cycle was set at 2–3 °C below the melting point of the PCR amplified product.

2.4. Effect of PCR mastermixes and of additives on the efficiency of LC qPCR

LightCycler PCR mastermixes manufactured by Roche, BioGene, Qiagen and ABGene, as well as an in-house laboratory modified Platinum Taq (Invitrogen) mix were compared. They are detailed in Table 2a. Each mix required a different magnesium chloride concentration that was determined in separate experiments. Amplifications were performed using a 3–6 mM range of magnesium chloride and the highest copy dilution (200 copies) used for our plasmid standard curve. The conditions that were used are shown in Table 1b. An extension time of 1 s was allowed for each 25 bp of PCR product being generated. Several compounds have been reported to enhance amplification of "difficult" target sequences as shown in Table 3a. The effect of each on LC-qPCR was evaluated. Also studied as potential substitutes for BSA were high-density lipoprotein (HDL), RNase A, glutathione, poly-lysine, poly-glutamic acid, poly-alanine, lysine, glycine and urea. The addition of each of these compounds reflects our attempts to deal with an intermittent problem; the formation of a precipitate in the glass capillary tube as the LC-qPCR progressed. Its appearance is probably due to the denaturation of BSA. A heat stable protein, peptide or amino-acid were candidate alternatives to BSA. RNase A was chosen because it is a heat stable protein, glutathione because it is representative of small peptides, and lysine and glycine because they are amino acids.

Table 2a
PCR mastermixes used for LC-qPCR

Mastermix	Supplied as	Final $MgCl_2$ used (mM)	Activation at 95 °C (min)	Other additions to the mix
Roche	10× mastermix with 1 mM $MgCl_2$	3–4	none	none
Roche Hotstart	10× mastermix with 1 mM $MgCl_2$	3–4	10	none
BioGene	2× mastermix with variable $MgCl_2$	3–4	none	Sybr Gold
BioGene Hotstart	2× mastermix with variable $MgCl_2$	3–4	3	Sybr Gold
Qiagen	2× mastermix with 1.5 mM $MgCl_2$	4–5	none	+250 ng/μl BSA, Sybr Gold
Qiagen HotStar	2× mastermix with 1.5 mM $MgCl_2$	4–5	15	+250 ng/μl BSA, Sybr Gold
AB Gene	2× mastermix with 3 mM $MgCl_2$	3–4	none	+250 ng/μl BSA, Sybr Gold
Platinum Taq in-house	10× buffer	3–4	3	1× buffer, 200–250 ng/μl BSA, HDL or RNase, 200 μM dNTPs, Sybr Gold, 0.8 units Taq

2.5. Application of cycle limited nested PCR for LC-qPCR

The current recommended limit of input DNA in Sybr Green based LC-qPCR is 50 ng. This corresponds to the DNA extracted from ~8000 human cells. The maximum volume of target DNA that can be incorporated in a 20-μl glass capillary PCR mix is 10 μl using the 2× BioGene or Qiagen PCR mastermixes. Roche also do not recommend exceeding this volume of DNA in a capillary. As many intracellular microorganisms persist in tissues at a frequency of less than one organism per 8000 host cells, this means that reliable LC-qPCR of the total DNA extracted is not realistically feasible for these pathogens using Sybr Gold detection. In the case of episomal HIV-1 DNA, it was possible to concentrate the extrachromosomal DNA before performing the LC-qPCR amplification. However, when the target DNA could not be easily separated from host chromosomal DNA, it has been possible to devise an alternative amplification strategy that was based upon cycle limited, nested PCR.

The first (outer) nested PCR amplification was performed in a conventional Perkin Elmer 9600 machine using a 50-μl standard PCR mix (Plat Taq, BioGene, Qiagen or Roche). Using this approach, it was possible to load 500 ng of target DNA compared to the 50 ng of DNA used in the LightCycler with Sybr Gold detection. We applied this methodology to the episomal HIV DNA whilst it was still present in a high background of genomic DNA (500 ng). The outer primers used were RCir3 and U3antisense (Table 1a) with the following amplification conditions: denaturation for 3 min at 95 °C, followed by 16 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. The small number of cycles used was to ensure that the target DNA was amplified without ever reaching the plateau phase of product formation. A 2-μl aliquot of this amplification mixture was then transferred to a LightCycler glass capillary tube containing 18 μl of the PCR mix and an inner pair of primers. LC-qPCR amplification was then performed as previously described. Standard PCR precautions were taken at all times to prevent carry over.

It was also important to determine whether the inclusion of large amount of non-target DNA in the conventional PCR amplification reduced the efficiency of the subsequent LC-qPCR. Consequently, in further experiments, purified HIV-1 2-LTR circles (of unknown value) as well as the plasmid quantification standards (starting at 200 copies) were serially diluted in water and amplified in 50 μl of a conventional PCR mix containing 300 ng of calf thymus DNA. After 16 cycles of amplification, 2 μl of the amplified product was transferred to a glass capillary tube for a further round of amplification in the LightCycler. For the purpose of generating equivalent standard curves, the unknown purified HIV-1 2-LTR circles were given a "normalised" nominal value of 200 copies. Standard curves were then constructed using the LightCycler software (manual "fit points" method) of the fluorescence threshold cross-over values against the log of the normalised target or quantification standards. The gradient of each of the standard curves was then calculated. This provided an absolute measure of the amplification efficiency for

each target. The relative efficiency of amplification of the target compared to that of the quantification standard was then determined by dividing the gradient of the target dilutional standard curve by that of the quantification standard curve.

3. Results

A large number of variables were found to influence our ability to perform efficient and accurate LC-qPCR. The cause of each problem identified and the solutions found are described below.

3.1. Primer design

There was considerable variation in the calculated primer melting temperature depending upon the method used to determine it. The basic melting temperature calculation on the Promega web site and that used on the Sigma-Genosys website (nearest neighbour analysis) gave a melting point of 55 °C for M669 and a melting point of 57 °C for M847. The base stacking melting temperature method gave a value of 70 °C for M669 and a value of 72 °C for M847. When experiments were performed, it became clear that there was considerable variation in the melting point temperature, which depended upon the PCR buffer being used. All of the nucleotide primers were therefore sequentially hybridized to their complementary sequences in each of the PCR buffers. Using this method, the melting point of the M669 primer was 69 °C and that of the M847 primer was 67 °C in the BioGene, Roche, Qiagen HotStar, and the Platinum Taq mixes. However, they were only 64 and 62 °C for M669 and M847, respectively in the new Qiagen Quantitect Sybr Green PCR kit. Failure to adjust for this difference of 5 °C in the melting temperature resulted in failure of the LC-qPCR amplification.

There was no discernable difference in the amplification efficiency of LC-qPCR when using either HPLC purified or standard desalted primers. The optimal magnesium chloride concentration also varied considerably for each PCR buffer as shown in Table 2a. In our case, the optimal magnesium concentration was 4–5 mM when using the Qiagen buffers, and 3–4 mM when using all other buffers.

3.2. Effect of different PCR mastermixes available commercially for the LightCycler

All of the commercially available PCR master mixes tested required 3–5 mM magnesium chloride for efficient amplification. In all cases, the ‘‘HotStart’’ versions of these PCR mixes were superior to the unmodified master mixes. They were therefore used routinely. The inclusion of Sybr Gold at a higher concentration than the one recommended by the supplier was detrimental to the efficiency of amplification. The Roche HotStart, BioGene HotStart and AB Gene mastermixes are specifically designed for use in the glass capillary system of the LightCycler, and they contain a Tris buffer, magnesium chloride and BSA. No sodium or potassium chloride is present. The AB gene buffer contains only 50 ng/μl BSA and requires supplementation with BSA (250 ng/μl) for efficient LC-qPCR amplification. The Qiagen HotStar buffer is a Tris-based ammonium sulphate, potassium chloride PCR buffer that also required supplementation with 250 ng/μl BSA, and 4–5 mM magnesium chloride for efficient LC-qPCR amplification.

The results of three different standard curves generated by amplification of 13 up to 200 copies of the plasmid standards using the Qiagen HotStar, Biogene HotStart and the Roche HotStart master mixes are shown in Fig. 1. Whilst amplification at the upper end of the standard curve was consistent, it was necessary to increase the number of low target copy number standards (i.e., 13 and 25 copy standards) that were amplified from a single to duplicate samples. This reflects the well-recognized problem of increased variability in target DNA sampling when very low copy target is present. Once corrections for each of these variables had been made, all of the PCR buffers used showed a good correlation between the input target copy number and the calculated theoretical value for that standard (r^2=0.94–1.0 (range)). The coefficients of variation of the plasmid standards for all four or the PCR mixes used are shown in Table 2b. They ranged from 8.9% for the 200-circle copy standard to 24.4% for the 13-copy standard.

Efficient amplification was also possible using a modified Platinum Taq based mix following the addition of extra magnesium (3–4 mM) and a blocking agent to reduce abstraction of PCR reagents to the glass capillary tube. BSA (250 ng/μl), HDL (250 ng/μl) and

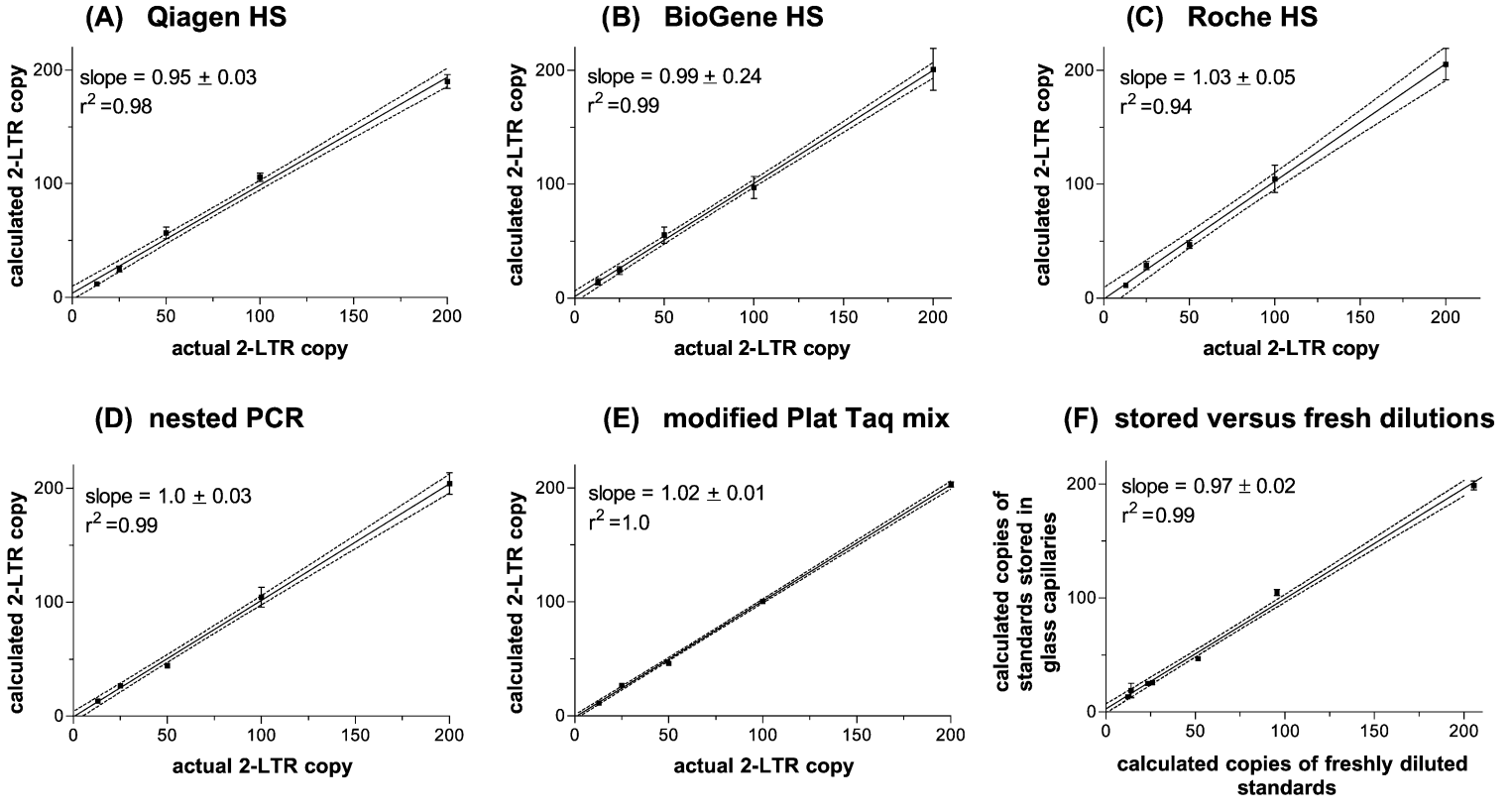

Fig. 1. Amplification of a dilutional series of HIV-1 2-LTR plasmid DNA standards was performed using several different PCR mixes (panels A–E). The measured HIV-1 2-LTR circle copy number used was plotted against the input (actual) number of copies. The dotted lines are the 95% confidence limits. Panel F demonstrates the effect of long-term storage (1 month) of the standards in appropriately treated glass capillary tubes. The Roche and BioGene buffer are supplied with a blocking agent. BSA was added to the Qiagen HotStar mix and HDL and RNase were added to the Platinum Taq mixes.

Table 2b
Coefficients of variation for the plasmid standards

Copies of plasmid	Mean±SEM	Coefficient variation (%)
200	199±4	8.9
100	102±4	15.7
50	51±2	17.2
25	26±1	21.7
13	12±1	24.4

n=20 experiments.

RNase (200 ng/μl) were found to be equally effective as dynamic blocking agents as described below.

3.3. Abstraction of PCR reagents and DNA onto glass surfaces

We have previously reported on the problem of abstraction of PCR reagents on glass surfaces when performing PCR in-situ on glass slides as well as within glass capillary microscope slide-chambers (Teo and Shaunak, 1995a,b). Target DNA, Taq polymerase and magnesium chloride are all abstracted by glass (Rasmussen, 1992; Al-Soud and Radstrom, 2001). Addition of BSA to the PCR master mix reduces the problem but does not prevent it completely. However, repeated cycles of heating and cooling denature the protein and can occasionally lead to the formation of a visible precipitate in the tube. Heat stable alternatives to BSA were therefore sought.

The addition of glutathione, poly-L-lysine, poly-aspartic acid, glycine or lysine at different concentrations did not block the adsorption of PCR reagents onto glass. Although we have previously shown that lecithin effectively blocks the abstraction of reagents onto glass surfaces when performing PCR in-situ (Teo and Shaunak, 1995a,b), HDL was selected for the studies described here because it combines the blocking effects of lipid and proteins and is a more stable alternative for long-term storage. At a concentration of 250 ng/μl, HDL was a good blocking agent. Heat stable RNase A (200 ng/μl) also reduced the abstraction of reagents by glass.

In further experiments, the effect of precoating the glass capillary tubes with each of the blocking agents prior to performing the PCR amplification was investigated. Capillaries were filled with 30 μl of each blocking agent (i.e., 2.5 mg/ml BSA, 2.5 mg/ml HDL, or 2 mg/ml RNase) and then incubated for 15 min, or overnight at room temperature. The blocking agent was removed by inverting the capillaries and centrifuging out the contents into microfuge tubes; 2000 rpm in a microfuge for 10 s. PCR amplification was then performed on the same day using a "homemade" PCR mix that did not contain any additional blocking agent. All three additives effectively blocked the abstraction of the PCR reagents onto the glass capillary tubes. A blocking time of 15 min was as effective as one of 24 h. Plasmid standards which had been stored in these tubes for 2 months at 4 °C were accurately amplified by LC-qPCR when compared with standards that had been prepared on the day of amplification (r^2=0.99).

3.4. The effects of additives on LightCycler qPCR

Additives that have been reported to enhance PCR reactions were individually evaluated for their effect on LC-qPCR. The results are summarized in Tables 3a and 3b. The addition of anti-Taq antibodies was beneficial because it reduced the frequency with which primer dimers formed in all the LC-qPCR master mixes tested. Consequently, either antibody-containing master mixes (BioGene and modified Platinum Taq) or chemically modified "HotStart" mixes (Roche and Qiagen) were used in subsequent experiments. The addition of transfer Ribonucleic Acid (tRNA) at a concentration of 5–100 ng/reaction reduced primer–dimer formation confirming the findings of Sturzenbaum (1999). However, the inclusion of RNase during the DNA extraction protocol precludes its general use. Surprisingly, the inclusion of RNase as a glass blocking agent also had the advantage that it significantly reduced the generation of primer–dimer artefacts when annealing was performed at a lower temperature (Fig. 2).

The addition of the PCR additives acetamide and betaine which have been used to amplify G–C rich sequences was found to be inhibitory for all of the LC-qPCR mixes tested. Ficoll, polyvinylpyrrolidone (PVP), trehalose and glycerol were neutral in their effects and they can therefore be included in the LC-qPCR mix for the uses specified in Table 3a. In some DNA extraction protocols, the precipitation of very small amounts of target DNA can be enhanced by the inclusion of additives such as "SeeDNA", blue dex-

Table 3a
Additives that have a positive or a neutral effect on LC-qPCR amplification

Additive	Source	Concentration	Mastermix	Effect	Comment
BSA (non-acetylated)	Sigma	250 ng/μl	Qiagen HotStar, AB Gene	++	Blocking agent necessary for amplification.
HDL	Sigma	250 ng/μl	Qiagen Hot Star, AB Gene	++	Blocking agent. Alternative to BSA.
RNase	Sigma	200 ng/μl	Qiagen HotStar, in-house buffer	++	Blocking agent. Heat stable alternative for BSA. Reduces primer–dimer formation.
Anti-Taq antibody	Sigma, CloneTech	0.16 μl /reaction	BioGene, Roche, AB Gene, Qiagen	++	Reduces primer–dimer formation.
tRNA	Sigma	50 pg/reaction	BioGene	+	Reduces primer–dimer formation.
DNA herring sperm	Promega	0–250 ng/reaction	BioGene, Roche	neutral/–	Used for diluting standards. No effect at < 50 ng. Deleterious effect at > 50 ng when using Sybr Gold.
Poly A	Sigma	500 ng/reaction	BioGene, Qiagen HotStar	neutral	Used for dilution of standards.
MS2RNA	Roche	0–50 ng/reaction	Roche	neutral	Used for dilution of standards.
Trehalose	Sigma	0.1 M	BioGene	neutral	Enzyme thermal stabilizer.
Ficoll	Pharmacia	1%	BioGene	neutral/+	Hybridization enhancer. Present in DNA gel sample loading buffer.
PVP	Sigma	1%	BioGene	neutral	Neutralizes the effects of phenolic contamination. PCR enhancer.
Glycerol	Sigma	4–8%	BioGene, homemade buffer	neutral	Used as an enzyme preservative. Reduces melting temperature.
Acetamide	Sigma	5%	BioGene, Qiagen HotStar	neutral/–	Used to minimise differences in primer melting temperature.
TMAC	Sigma	2 mM–20 mM	BioGene	neutral	PCR enhancer.
Agarose LGT SeaKem	FMC	0.05–0.2%	BioGene	neutral/+	Samples amplified from gel slices. Can reduce primer–dimer formation.

Key: ++=strong positive effect, +=positive effect, –=negative effect.

tran'' or acrylamide. All of these co-precipitants were found to be detrimental to the efficiency of LC-qPCR (Table 3b). Furthermore, although ''SeeDNA'' did not interfere with Sybr Gold detection of the LC-qPCR amplified products, it did interfere with the use of fluorescent probes such as Flourescein-LC640.

Table 3b
Additives that have an inhibitory effect on LC-qPCR amplification

Additive	Source	Concentration	Mastermix	Effect	Comment
Betaine	Sigma	0.2–1.6 M	BioGene	–	PCR enhancer.
SeeDNA	Amersham Pharmacia	1 μl per 50 μl precipitation	BioGene	neutral/–	DNA precipitant. Interferes with fluorescent hybridization.
Acrylamide	National Diagnostics	0.1%	BioGene	–	Used as a DNA precipitant.
Phosphate	Sigma	0.1 mM	BioGene	–	Present in buffers.
Glutathione	Sigma	250 ng/μl	BioGene	–	Potential substitute for BSA in buffer.
Lysine	Sigma	250 ng/μl	BioGene	–	Potential substitute for BSA in buffer.
Glycine	Sigma	250 ng/μl	BioGene	–	Potential substitute for BSA in buffer.
Poly-lysine	Sigma	250 ng/μl	Platinum Taq in-house	–	Potential substitute for BSA in buffer.
Poly-aspartic acid	Sigma	250 ng/μl	Platinum Taq in-house	–	Potential substitute for BSA in buffer.
Histone 2B	Roche	25–200 ng/μl	Platinum Taq in-house	–	DNA binding protein.

Key: –=negative effect.

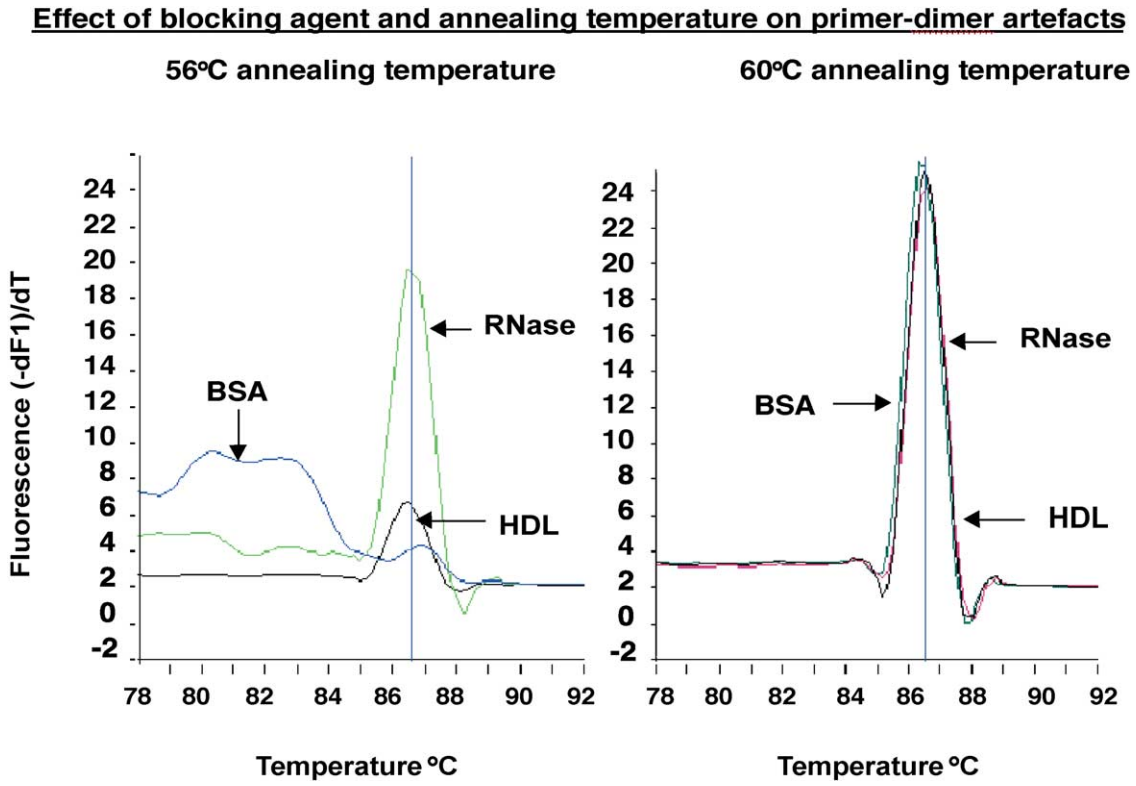

Fig. 2. The effects of different blocking agents on primer–dimer formation during amplification of 13 copies of target DNA at different temperatures. For the purpose of clarity, the scales of the graphs have been redrawn. The inclusion of RNase or HDL in the Platinum Taq PCR mix enables amplification of specific products at lower annealing temperatures than is possible with BSA.

The dilution of the DNA standards in a carrier DNA resulted in a significantly higher basal fluorescence when more than 50 ng of exogenous DNA was present. It may, in part, reflect the incomplete denaturation of carrier DNA that is a consequence of the very short denaturation times used by the LightCycler. MS-2 RNA did not give rise to this artifact because it is a single stranded polynucleotide, but it is sensitive to RNase. As an RNase resistant alternative to MS-2 RNA, single stranded Poly dA can be used as a diluent at concentrations of up to 250 ng/μl without it interfering with the PCR amplification.

3.5. Amplification of low copy number target DNA from cells

In conventional PCR, the problem of low copy number target DNA in the sample is usually circumvented by increasing the input of total DNA. Up to 1 μg of DNA can be added to a conventional PCR reaction without compromising its efficiency. However, if the LightCycler is used with Sybr Gold as the method of detection, this leads to a higher level of basal fluorescence which, in turn, masks the increase in fluorescence that is generated by the amplification of the target DNA. In the model system described here, the low copy number target DNA was present in an episomal form. DNA extraction procedures were therefore devised which eliminated chromosomal, non-target DNA whilst retaining the extrachromosomal episomal target. This is described in detail in the next paper. Such target enrichment procedures are more difficult to devise if the target is within genomic DNA, and this will require the use of hybrid capture techniques.

A low copy number of target DNA is associated with other problems. Primer–dimer formation and non-specific products of amplification are more read-

ily generated. These amplification artefacts will then compete with the "real" target DNA sequences for the available PCR reagents and will lead to anomalous quantification values. Unless due care and attention is exercised, this can become a serious problem.

Cycle-limited, nested PCR using a combination of conventional PCR and LC-qPCR enabled the detection of low copy number target DNA (plasmid standards and patient samples) when it was present in a high background of non-target DNA. The first round PCR amplification allowed sufficient product to be generated from the low copy number target DNA for quantitative detection using the LightCycler (r^2=0.99, Fig. 1, panel D). Using this approach, primer dimers and/or artifacts were not detected. The relative efficiency of amplification of the target DNA versus that of the quantification standards used was 0.99 in this two round PCR amplification (Fig. 3). Addition of 500 ng of non-target DNA to the low copy number standards (i.e., 25 and 13 copies of target DNA) had no adverse effect on the PCR amplification of either standard. Provided suitable care was taken during the transfer of the first round amplification products to the glass capillary tubes used for the LC-qPCR, and all reaction vessels were then kept closed, carry-over did not occur.

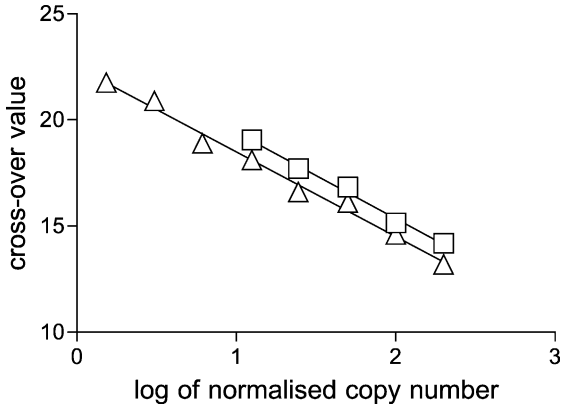

Fig. 3. The effect of adding calf thymus DNA on the efficiency of amplification of extracted circles and of plasmid standards in a nested PCR amplification. An amount of 300 ng of calf thymus DNA was added to the samples at the start of the first round of PCR (17 cycles). A 2 μl aliquot of the product was then removed for the secondary amplification in the LightCycler. The open triangles correspond to the extracted circles. The open squares correspond to the plasmid standards. The dilutional series of extracted circle samples has been normalised so that it is equivalent to the plasmid standards. The slope for extracted circles was -4.01 ± 0.16; r^2=0.99. The slope for the plasmid standards was -4.06 ± 0.16; r^2=0.99.

4. Discussion

The major outstanding problem for PCR based assays is the accurate quantification of amplified target DNA. With conventional technology, the minimum difference in amplified target DNA concentrations that are regarded as being significant in clinical assays (e.g., diagnostic and therapeautic monitoring) is 0.5 log units. This has hampered the effective use of DNA PCR quantification in many clinical situations where accurate, reliable and reproducible quantification is critical. The LightCycler offers the potential of PCR amplification with a degree of quantitative accuracy that exceeds that of enzyme immunoassay with data analysis of both product and artefact generation provided in real-time.

In our case, this technique was applied to the amplification of low copy number target DNA from HIV-1 infected samples. Our experience has shown that standard LightCycler methods on <50 ng total DNA are accurate when >20 copies of target DNA are present or in cases where there is one copy of target DNA per cell. However, problems arise when the target DNA copy number being amplified is low. Typically this is seen when the technology is applied to the detection of infectious pathogens and the DNA has been extracted from human or animal tissues. At the high amplification cycle numbers needed for detection of low target copy number DNA, the competitive formation of primer dimers occurs readily and can inhibit target DNA amplification. In such circumstances, protocols associated with sample preparation and LightCycler amplification have to be amended.

The major difference between conventional PCR (as performed using the Perkin-Elmer 9600 machine) and the LightCycler is the format of the vessel in which the amplification of DNA is performed. It has changed from a polypropylene plastic vessel to a glass capillary tube. This allows for very rapid cycling times. However, it has also led to a set of problems that are not encountered or detectable in conventional PCR. Almost all of the cycling conditions have to be recalibrated. When annealing and extension times are

very short, using the correct annealing temperature is essential in order to reduce mis-priming and promote efficient amplification of the target DNA. We have noted a wide discrepancy (up to 10 °C) between calculated primer melting temperatures and those obtained by a melting point analysis of primers hybridised to their complementary sequences. In part, this is because the less sophisticated primer analysis software programs often do not take the salt and the magnesium ion concentration into account. The calculated temperatures should be used as a rough guide only, although the most recent primer design software available from Roche tries to address this problem. Complementary primers should therefore be synthesised, annealed to the amplification primers, and their exact melting temperature determined in the PCR amplification buffer that will be used. The benefits far outweigh the modest extra cost and time involved.

Using HPLC purified primers did not lead to more consistent or reliable PCR amplification than could be achieved with standard, desalted primers. The reported artefacts using non-HPLC purified primers probably relate to poor desalting and they are less likely to arise if an ammonium sulphate containing buffer system (e.g., Qiagen HotStar) is used. In addition, the use of primers that have been modified to include sequence specific –AA at their 3′ termini can reduce artefactual amplification.

Some of the other difficulties that have been encountered with LC-qPCR relate to the modified Taq polymerase buffer that is being used. Firstly, unmodified conventional mixes used in block thermocyclers do not work in the LightCycler. All of the commercial LC-qPCR buffers tried worked, but they all required further optimisation of their magnesium chloride and BSA concentrations. A more detailed analysis of the core chemistry that affects PCR amplification efficiency was not possible because the exact formulations of the different mixes remain proprietary information. In addition, a modified Qiagen HotStar mix and an in-house mix based upon Platinum Taq polymerase were also evaluated. Both mixes worked well and were considerably cheaper than the other commercial mixes available. With the modifications discussed below, we were able to get consistent amplification over the range of 13–200 copies of target DNA with coefficients of variation that ranged from 8.9% (200 copy standard) to 24.4% (13 copy standard).

Most commercially available LC-qPCR mastermixes do not contain sodium or potassium chloride because their presence inhibits LC-qPCR (Wittwer, 1991) or leads to a substantial increase in PCR primer–dimer formation and to the generation of artefacts during the PCR amplification. The presence of potassium chloride in the in-house Platinum Taq mix probably contributes to the excessive formation of primer–dimers at low, sub-optimal annealing temperatures as shown in Fig. 2. Interestingly, this was not seen with the Qiagen buffer, probably because ammonium sulphate counteracts the effect of potassium chloride, by destabilising the weak hydrogen bonds that form when mismatched primer binding occurs (Qiagen PCR handbook). However, it should be noted that *Thermus aquaticus* from which Taq DNA polymerase is isolated, is found in a high temperature, high salt, ion rich environment. Taq polymerase is therefore adapted to work under conditions of relatively high salt such as those typically used in a conventional PCR amplification. In our hands, some primer pairs and PCR mixes that performed well in conventional PCR machines gave poor PCR amplification in the LightCycler. We were able to get reliable and reproducible LC-qPCR at low copy number target DNA using a BSA-supplemented Qiagen HotStar PCR mix which contained Tris, ammonium sulphate and potassium chloride. The combination of ammonium sulphate and potassium chloride also reduced the dependency of the LC-qPCR on a narrow magnesium concentration range. It had the added advantage that the buffer could also be used to perform a limited number of restriction enzyme and DNase digests prior to the LC-qPCRs. This is described in detail in the next paper.

A significant problem that emerged with the use of glass capillary tubes as the amplification vessel in the LightCycler was the abstraction of PCR reagents and of DNA by the glass. This problem was recognised when PCR in-situ was performed on glass capillary microscope slide chambers (Teo and Shaunak, 1995a,b), in capillary PCR (Rasmussen, 1992; Wittwer et al. 1997a,b; Morrison et al., 1998) and has been reported with flow through microchip PCR (Shoffner et al., 1996; Cheng et al., 1996; Kopp et al., 1998; Giordano et al., 2001). In the case of PCR in-situ, we found that the problem was reduced by the use of phospholipids such as lysolecithin in PCR mixes. The phosphatidylcholine moiety in lysolecithin

binds to glass through an ionic interaction, eliminating the charged polar surface and replacing it with a hydrophobic surface because of the alkyl side chains. This hydrophobicity reduces the adsorption by glass. Dialkyl lipids (i.e., lecithins) behave in a similar but less effective manner. With microchip PCR amplification, other solutions have included pre-treatment of the glass surface with sulphuric acid and hydrogen peroxide (to remove organic and metal ion contamination) followed by silanisation and coating with reagents such as poly-L-lysine, polyadenylic acid, polyvinylpyrrolidone and PEG 800 (Shoffner et al., 1996; Kopp et al., 1998; Giordano et al., 2001; Lee et al., 2000). Silanization is a laborious and tedious process that cannot be easily applied to the glass capillary tubes used in the LightCycler. Experiments were performed in which glass capillary tubes were filled with 30 μl of dimethyl-dichlorosilane, left for 10 min at room temperature, inverted, centrifuged and then baked at 70 °C for 1 h. This did not reduce the requirement for a blocking agent in the subsequent PCR amplification (data not shown).

DNA, Taq polymerase enzyme and magnesium ions are thought to bind strongly to certain types of glass and silica, particularly sodium glass. This can be reduced by the use of no-salt buffers. The formation of primer–dimers and other mispriming artefacts is exacerbated with a higher concentration of the primers. In many of our PCR amplifications, the target DNA copy number is very low and there is a large excess of primer and non-target DNA. Abstraction may cause high local concentrations of these reagents on the surface of the glass that facilitates anomalous PCR amplification. In our experiments, the use of good glass blocking agents coupled with "Hot Start" methods based upon the use of either anti-Taq antibodies or a chemically modified Taq polymerase was the most effective way to reduce these glass surface related problems. The antibody based approach had the advantage that the recommended activation times is only 1–3 min compared to the 15 min required for chemically modified Taq polymerase. This significantly shortens the overall PCR amplification time and can be useful when a LightCycler is used in a multi-user clinical diagnostic pathology context.

"Hot Start" methods reduce but do not eliminate the problem of anomalous PCR amplification. This suggests that mis-priming can occur during each cycle of the PCR amplification, or that there is a gradual and progressive reduction in the glass blocking ability of BSA. High-density lipoprotein was evaluated as a more stable alternative to lysolecithin because it is readily soluble in water at high concentrations, and it can replace BSA in the LC-qPCR mix. High-density lipoprotein was effective at blocking abstraction of reagents onto glass at a concentration of 125–500 ng/μl in the absence of BSA. Fig. 2 illustrates the specificity of the products generated when LC-qPCR was performed in the presence of HDL using 13 copies of target DNA. In the case of LC-qPCR, our data indicates that HDL as well as RNase is effective. They can be used to pre-coat the capillaries or they can be used as dynamic blocking agents. Successful amplification of the target after pre-coating of the capillary tubes suggested that the blocking was effective throughout all of the cycles of the PCR amplification.

Surprisingly, the use of RNase as a blocking agent reduced the incidence of primer–dimer formation compared to BSA when it was used in conjunction with Platinum Taq buffer at sub-optimal annealing temperatures. As well as being more heat stable, it had the potential to allow specific amplification to occur over a wider range of annealing temperatures. The mechanism for reducing primer–dimer formation is not known, but it could involve the binding of RNase to the single stranded DNA primers. Scrambled RNase, which is functionally inactive or hydrogen peroxide inactivated RNase did not possess the same activity. Primer–dimer formation was also reduced by the addition of tRNA.

A number of other PCR additives that were potentially useful for enhancing PCR amplification or for long-term storage of LightCycler PCR reagents were tested and shown to be compatible with LightCycler PCR mixes. For instance, glycerol can be used to stabilise PCR reagents during long-term storage (Lee et al., 1999). Trehalose confers thermal stability and prevents heat induced protein aggregation of *Eco*RI and reverse transcriptase (Hottiger et al., 1994; Carninci et al., 1998). Glycerol and tetramethyl-ammonium chloride (TMAC) are useful when the amplified sequences have a high G–C content because they alter their melting characteristics (Williams, 1989; Lu and Negre, 1993; Kovarova and Draber, 2000). PVP has been used as a glass passivation/blocking agent to

reduce the interactions of glass microchips with PCR reagents (Giordano et al., 2001) and to reduce the inhibitory effect of polyphenolic contaminants (Koonjul et al., 1999). These reagents can therefore be used selectively in LC-qPCR.

When performing LC-qPCR for low copy number target DNA, as is typically the case with pathogens in human or animal tissues, a major limitation of the system is the amount of DNA that can be loaded into the PCR reaction tube. In our experience, the addition of >50 ng of DNA per capillary tube leads to an unacceptably high baseline level of fluorescence and to less efficient PCR amplification of the target. As quantification is based upon back extrapolation of the amplification curves to generate a fluorescence threshold cross-over value, these deviations have a detrimental effect. Two approaches were tried to circumvent this problem. The first involved a limited nested PCR approach. The second required the removal of most of the non-target high molecular weight DNA from the sample and is the subject of the accompanying paper.

When performing cycle limited, nested PCR, the relative amplification efficiency of the target DNA and of the quantification standards was almost identical. Inclusion of total sample DNA up to 500 ng in the first polypropylene tube based PCR was not detrimental to the amplification efficiency of the subsequent LightCycler based PCR. This is because only a small proportion of the first round PCR containing amplified target was required for the second round LightCycler PCR amplification. As a general principle, quantifying a sample with a relatively high DNA target copy number (>50 copies) was more reliably achieved by performing several serial dilutions and amplifications of the sample than by simply performing multiple replicates of a single dilution of the sample. This has the added advantage that the amplification efficiency and its linearity can be checked over a range of concentrations. The amplification profiles generated by cycle limited nested PCR have low basal fluorescence and minimal primer–dimer formation. This approach also confers a specificity that can otherwise only be achieved by the use of expensive hybridization probes. Furthermore, as the first PCR is performed in a conventional PCR machine with conventional PCR mixes, 96 samples can be amplified simultaneously. One set of eight standards can therefore be used to quantitate all of the other 88 samples. The LightCycler 3.5 software has the facility to import a reference standard curve that can be used to calibrate an internal reference standard. Using this approach, it is possible to quantify many more samples in a single run.

In conclusion, LC-qPCR is a powerful and increasingly widespread technique that requires significant modification of currently used protocols in order to achieve reliable and reproducible quantification of low copy number target DNA. Our observations will have particular relevance when applying this technology in a clinically relevant context to the study of pathogens in human or animal tissue samples.

Note added in proof

Since the completion of these sets of experiments we have noted that in some instances increasing the concentration of BSA up to 600ng/μl can improve the amplification of target DNA in the LightCycler when using modified mixes e.g. Qiagen HotStar.

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