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Research Paper

Real-time RT-PCR: considerations for efficient and sensitive assay design

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Abstract

Real-time RT-PCR has been recognised as an accurate and sensitive method of quantifying mRNA transcripts. Absence of post amplification procedures allows rapid analysis with a greater sample throughput, yet with less risk of amplicon carry-over as reaction tubes are not opened. In order to maximise sensitivity, careful reaction design and optimisation is essential. Several aspects of assay design for real-time RT-PCR are discussed in this paper.

We demonstrate the effect of amplicon secondary structure on reaction efficiency and its importance for primer design. Taq-man probes with a deoxyguanosine base at the 5′ end fluoresce weakly when labelled with FAM, although weak fluorescence is not a problem when probes are labelled with Texas Red. DNA contamination of RNA samples purified using silica membrane columns is a significant problem but DNase digestion can be used to reduce this, particularly in-solution. MMLV and AMV enzyme systems using a variety of RT priming methods are compared and the problem of primer–dimer formation associated with RT enzymes is described.

Keywords: Canine; Real-time RT-PCR; Primer–dimers; Reverse transcription; Genomic contamination; Taq-man probes

1. Introduction

Real-time RT-PCR has been recognised as an accurate and sensitive method of quantifying mRNA transcripts (Bustin, 2000, 2002). The method allows the detection of amplicon accumulation since it is performed using fluorogenic probes or intercalating dyes such as SYBR Green I, rather than by conventional end-point analysis. As there is no need for post amplification procedures, e.g. gel electrophoresis or Southern blotting, analysis can be completed rapidly allowing a greater sample throughput. In addition there is less risk of amplicon carry-over as reaction tubes are not opened. Real-time measurement of amplicon accumulation also allows determination of reaction efficiency and thus permits the selection of more sensitive assays.

Intercalating dyes such as SYBR Green I only fluoresce intensely when associated with double

Abbreviations: AMV-RT, Avian myeloblastosis virus reverse transcriptase; gDNA, Genomic DNA; MMLV-RT, Moloney murine leukaemia virus reverse transcriptase; PCR, Polymerase chain reaction; RT, Reverse transcriptase; RT-PCR, Reverse transcriptase polymerase chain reaction.

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stranded DNA. Therefore, as double stranded PCR product accumulates, the level of fluorescence increases mirroring the template accumulation. The major drawback is that any double stranded product, including primer–dimers, will be detected and therefore false positives can occur. Intercalating dyes should therefore be avoided when targets are present at low copy number (Yin et al., 2001).

Fluorescent probes offer a more specific way of detecting the accumulation of amplicon, since these probes use sequence specific oligonucleotides coupled to fluorescent dyes. There are several types of probe available, including hydrolysis probes (Taq-Man Probes) (Livak et al., 1995; Heid et al., 1996), molecular beacons (Tyagi and Kramer, 1996; Fang et al., 2000) and scorpions (Whitcombe et al., 1999). Probes of all types are more expensive than reporter dyes but they allow sequence specific reporting, eliminating false positives due to non-specific product formation.

Critical to the successful application of real-time RT-PCR is the prevention of amplification of contaminating genomic DNA and resulting overestimation of the amount of RNA present. Genomic contamination is a significant problem when housekeeper genes such as G3PDH (Overbergh et al., 1999; Peters et al., 2003) and β-actin (Overbergh et al., 1999; Stordeur et al., 2002) are used in RT-PCR, as these genes are associated with the presence of multiple pseudogenes (Hanauer and Mandel, 1984; Ng et al., 1985). DNase digestion either during the purification step (Mena et al., 2002; Peters et al., 2003) or on the purified RNA (Leutenegger et al., 1999; Mena et al., 2002; Stordeur et al., 2002) has been employed to minimise genomic contamination but no attempt has been made to quantify the effect of such digestion on either the genomic DNA contamination or the RNA transcripts themselves.

This paper describes problems associated with successful design and optimisation of real-time RT-PCR reactions for application to clinical material. Topics considered and discussed here include the effects of secondary structure, 5′-deoxyguanosine quenching in Taq-man probes, minimisation of primer–dimers associated with RT, sensitivity of different RT protocols and minimisation of gDNA contamination of RNA by the use of DNase.

2. Materials and methods

2.1. Sample collection

Endoscopic biopsies of duodenal mucosa were obtained from dogs presented to the School of Clinical Veterinary Science, University of Bristol for investigation of gastrointestinal disease. Gastroduodenoscopy was performed using a GIF-XQ230 flexible video endoscope (Olympus Keymed, Southend-on-Sea, UK). Multiple mucosal biopsies were taken at the level of the caudal duodenal flexure using FB-25K biopsy forceps (Olympus Keymed). Biopsies were placed in a 1.0-ml cryotube (NUNC, Fischer Scientific, Loughborough, Leicestershire), snap frozen in liquid nitrogen and stored at −70 °C.

2.2. Primer and probe design

The primers and hydrolysis probes (Taq-Man Probes) were designed using Primer 3 (Rozen and Skaletzky, 2000) (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) using the Genbank sequences for canine α-chain, G3PDH, J-Chain and IL-18 (accession numbers:L36871, AB038240, AY081058 andY11133) (Table 1). The primer and probe sets were designed such that the annealing temperatures of the primers were 60 °C and the probes 8–10 °C higher with an 80–200 bp product. In order to minimise primer–dimer formation, the maximum self-complementarity score was 4 and the maximum 3′ self-complementarity score was 2.

The targets amplified by the primer pairs were characterised using M-fold (SantaLucia, 1998) http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi in order to predict the nature of any secondary structures which might form at the site of primer or probe binding. Primers were synthesised by Invitrogen (Paisley, Scotland) and the probes were synthesised by Cruachem (Glasgow, Scotland) or Eurogentech (IL-18) (Romsey, Hampshire, UK).

2.3. RNA isolation

Two endoscopic biopsies (total tissue mass 9–16 mg) were added to a green Ribolyser tube (Ribolyser System, Thermo-Hybaid, Ashford, Middlesex, UK) containing 1 ml of lysis buffer from the RNA isolation

Table 1
Primer and probe sequences

Target	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	5′-Fluorophore	Probe Sequence (5′–3′)	3′-Quencher	Product Size (bp)
G3PDH	TCAACGGATTTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	Hex	CAGGGCTGCTTTTAACTCTGGCAAAGTGGA	BHQ-1	90
α-chain	TGTGCCCTGCAAAGATAACA	AGGGCTGGCTTCTGTAGTGA	Texas Red	GTCATCCATGTCCCTCGTGCAATGAG	BHQ-2	84
J-Chain	TCTGATCCCACCTCACCAGT	TGCGAGGCAATAACTACTTGA	–	–	–	94
IL-18	TTAAAGCGGAAAGTGATGAAGG	TCGGGCATATCCTCAAATACA	FAM Texas Red	GAAATTTGAACGACCAAGTCCTCTTCG	BHQ-1 ELLE	139

This table shows the combinations of forward and reverse primers as well as the probe sets. All primers were desalted when purified and the probes were HPLC purified.

kit (see below) and processed for 45 s at 6.0 m/s to homogenise the biopsies. An aliquot of 200 μl of this lysate was processed using the SV Total RNA Isolation System (Promega) or the RNeasy Mini Kit (Qiagen, Crawley, UK). Samples were processed as per the manufacturer's protocol, except that the RNA was eluted with 2×50 μl of nuclease free water. A negative control of nuclease free water was included with all extractions. The concentration of RNA was calculated by measuring the absorbance of the pooled elution at $A_{260\ nm}$. The mean (range) concentration of RNA was 62 μg/ml (24.8–76.0 μg/ml).

2.4. DNase digestion

DNase digestion was carried out either on the extraction column using the DNase provided with the SV Total RNA Isolation System or using the RNase-Free DNase Set (Qiagen) both as per the manufacturer's protocol.

In-solution DNase digestion was carried out by eluting the RNA from the extraction column in 60 μl (2×30 μl) and treating it with six units of Amplification Grade DNase 1 (Invitrogen) as per the manufacturer's instructions. In order to remove residual DNase and EDTA from the treated RNA, the solution was passed through the RNeasy system for a second time using the RNA clean-up protocol. A second DNase digestion was carried out on this column using the RNase-Free DNase Set (Qiagen). For all DNase digestion steps no-DNase controls were performed by the addition of nuclease-free water in place of the DNase enzyme.

2.5. PCR

PCR was performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) or HotStarTaq Master Mix Kit (Qiagen) as per the manufacturer's instructions using 3 mM $MgCl_2$, 200 nM of each primer, 100 nM of probe or 1/50,000 SYBR Green 1 (Sigma-Aldrich, Poole, Dorset) and 5 μl of cDNA or purified sequenced PCR product in a final volume of 25 μl. All reactants were mixed together as a master mix and aliquotted into a 24 or 96-well PCR plate (Thermofast, Abgene, Epsom, Surrey) prior to addition of the 5 μl sample. A no template control of nuclease-free water was included in each run.

The PCR was performed in an iCycler iQ (Bio-Rad Laboratories, Hercules, CA) with an initial incubation of 95 °C for 15 min (Qiagen) or 5 min (Invitrogen), and then 45 cycles of 95 °C for 10s and 60 °C for 15 s during which the fluorescence data were collected. The threshold cycle (Ct value) was calculated as the cycle when the fluorescence of the sample exceeded a threshold level corresponding to 10 standard deviations from the mean of the baseline fluorescence.

A melt curve was produced by heating the samples from 75 to 95 °C in 0.2–0.5 °C increments with a dwell time at each temperature of 10 s during which the fluorescence data were collected. The melting temperatures of the products was determined with the iCycler iQ Optical System Software (version 3: Bio-Rad Laboratories) using a rate of change of fluorescence ($-$ d(RFU)/dT) vs. temperature graph.

2.6. Gene specific single-step RT-PCR

Gene specific single-step RT-PCR amplification was performed using the Platinum Quantitative RT-PCR Thermoscript One-Step System (Invitrogen) using 5 μl (mean: 0.31 μg) of RNA, 3 mM $MgCl_2$, 200 nM of primers and 100 nM of probe or (1/50,000) SYBR Green I (Sigma-Aldrich) in a final volume of 25 μl. No-RT reactions were made by substituting the Thermoscript/Platinum Taq enzyme mix with 2 units of Platinum Taq DNA Polymerase (Invitrogen). All reactions were made up on ice as a master mix prior to template addition and were then placed in the iCycler iQ held at 55 °C. RT was performed by incubation at 55 °C for 20 min, after which the same protocol as for PCR was followed.

This protocol was subsequently modified by omitting the addition of the forward primer to the mastermix. The 25 μl samples were incubated at 55 °C for 20 min, 85 °C for 5 min (to inactivate the RT enzyme) and then cooled to 5 °C. The plate was opened and 200 nM of the forward primer was added in RT buffer to increase the reaction volume to 30 μl and the reaction protocol was completed as before.

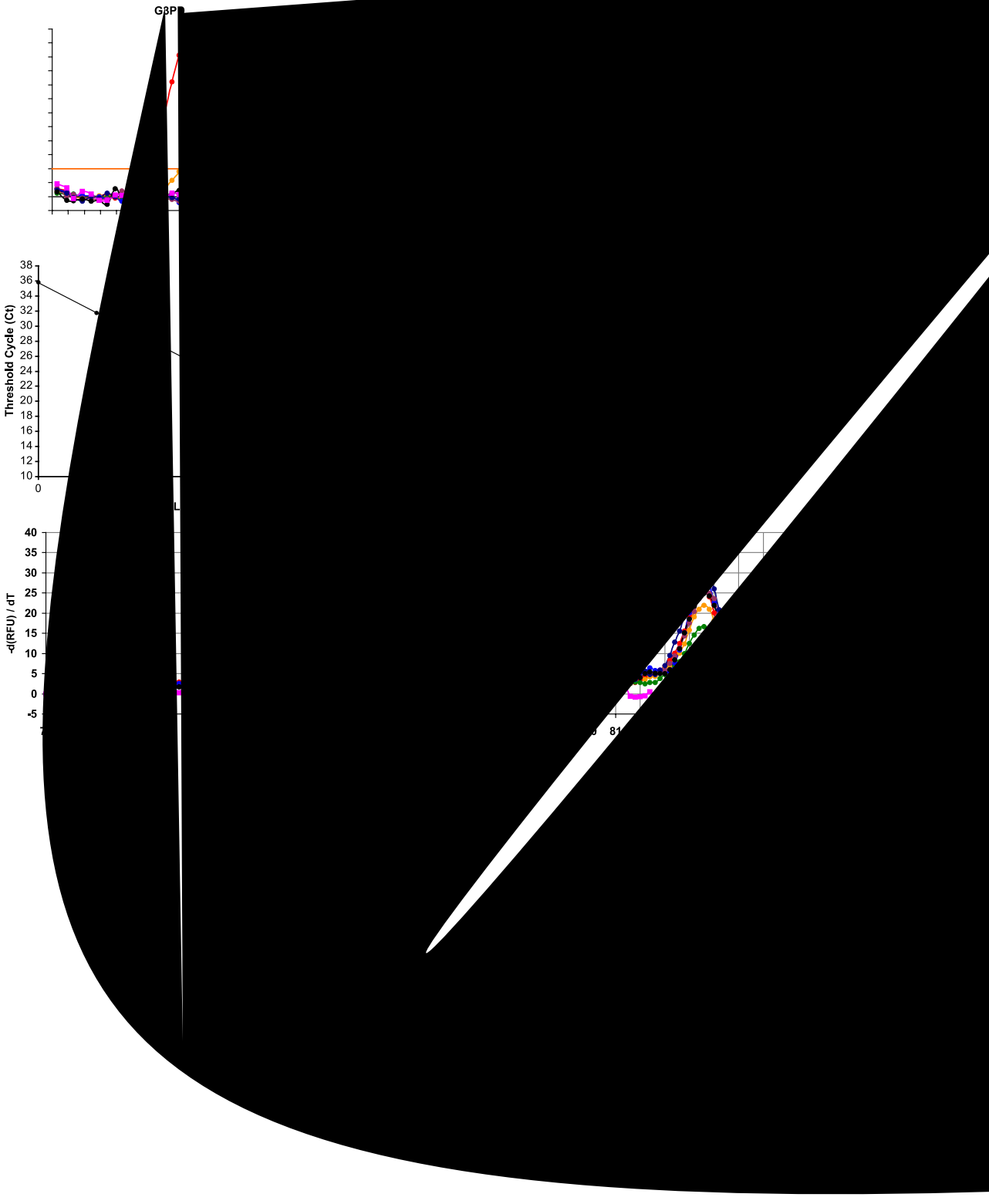
2.7. Two-step RT-PCR

First strand cDNA synthesis was carried out using either a gene specific reverse primer (200 nM), oligo dT primers (500 ng) or random primers (hexamer or decamer) (500 ng) using either the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), Platinum Quantitative RT-PCR Thermoscript One-Step System (Invitrogen), ImProm-II Reverse Transcription System (Promega) or Reverse-iT 1st Strand Synthesis Kit (ABgene, Epsom, Surrey) using 9 μl (mean: 0.56 μg) RNA in a final volume of 20 μl. All reactions were made up according to the manufacturer's instructions giving a final magnesium chloride concentration of 3 mM in the Superscript, Thermoscript and ImProm-II reactions and 1.5 mM in the Reverse-iT reactions.

Thermoscript gene specific and oligo dT cDNA synthesis was carried out by mixing all reactants together as a master mix on ice prior to template addition. Reaction tubes were cooled in an ice block prior to addition of the master mix, and then RNA was added. Reactions were placed in an MJ Research PTC-200 DNA engine (GRI) held at 50 °C (oligo dT) or 55 °C (gene-specific), at which temperature they were incubated for 30 min before heating to 85 °C for 5 min. Superscript oligo dT and gene-specific cDNA synthesis was carried out according to the protocol provided by the manufacturer.

Random hexamer (Thermoscript and Im-Prom-II) and random decamer (Reverse-iT) cDNA synthesis was carried by mixing 9 μl of RNA with the appropriate primer in a reaction tube. Samples were heated to 70 °C for 5 min in the PTC-200 DNA engine (GRI) before cooling to 4 °C for 5 min. Tubes were then placed in a cold block before addition of the remaining reaction components including the reverse transcriptase enzyme to make a total volume of 20 μl. Reverse transcription was then completed by heating the samples to 25 °C for 5 min, 47 °C (Thermoscript and Reverse-iT) or 42 °C (IM-Prom-II) for 30 min and finally 75 °C for 10 min in the PTC-200 DNA

Fig. 1. Effect of secondary structure on reaction efficiency. These reactions were performed using a 1/10 dilution of a sequenced purified PCR product for G3PDH using the Platinum Quantitative PCR system (Invitrogen) and SYBR Green I. Representative dilution series are shown for each primer set. The same reverse primer is used in each system but the original forward primer binds at the site of a predicted loop when the product is folded in m-fold (C). The original primer set produced a reaction which was only 75–80% efficient (A). Moving the forward primer by 14 bases such that the 3′-end is no longer in the loop (C) results in a 100% efficient reaction (B). Similar results were obtained using the Taqman probe (not shown).


G3P
Threshold Cycle (Ct)
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
0
-d(RFU) / dT
40
35
30
25
20
15
10
5
0
-5

engine (GRI). No-RT controls were performed by omitting addition of the reverse transcriptase enzyme, and no template controls were performed by addition of nuclease free water. All products were stored at − 20 °C for future use.

2.8. Purified and sequenced PCR products

Purified and sequenced PCR products were obtained for G3PDH and α-chain by gel purification of a PCR product (QIAquick PCR Purification Kit, Qiagen) which had been sequenced to check specificity. These products were diluted 1/1000 in nuclease free-water prior to addition to the reaction.

2.9. Standard curve production

Standard dilution curves (1/10 dilution) of RNA or PCR product were produced by dilution in nuclease-free water. A master mix was made up and aliquotted into the PCR plate prior to addition of the template into each reaction tube individually. A graph of threshold cycle (Ct) vs. $\log_{10}$ copy number of the sample from the dilution series was produced (Fig. 1). The slope of this graph was used to determine the reaction efficiency.

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

3. Results

3.1. Secondary structure

During the development of the assay for G3PDH the initial primer and probe combination produced reactions which consistently had an efficiency of 75–80% when used to produce standard curves with purified PCR product using the Platinum Quantitative PCR system (Fig. 1A). This was despite the formation of only a single product in all dilutions. This efficiency could not be improved by altering the annealing temperature of the reaction nor the magnesium concentration. The product sequence was folded using the M-Fold server, and a predicted loop formed at the site where the 3′-end of the forward primer annealed (Fig. 1C). Therefore, this reaction was redesigned using the same reverse primer and probe combination, but the forward primer was moved by 14 bases such that the 3′-end of the primer was no longer in the loop. The subsequent reaction had an efficiency consistently greater than 95% (Fig. 1B).

3.2. Fluorescence quenching by 5′-deoxyguanosine

Two separate Taq-man probes with the same oligonucleotide sequence containing a 5′-deoxyguanosine and labelled with either 5′ Texas red or fluorescein were obtained to investigate the quenching effect that a 5′-deoxyguanosine residue would have on these fluorophores when used in a Taq-man system to detect canine IL-18 mRNA. The underlying PCR used for the assay was 100% efficient with $R^2 = 0.99$ when used on a standard dilution curve of sequenced PCR product with SYBR Green 1 (Fig. 2A,D). A 5′-FAM fluorophore and 3′-BHQ-1 quencher combination failed to produce fluorescence changes greater than 50 units despite the probe being used at 400 nM (Fig. 2B).

The same probe sequence with a Texas-Red/ELLE combination resulted in reactions with a good level of fluorescence change (Fig. 2C), therefore the quenching effect of 5′-deoxyguanosine was not seen with Texas red. The α-chain Taq-man probe also has a similar 5′-deoxyguanosine/Texas red combination and it also produced good levels of fluorescence change.

3.3. Primer dimer formation associated with RT-enzyme

Primer–dimer formation was a problem with the G3PDH assay when applied to RNA dilutions in the Platinum Quantitative RT-PCR system, particularly in the low copy number samples and negative control (Fig. 3A) despite this problem not being evident with the PCR based systems (Fig. 1B). This was most apparent in the negative control and was not due to contamination since the PCR products had a lower melting temperature and were smaller than the specific target when separated by agarose gel electrophoresis. Identical reactions using the Taq-man probe did not detect the primer–dimers but the traces from the samples with lower copy number had reduced fluorescence, reduced slope and later Ct (Fig. 3C). The annealing temperature of the reaction had no

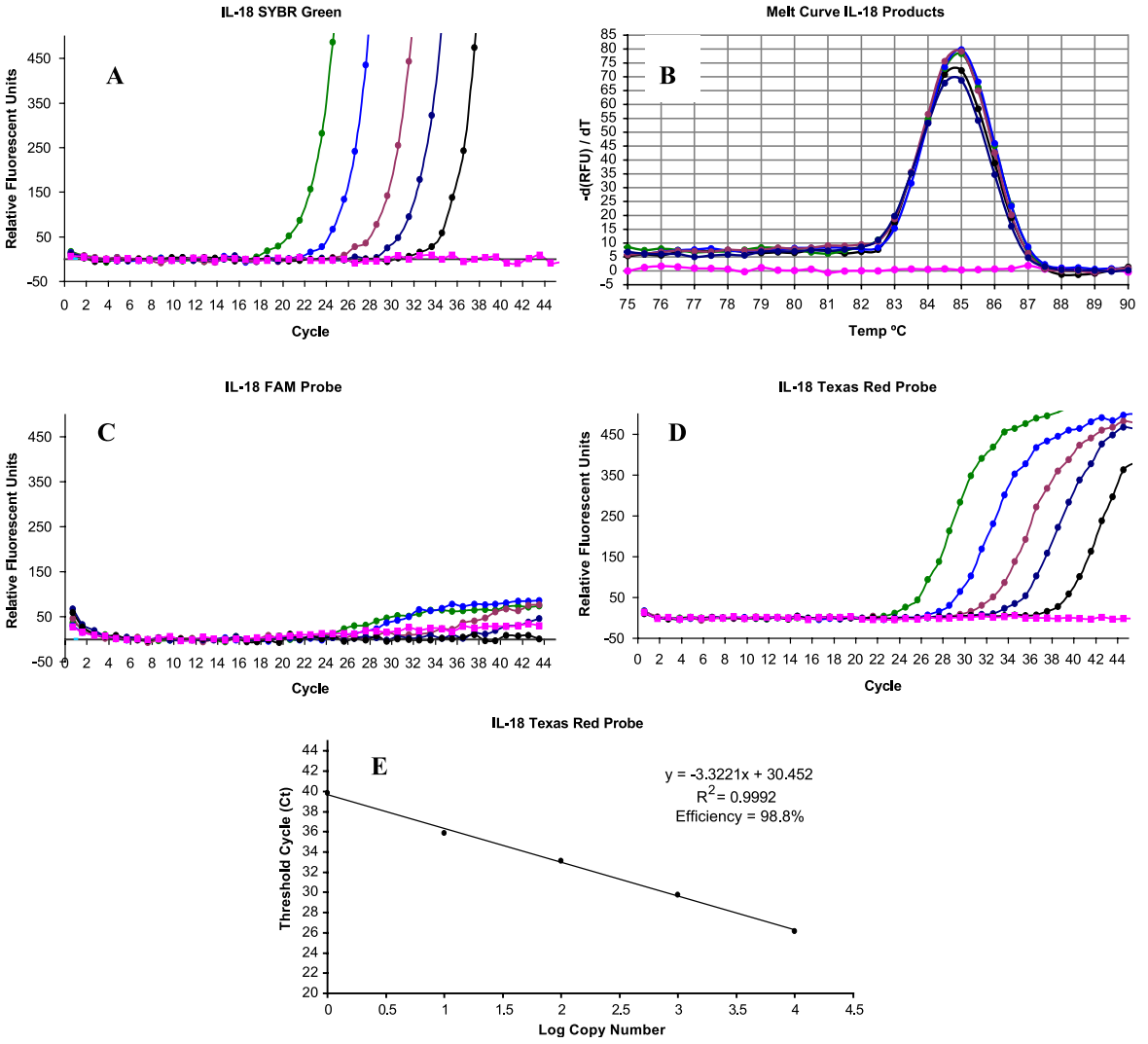


Fig. 2. Effect of 5′-G on Fluorescein and Texas Red fluorophores on 1/10 dilution of sequenced PCR product. The primer and probe set used for the amplification of canine IL-18 demonstrates the poor fluorescent changes seen with Fluorescein labelled Taq-man probes (C) with a 5′-G despite the efficient underlying PCR as seen with the SYBR Green I traces (A). Examination of the melt curve (B) from the reaction with SYBR Green I demonstrated that only a single product was formed in the reactions. Synthesis of the probe with Texas Red as the fluorophore had no such problem with poor fluorescence (D). The standard curve from the dilution series with the Texas-red probe (E) shows the reaction was 98.8% efficient.

effect on the dimer formation, nor did cooling the reactions during set up or placing them directly into the i-Cycler held at the initial incubation temperature. In order to investigate which primers were required for dimer formation, reactions of each primer alone and in combination were added to the same total primer concentration (200 nM) in separate RT-PCR reactions. Both the forward and reverse primers were required for dimer formation as no dimers were formed in the samples with forward or reverse primers alone (data not shown). The RT enzyme was required for dimer formation since when the same reaction buffer was used with platinum Taq (Fig. 4), primer–dimer formation did not occur with the for-

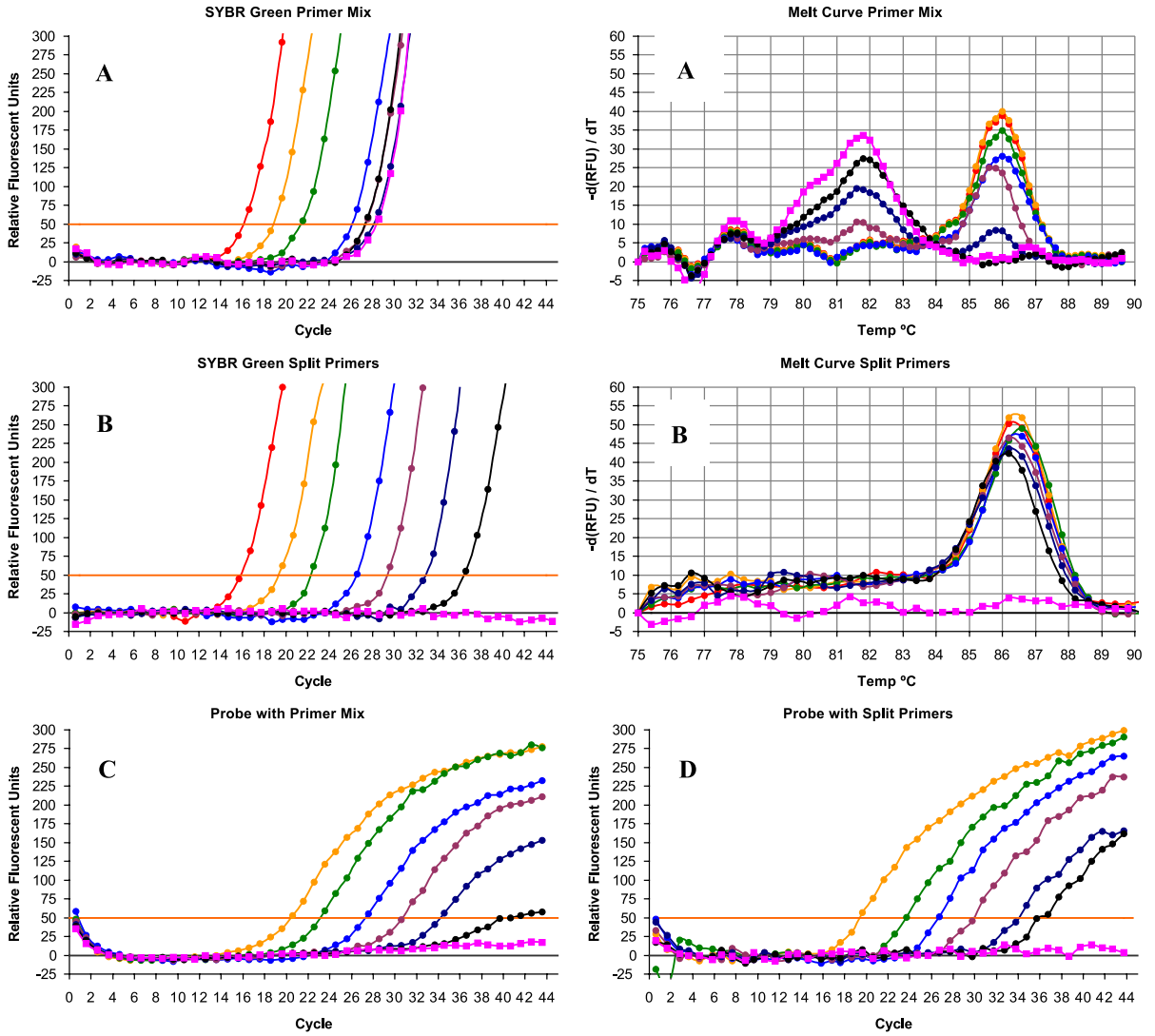


Fig. 3. Primer–dimer Formation Associated with RT Enzyme. These curves were produced with the G3PDH primer set with either SYBR Green I (A) or probe (C) used in one-step RT-PCR reaction on 1/10 dilution of RNA. The forward and reverse primers were added either as a mix prior to the RT step or the forward and reverse primers were split such that only the reverse was added prior to the RT reaction. Melt curves show that addition of both primers prior to the RT step leads to the formation of non-specific products. A widening of the distance between the traces and an alteration in the slope occurs when the probe is used (C). The addition of the reverse primer alone to the reaction for the RT step results in parallel traces with both SYBR Green I (B) and the probe (D) with a linear standard curve (not shown) with efficiencies of 96.4% and 96.1%, respectively.

ward and reverse primer combination. A similar effect has been seen with other primer sets (data not shown) and is usually associated with forward-reverse primer combinations.

The primer–dimer formation was prevented by delaying the addition of the forward primer to the reaction mix until after the RT-step and incubation of the reaction at 85 °C to reduce the reverse transcriptase activity but prior to Taq activation at 95 °C. Splitting the primers until after the RT step prevented primer–dimer formation and resulted in an efficient amplification of G3PDH template with no primer–

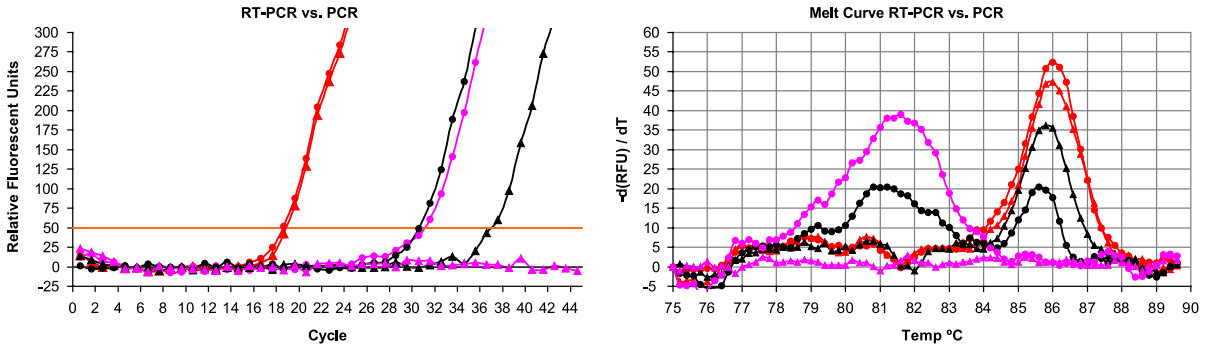


Fig. 4. RT-PCR Enzyme Mix vs. Platinum Taq Alone. Two dilutions of a purified and sequenced PCR product of G3PDH (red and black) and a no template control (pink) were amplified by RT-PCR (●) or the RT buffer supplemented with Platinum Taq alone (▲). The high copy number sample (red) was unaffected by the type of enzyme used and only a single product of the correct melt temperature was produced. The low copy number sample produced two products with the RT-PCR mix but only a single product with platinum Taq and this increased the Ct by 8 demonstrating the effect of the non-specific product formation in the RT-buffer on Ct value. Primer–dimer formation did not occur in the negative control with the platinum Taq alone indicating the need for the RT enzyme in the system to cause primer dimer formation.

dimer formation (Fig. 3B). Reactions with the probe using this technique showed that all were parallel with no reduction in reaction efficiency due to dimer formation (Fig. 3D).

This strategy is beneficial when amplifying RNA samples as many may contain small numbers of the target mRNA, and false negatives and underestimation of copy number will be a problem. An assay for quantifying J-chain in RNA from canine duodenal mucosa utilised SYBR Green I (Peters et al., 2003) manifested a similar problem with primer dimer-formation (Fig. 5). This non-specific product and primer–dimer formation made accurate quantification impossible as identification of samples with J-chain specific product was impossible. Splitting the primers resulted in samples with single products with the predicted melting temperature (Fig. 5).

3.4. Comparison of reverse transcription priming methods and enzymes

In order to determine the optimum enzyme type and priming method for production of cDNA, a 1/10 dilution of RNA was analysed by RT-PCR for α-chain using either oligo dT or gene specific primers in combination with either avian myeloblastosis virus reverse transcriptase (AMV-RT) (Thermoscript as part of Platinum Quantitative RT-PCR system) or Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Superscript II). Gene specific priming of the RT

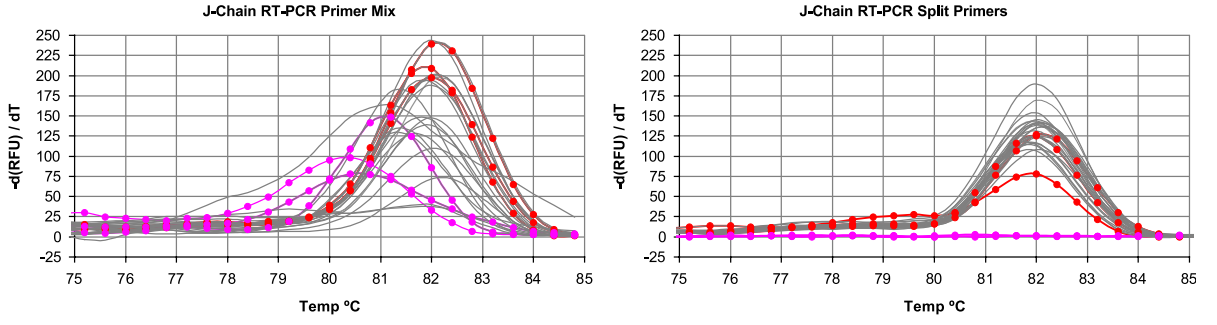


Fig. 5. Effect of Split Primers on 30 RNA Samples. The effect of primer–dimer formation in 30 RNA samples, primer–dimers and non-specific products are formed in low copy number samples making accurate quantification impossible. When the primers are split, the triplicate repeats of the negative control (pink) and specific RNA sample (red) no longer form primer–dimers and products with a single melting temperature are formed as seen with G3PDH assay. This leads to accurate quantification of the template quantity even in low copy number samples.

Table 2
Comparison of Ct values and reaction efficiencies for RT priming methods

Method	Number of Relative Copies in RNA						
	100 000	10 000	1000	100	10	1	Efficiency (%)
Oligo dT two-step thermoscript	20.4 (0.21)	24.1 (0.19)	27.8 (0.25)	32.1 (0.38)	36.6 (0.42)	–	76.8
Gene-specific modified one-step	18.7 (0.15)	21.1 (0.10)	25 (0.26)	28.3 (0.17)	31.7 (0.22)	35.9 (0.25)	95.0
Gene-specific two step thermoscript	19.4 (0.09)	22 (0.24)	24.9 (0.30)	28.5 (0.21)	31.9 (0.19)	36.2 (0.35)	98.8
Gene-specific two step superscript	22.7 (0.19)	24.4 (0.22)	29.4 (0.27)	34.9 (0.43)	–	–	73.9

The mean Ct (standard deviation) of triplicate repeats of RT-PCR for α-chain on a 1/10 RNA dilution using the four alternative methods of RT-PCR. Similar Ct values and reaction efficiencies were seen when the Thermoscript enzyme was used with gene-specific priming either as a one-step (delayed addition of forward primer) or as a two-step method. Oligo dT priming was inferior to the gene specific method as the Ct values were later and the reaction efficiency was much poorer leading to negative results in the last RNA dilution. The Superscript enzyme system performed the poorest of all the methods tried and the poor sensitivity and reaction efficiency combined to give negative results in the last two RNA dilutions.

was more sensitive (Ct 19.4 vs. 20.4) and efficient (98.8 vs. 76.8%) when compared with oligo dT when used with the AMV-RT enzyme particularly at lower template concentrations (Table 2). The one-step RT-PCR method with delayed input of the forward primer gave comparable results to the two-step method using AMV-RT for cDNA synthesis (Table 2) as both reactions had similar Ct (18.7 and 19.4) values for the RNA dilutions and reaction efficiencies (95% and 98.8%).

The MMLV-RT enzyme system was the least successful of all the methods used as the initial reaction had a later Ct when compared with the Thermoscript system (22.7 vs. 19.4) and was inefficient with poor linearity of the standard curve, leading to a negative result in the last two RNA dilutions (Table 2). Therefore the best method for the RT reaction was a combination of gene-specific priming and AMV-RT enzyme as either a one or two-step system.

A number of RT enzyme mixes have recently become available. Their exact composition is not available, but is likely to be a combination of AMV and MMLV. Two enzyme mixes were compared, Reverse-iT (ABgene) and ImProm-II (Promega). Random decamers were supplied with the Reverse-iT and they were used with this enzyme system, whereas random hexamers were used with the others.

The results of a 10-fold dilution series of RNA submitted to parallel RT and PCR reactions for canine α-chain are shown in Table 3. The Thermoscript one-step RT-PCR system was compared using gene specific priming with the modified one-step protocol and random hexamer primers to prime the RT reaction before addition of the gene-specific primers for the PCR. The reactions produced similar efficiencies (115% vs. 110%) but the Ct values for the random hexamer priming were approximately 2 cycles later . The Reverse-iT enzyme mix had a very poor efficiency (61.4%) which resulted in the last RNA dilution

Table 3
Comparison of Ct values and reaction efficiencies for RT enzyme mixes vs. thermoscript

Method	Number of Relative Copies in RNA					
	10 000	1000	100	10	1	Efficiency (%)
Thermoscript modified one-step (GSP)	24.8 (0.14)	27.6 (0.23)	31.4 (0.20)	34 (0.21)	37 (0.26)	115.2
Thermoscript two-step (Random hexamer)	26.7 (0.14)	28.7 (0.20)	32 (0.12)	35.4 (0.26)	38.9 (0.28)	110.0
Reverse-iT (ABgene) (Random decamer)	23.8 (0.43)	28.4 (0.48)	34.6 (0.56)	38 (0.63)	N/A	61.4
IM-Prom-II (Promega) (Random hexamer)	24.8 (0.10)	28.9 (0.20)	31.4 (0.26)	35 (0.30)	38 (0.29)	100.8

Two RT-enzyme mixtures (Reverse-iT and IM-Prom-II) with random primers were compared with Thermoscript (AMV) using both gene specific and random hexamers on a 10-fold dilution series of RNA using the α-chain assay. The reaction efficiencies using the Thermoscript enzyme with gene-specific and random hexamers were similar (115% vs. 100%) but the Ct values were two cycles later for the random hexamer samples. The reaction efficiency of the Reverse-iT mix was poor at only 61.4% resulting in the last RNA dilution not being detected. The IM-Prom-II enzyme system produced a similar efficiency and Ct value as the modified one-step system with gene-specific priming. The mean Ct (standard deviation) of triplicate repeats of each reaction is shown.

being negative, although the Ct value of the first dilution was similar to the modified one-step reaction. The Im-Prom-II enzyme mix produced similar results to the modified one-step reaction in terms of reaction efficiency (100%) and Ct values.

3.5. Minimisation of genomic contamination

Genomic contamination of RNA samples can be a significant problem especially with housekeeper pseudogenes or when the intron-exon structure of the gene is unknown. In order to investigate the ability of different systems to preferentially purify RNA over DNA, equal volumes of lysate were added to separate columns and the on-column DNase step was applied to half of the columns for 15 min at room temperature (Table 4). In the absence of DNase digestion there was a significant level of gDNA contamination as only a two to three cycle difference between the RT and No-RT samples was observed, indicating that 12.5–25% of the template was DNA. The application of DNase reduced this contribution to <2% of the total template (Ct difference >6). Qiagen columns were more efficient at isolating RNA from the lysate as the sample had a Ct value two cycles before the Promega column samples.

The length of on-column DNase digestion had no effect on the amount of gDNA contamination of the RNA sample. Application of DNase for up to 60 min had no greater effect than application for 15 min (Table 5). The incubation of the DNase at 37 °C did not reduce the amount of gDNA contamination vs. incubation at 25 °C (data not shown). Therefore DNase digestion was successful at reducing genomic contamination but increasing the duration of application over 15 min or using temperatures greater than 25 °C did not reduce the contamination further. Since the RNeasy columns isolated a greater amount of the RNA from the sample, these were used for subsequent isolations.

Table 4
Effect of column type and DNase treatment on genomic contamination

	Threshold Cycle			
	Promega DNase	Qiagen DNase	Promega No DNase	Qiagen No DNase
RNA	22.5 (0.28)	20.5 (0.32)	22.2 (0.33)	20.1 (0.27)
DNA	28.6 (0.29)	27.2 (0.37)	24.5 (0.26)	23.2 (0.31)
Difference	6.1	6.7	2.3	3.1

The amount of genomic contamination from both column types was comparable, with DNase digestion resulting in an approximate 16-fold decrease in the amount of genomic contamination. Qiagen columns were slightly more efficient at isolating RNA as there was a fourfold greater amount of RNA isolated from the sample compared with the Promega system. The mean Ct (standard deviation) of triplicate repeats of each reaction is shown.

Table 5
Effect of the duration of on-column DNase treatment on genomic contamination with Promega columns

Sample Treatment	Threshold cycle			%Signal From DNA
	PCR	RT-PCR	Difference	
No DNase	24.5 (0.26)	22.2 (0.33)	2.3	20.3
15 min DNase	33.6 (0.31)	21.2 (0.31)	12.4	0.02
30 min DNase	31.9 (0.25)	20.7 (0.23)	11.2	0.04
45 min DNase	33.7 (0.21)	21.0 (0.22)	12.7	0.02
60 min DNase	31.8 (0.34)	21.0 (0.36)	10.8	0.05

The length of DNase digestion had little effect on the amount of genomic contamination and on the amount of RNA isolated from the spin-column as there was no effect on the G3PDH RT-PCR Ct value or Ct difference when the time was increased. Increasing the temperature of incubation to 37 °C rather that 25 °C also had no effect (data not shown). The mean Ct (standard deviation) of triplicate repeats of each reaction is shown.

DNase digestion can be performed either on the spin-column or in the solution of isolated total RNA. The two methods of digestion were compared by adding equal aliquots of lysate from four duodenal biopsies to eight columns and different combinations of DNase digestion were performed (Table 6). The eluted RNA was used for quantification of α-chain transcript since this primer set will not amplify gDNA, therefore measuring the effect that DNase has on RNA alone. The G3PDH assay was used to quantify the level of genomic contamination. DNase digestion had little effect on the amount of α-chain mRNA present in the RNA samples as the variation in Ct (14.3–15.6) was no greater than the intra-plate variation in this assay reported previously (Peters et al., 2003). The highest Ct was seen in the sample with no DNase digestion and this may be due to the maximum binding capacity of the column being exceeded due to the combination of RNA and gDNA. The greatest reduction in genomic contamination was seen when a combination of on-column and in-solution DNase digestion was used (Ct = 41.8). One vs. two on-column DNase digestions in combination with in-solu-

Table 6
Comparison of on-column and in-solution DNase treatment with qiagen columns

Sample	DNase			IgA	G3PDH		
	Column	Solution	Column		RT-PCR	No-RT	Difference
1	+	+	+	15.2 (0.17)	18.9 (0.27)	41.8 (0.35)	22.9
2	–	–	–	15.6 (0.22)	18.5 (0.21)	20.2 (0.27)	1.7
3	+	–	–	15.2 (0.11)	18.8 (0.21)	26.5 (0.22)	7.7
4	+	–	+	14.8 (0.18)	18.7 (0.17)	34.4 (0.27)	15.7
5	+	+	–	14.3 (0.15)	18.4 (0.19)	41.2 (0.44)	22.8
6	–	+	–	14.9 (0.17)	18.6 (0.16)	35.8 (0.25)	17.2
7	–	–	+	15.0 (0.16)	18.7 (0.11)	32.7 (0.27)	14.0
8	–	+	+	14.6 (0.11)	18.9 (0.20)	40.8 (0.36)	21.9

DNase digestion had little effect on the amount of RNA isolated from the column, as demonstrated by the relatively constant Ct value for the IgA primer set which did not amplify genomic DNA and were thus unaffected by genomic contamination. In-solution DNase digestion was more effective than on-column digestion as this results in a greater Ct difference that on-column digestion alone. A combination of the two methods of digestion resulted in the largest Ct difference and in some samples would eliminate the genomic contamination (data not shown). The mean Ct (standard deviation) of triplicate repeats of each reaction is shown.

tion DNase digestion had no greater effect on genomic contamination. The DNase digestion in-solution was more effective than on-column digestion at reducing contamination (Ct = 35.8 vs. 32.7/26.5), although digestion on the second column had a greater effect than on the first when in-solution digestion was not used.

4. Discussion

The formation of secondary structure by association of complementary bases within a nucleotide sequence by hydrogen bonding between the bases will render them unavailable for interaction with the primer annealing at the same site. This interaction will be dependent upon the predicted melting temperature of the loop since if the primer anneals prior to the loop forming, there will be no such competition. The melting temperature of the predicted loop in the original G3PDH product was higher than the 60 °C melting temperature of the forward primer and therefore it was likely to interfere with primer annealing. Since the 3′ end of the primer is critical for the initiation of Taq-mediated polymerisation, loops forming at this end may well have a greater effect on reaction efficiency than those forming towards the 5′-end, as shown with the G3PDH forward primers. Similar effects have been seen with loops forming at the site of the reverse primer (data not shown). Use of the M-fold server, which was used in subsequent reaction design, resulted in much more efficient reactions. By definition, reactions with poor efficiency will have poor sensitivity, particularly in low copy number samples due to the later Ct values in these samples (Fig. 1A).

The quenching effect of deoxyguanosine in close proximity to fluorescein has been well documented (Crockett and Wittwer, 2001; Nazarenko et al., 2002a,b). Other dyes have also been associated with this phenomenon including HEX, ROX and TAMRA but Texas Red, Cy3 and Cy5 are not affected by coupling to this base (Nazarenko et al., 2002b). Some authors have suggested that 5′-deoxyguanosine should be avoided when designing Taq-man probes as after hydrolysis, the 5′ base remains attached to the fluorophore in solution (Bustin, 2000). Our findings indicate that fluorescein associated with a 5′-deoxyguanosine is associated with quenching in solution, but coupling to Texas Red (and presumably Cy5) is a viable alternative in systems where this base combination cannot be avoided due to sequence constraints. We have successfully designed fluorescein labelled probes with each of the other three remaining base types with no such base quenching problems.

One-step RT-PCR systems have been previously associated with poor sensitivity when compared with two step methods (Leutenegger et al., 1999). In this study the lack of sensitivity was particularly apparent in the samples with increased Ct values. This finding is similar to the effect seen with the G3PDH assay where samples with a Ct between 20 and 30 were relatively unaffected by primer–dimer and non-spe-

cific product formation, but samples with later Ct values were. Our data indicate that this problem is associated with the RT enzyme, and the primer–dimers and non-specific products are formed prior to the onset of the PCR phase of the reaction. The Taq enzyme in both the PCR and RT-PCR master-mix systems is inactivate until heated to 95 °C, after which the reactions are not cooled below 60 °C until the end of the analysis. In the case of the RT enzyme, there are no 'hot-start' enzymes available and thus they have activity during the reaction set-up. Therefore, it is likely that the primers will anneal to one another and to non-target RNA during set-up and can thus form primer–dimers and non-specific products. We have observed similar effects using non-'hot start' Taq enzymes. Cooling the reactions on ice did reduce the problem but did not eliminate it. Two step enzyme systems do not have the same problem as only a single primer is added to the mixture for the RT reaction. The forward primer is added only when the PCR reaction is carried out with 'hot-start' Taq. One-step multiplex RT-PCR would be very difficult due to the requirement for multiple primer sets, which would lead to increased primer–dimer formation and loss of sensitivity.

The use of a one-tube system for multiple replicates of the RT-PCR has the advantage over a two step system since each replicate repeats the reverse transcriptase reaction as well as the PCR. This system would be particularly useful when a single gene product is to be quantified from each RNA sample. In order to reduce the number of non-specific products produced during the set-up of the reactions, a modified one-step method was produced where the addition of the forward primer was delayed. Opening of the reaction tubes after the RT step and the addition of the forward primer does increase the risk of sample contamination but occurs prior to the production of large amounts of amplicon. This does not negate the beneficial effects of real-time PCR in minimising amplicon carryover and is a viable means of reducing primer dimer formation and maximising sensitivity. In our hands we have previously used this system to produce reactions with 90–100% efficiency over a wide range of RNA dilutions (Peters et al., 2003).

A two step-system utilising a single RT reaction and multiple PCR reactions requires the RT step to be equally efficient between all samples and at different template concentrations. Several strategies and enzyme types are available for the reverse transcriptase reaction. In our hands, pure AMV-RT was superior to MMLV-RT when used with gene-specific primers, especially in samples with low copy numbers. More recently mixtures of the two types of enzyme have become available and some can produce results comparable to pure AMV-RT enzyme but have the advantage of reduced cost. When standardising RT-PCR reactions it is important to use dilutions of RNA rather than cDNA for both one-step and two-step systems since PCR carried out on a cDNA dilution does not confirm that the system amplifies dilutions of RNA in a linear fashion.

Single gene specific priming was superior to oligo dT priming in our hands, particularly with low copy number templates where there was a reduction in reaction efficiency. This may be due to reverse-transcription of non-target RNA at the expense of target RNA. Random hexamers produced similar results to gene specific primers, although they have previously been associated with overestimation of template quantity up to 19 fold (Zhang and Byrne, 1999), although this study utilised competitive RT-PCR quantification. We also found that when a combination of reverse primers was used to make a pool of cDNA for multiple PCR assays, a similar loss of sensitivity was seen in the lower copy number samples. This was possibly due to primer–dimer formation resulting from the interaction of the primers and RT enzyme. Therefore random hexamers would be the priming method of choice for producing a 'pool' of cDNA especially when multiple mRNA types are to be quantified from a single RNA or where a multiplex PCR is to be used.

Many studies have utilised DNase digestion for minimisation of genomic contamination (Leutenegger et al., 1999; Mena et al., 2002; Stordeur et al., 2002; Peters et al., 2003) but no attempt has been made to quantify the effect on the RNA and genomic DNA. Neither type of column used was able to exclusively isolate RNA, as found in a previous study (Bustin, 2002), thus DNase digestion was imperative for housekeeper gene quantification. Two strategies were used for DNase digestion, with digestion in solution being superior to digestion on column. This may be due to the free association of the DNase with the gDNA in solution rather than relying on the penetra-

tion of the column membrane by the DNase to digest the bound gDNA. The gDNA associated with the silica may not be accessible or in the correct orientation for DNase digestion.

Digestion on the second column was more effective than on the first, possibly due to the absence of protein contamination on the column. The centrifuged lysates added to the first column had visibly more protein than those added to the second. This protein may limit the penetration of the DNase to the gDNA bound to the column membrane.

Increasing the length of DNase digestion or the temperature at which it was carried out had little effect on the level of gDNA contamination. These findings indicate that in order to reduce genomic contamination to the lowest level possible, a combination of digestion in solution and on the spin-column is superior to either procedure alone, although a single in-solution digestion would be preferable to on-column alone. This is particularly important for quantification of housekeeper genes associated with pseudogenes, where the intron–exon structure of the gene is unknown, or when an intron spanning probe is used. We have found that assays with primers in separate exons but with a relatively short intermediate intron can still amplify gDNA utilising the relatively short annealing times of real-time PCR. Intron spanning probes will not report the production of genomic products but these products will still be present in the reaction and will reduce sensitivity (as with primer–dimers), since genomic products will not lead to probe hydrolysis but will compete with cDNA in the reaction. This loss of sensitivity will be most apparent with low copy RNA or samples with large amounts of genomic contamination. Intron spanning primers (e.g. forward primer used in the α-chain assay) will not amplify gDNA and will have no loss of sensitivity.

In conclusion, the introduction of real-time RT-PCR has revolutionised the quantification of mRNA but requires careful assay design and reaction optimisation to maximise sensitivity. This paper has demonstrated the effects of secondary structure, 5′-deoxyguanidine quenching in Taq-man probes and primer–dimer formation on the sensitivity of RT-PCR assays. The design of assays with intron spanning primers offers the most efficient way of minimising the effect of genomic contamination but DNase digestion, particularly in solution, offers an alternative method of gDNA reduction. The problem of primer–dimer formation and loss of sensitivity with one-step RT-PCR enzyme systems highlights the need for the development of a ‘hot-start’ RT enzyme for use in sensitive one-step or multiplex RT-PCR systems.

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References

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23–39.

Crockett, A.O., Wittwer, C.T., 2001. Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. Anal. Biochem. 290, 89–97.

Fang, X., Li, J.J., Perlette, J., Tan, W., Wang, K., 2000. Molecular beacons: novel fluorescent probes. Anal. Chem. 72, 747A–753A.

Hanauer, A., Mandel, J.L., 1984. The glyceraldehyde 3 phosphate dehydrogenase gene family: structure of a human cDNA and of an X chromosome linked pseudogene; amazing complexity of the gene family in mouse. EMBO J. 3, 2627–2633.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Leutenegger, C.M., Mislin, C.N., Sigrist, B., Ehrengruber, M.U., Hofmann-Lehmann, R., Lutz, H., 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Vet. Immunol. Immunopathol. 71, 291–305.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K., 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4, 357–362.

Mena, A., Ioannou, X.P., Van Kessel, A., Van Drunen Little-Van Den Hurk, S., Popowych, Y., Babiuk, L.A., Godson, D.L., 2002. Th1/Th2 biasing effects of vaccination in cattle as determined by real-time PCR. J. Immunol. Methods 263, 11–21.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., Rashtchian, A., 2002a. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res. 30, e37.

Nazarenko, I., Pires, R., Lowe, B., Obaidy, M., Rashtchian, A., 2002b. Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. Nucleic Acids Res. 30, 2089–2195.

Ng, S.Y., Gunning, P., Eddy, R., Ponte, P., Leavitt, J., Shows, T.,

Byrne, C.D., 1985. Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol. Cell. Biol. 5, 2720–2732.

Overbergh, L., Valckx, D., Waer, M., Mathieu, C., 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 11, 305–312.

Peters, I.R., Helps, C.R., Batt, R.M., Day, M.J., Hall, E.J., 2003. Quantitative real-time RT-PCR measurement of mRNA encoding alpha-chain, pIgR and J-chain from canine duodenal mucosa. J. Immunol. Methods 275, 213–222.

Rozen, S., Skaletzky, H.J., 2000. Primer3 on the WWW for General Users and for Biologist Programmers. Humana Press, Totowa, NJ.

SantaLucia Jr., J., 1998. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc. Natl. Acad. Sci. U. S. A. 95, 1460–1465.

Stordeur, P., Poulin, L.F., Craciun, L., Zhou, L., Schandene, L., de Lavareille, A., Goriely, S., Goldman, M., 2002. Cytokine mRNA quantification by real-time PCR. J. Immunol. Methods 259, 55–64.

Tyagi, S., Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303–308.

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S., 1999. Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. 17, 804–807.

Yin, J.L., Shackel, N.A., Zekry, A., McGuinness, P.H., Richards, C., Putten, K.V., McCaughan, G.W., Eris, J.M., Bishop, G.A., 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol. Cell Biol. 79, 213–221.

Zhang, J., Byrne, C.D., 1999. Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR. Biochem. J. 337, 231–241.