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Factors Influencing Real-Time RT-PCR Results: Application of Real-Time RT-PCR for the Detection of Leukemia Translocations

John D. Curry[2,*], Cliona McHale[1], and Martyn T. Smith[1]

[1]Division of Environmental Health Sciences, School of Public Health, University of California,
[2]Department of Immunology, Molecular and Cellular Biology, 439 Life Sciences Addition, University of California, Berkeley, CA 94720-3200, USA

Abstract

A variety of factors that influence real-time reverse transcriptase (RT)-PCR results were examined. Real-time reaction volumes can be decreased from the manufacturers suggested volumes (50μl) to as little as 5μl. Errors introduced through volume handlings can be significantly mitigated through the use of electronic pipettes. Random hexamers are compared against the use of a specific reverse transcription primer. Negative effects of non-target total RNA on the real-time RT-PCR results are detailed. Finally the amount of the RT reaction that can be successfully loaded into the real-time PCR reaction was determined and results indicate that a considerable amount can be successfully utilized. It was determined that the RT reaction buffer alone impacts on the quality of the real-time PCR results. The results demonstrate simple and effective methods to increase the resolution and reliability of the real-time RT PCR protocols.

Introduction

Real-time polymerase chain reaction (PCR) is now being widely used for sequence detection. Real-time PCR's ability to not only detect but also to quantify initial template amounts is the main reason for deployment of this assay. In addition, real-time PCR offers exceptional resolution abilities afforded by the laser detection system compared with conventional gel based visualizations. When real-time PCR is coupled with a reverse transcription (RT) assay, one has a high resolution system that can accurately quantify specific mRNA expression from any type of biological sample (Review, Bustin 2000). Real-time RT-PCR assays are currently utilized for the detection and quantification of leukemogenic fusion genes generated by specific translocations (Amabile *et al*. 2001; Curry *et al*. 2001; Marcucci *et al*. 1998; van Dongen *et al*. 1999). Such assays are becoming the norm when attempting to detect levels of minimal residual disease following patient treatment (Dolken 2001; Krauter *et al*. 2001). Quantification of residual leukemic cells offers the ability to monitor the progression of any potential relapse (Sugimoto *et al*. 2000; Tobal *et al*. 2000).

Drawbacks of real-time PCR are limited primarily to the cost of the reagents required for the assay. Fluorogenic probe, which is used for the actual detection, is considerably more expensive than regular primers. The other major expense, aside from the cost of the instrumentation, is the enzyme mixes sold by the machine manufacturers. It is possible to reproduce this PCR enzyme mix at a considerable cost saving (Marcucci *et al*. 1998). However, the manufacturer's incorporation of a proprietary reference dye into their commercial PCR enzyme mix and then its use as a standard in their software produces far superior and reproducible results. As such, the use of these pre-made PCR enzyme mixes is preferable. However, cost of the individual real-time PCR reactions can be decreased by optimizing the amount of fluorogenic probe used, and by decreasing the size of the PCR reactions from the volumes suggested by the manufacturer. Several factors that likely influence the quality and resolution of the real-time RT-PCR assay are explored here. The influence of neutral background RNA (ratio of non-target to target RNA) on the sensitivity of the assay is examined in detail. Other factors investigated are the decision to use specific versus random hexamers to prime cDNA synthesis, levels of RT reaction that can be loaded into PCR reactions before inhibition of PCR occurs, and appropriate volume handling equipment to ensure reproducibility. Another crucial factor, which is not explored here, is the RT enzymes efficiency in converting primed RNA into cDNA. We had previously reported that the Moloney murine leukemia virus (MoMLV) RT enzyme exhibits a relatively low conversion efficiency (Curry *et al*. 2002).

A fusion gene formed by a translocation (t(8;21)) between chromosomes 21 (acute myeloid leukemia 1 gene, *AML1*) and 8 (myeloid translocation gene on chromosome 8, *MTG8*) was chosen, as a real-time RT protocol had previously been described (Marcucci *et al*. 1998) and is currently being utilized in house. The assay is highly sensitive and previous reports purport to detect 3 copies of cDNA (Marcucci *et al*., 1998).

Results

Effect of reaction volume on PCR assay.

Applied Biosystems, the manufacturer of the ABI Prism 7700 Sequence Detection System, suggests that 50 μl PCR

*For correspondence. Email curry@uclink4.berkeley.edu; Tel. (510) 642-6858; Fax. (510) 642-0427.

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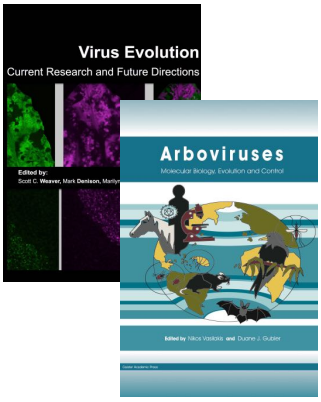

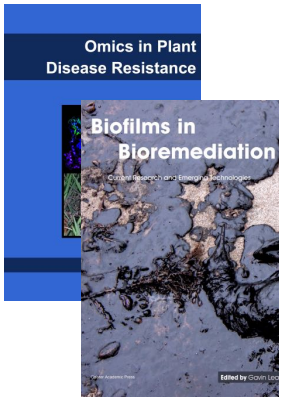

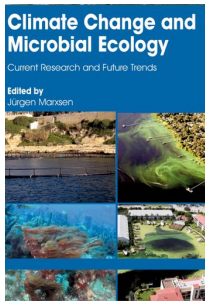

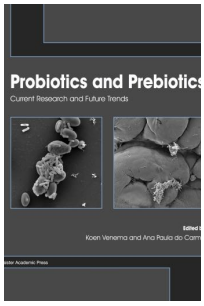

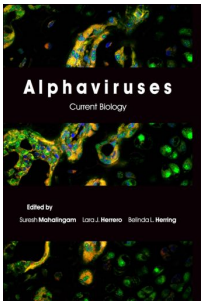

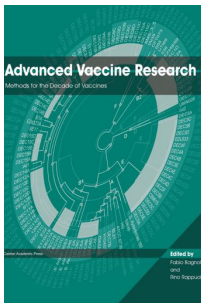

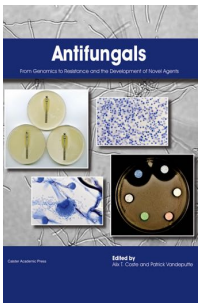

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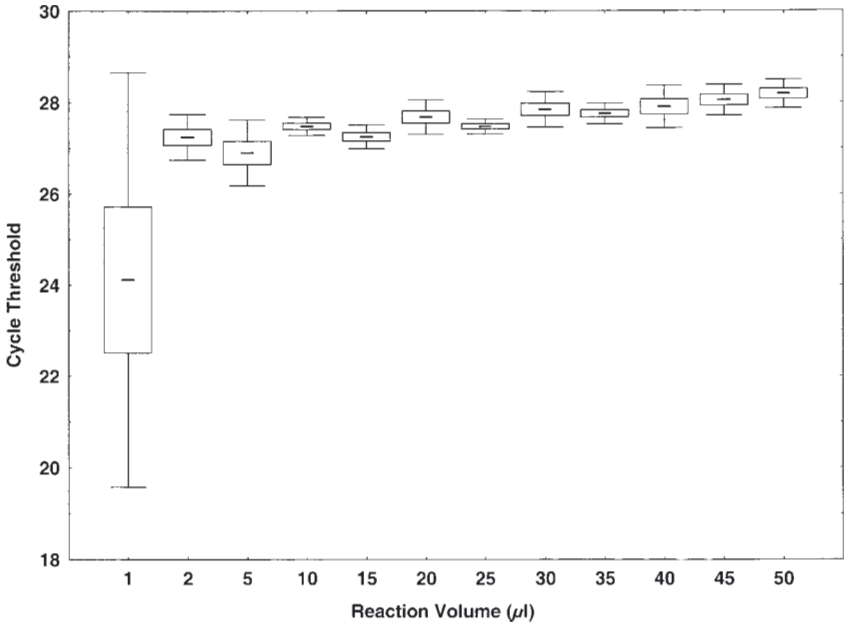

Figure 1. The effect of reaction volume sizes on the reproducibility of real time PCR. Mean Ct values (eight replicates for each volume) are indicated by bars with standard errors (boxes) and deviations (whiskers). Real time PCR volumes can be substantially reduced from the manufacturer's recommended volumes.

volumes be used. We wanted to determine if reaction volumes could be considerably reduced and significant cost savings realized through use of reduced amounts of reagents and expensive TaqMan™ probe. To demonstrate that smaller reaction volumes are as reliable as the suggested 50μl volumes, a single large volume reaction was prepared (containing plasmid DNA as template) and a series of twelve different volumes (1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 μl) each with 8 replicates was aliquoted onto a 96 well PCR plate. After the real-time reaction was complete, the mean cycle threshold (Ct) values for each starting volume was determined as well as standard deviations and errors (Figure 1). For the volumes 10 to 50 μl, very little deviation was noted, however for the smaller volumes (1, 2 and 5 μls) considerably more deviation from the mean was noted. Clearly such small reaction volumes are impractical where a fraction of the volume will comprise cDNA from the RT reaction. Volume sizes between 5 and

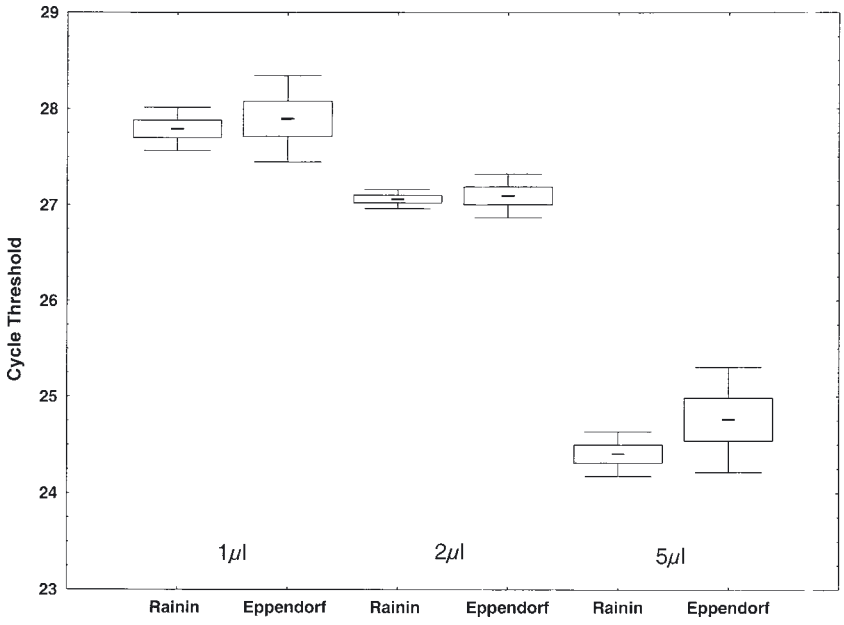

Figure 2. Comparison of a manual (4710, Eppendorf) and an electronic (E3-10, Rainin) 10 μl pipettes. Both pipettes had been recently calibrated prior to use. Three volumes sizes containing a cDNA template were delivered to identical PCR volumes, with 6 replicates for each point. The mean Ct value is given, with standard error (box) and deviations (whiskers).

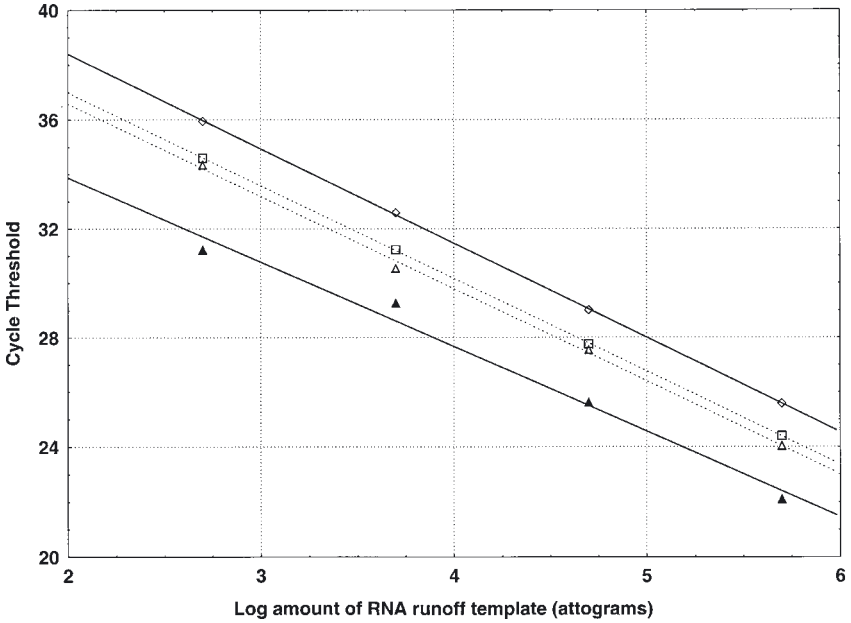

Figure 3. Random hexamers versus specific oligos as RT primers. Solid points indicate the use of a non-target background RNA in the RT reaction, while open points do not. Squares indicate that the T12 specific primer was used, while triangles indicate the use of random hexamers. More efficient RT reactions are indicated by lower Ct intercepts as they would contain more cDNA. Each linear regression had a R^2 of 0.99, $p < 10^{-4}$, except the random hexamers with background RNA (closed triangles) which was R^2=0.98, $p<10^{-3}$. Statistical comparison of the four regression lines was not attempted as there was not enough power.

10 μl are feasible when the template can be mixed with a larger PCR volume and then smaller aliquots dispensed. Reaction volumes of 20 μl offered considerable cost savings, while still permitting a large amount of cDNA or other template to be accurately added. All subsequent PCR volumes used were 20 μl.

Error induced by pipette errors

Some error can be introduced due to pipetting inaccuracies. Such errors will be particularly apparent when loading some fraction of the RT cocktail or other template into the real-time PCR. In an attempt to mitigate errors associated with volume handling, electronic pipettes were utilized. To demonstrate the effectiveness of such devices, either 1, 2, or 5 μls of a single RT cocktail was loaded into identical PCR reactions using an electronic pipette (Rainin Scientific) or a manual pipette (Eppendorf). Six replicates of each load were reacted. The mean Ct values with standard deviations and errors are shown in Figure 2. More deviation was obtained with the manual pipette then the electronic one. Thus to reduce pipette associated errors, using an electronic device is preferable and all subsequent volume handlings were carried out with electronic pipettes.

Specific primer versus random hexamers

Efficiency of cDNA generation in the RT step, with either a specific primer or random hexamers was compared. Ultimately the choice to employ either of the primers will be determined by need. Using random hexamers produces cDNA for all RNA species within a sample RNA, and as such, a single RT reaction could serve as template for a variety of different PCR assays. Use of specific primers in a single RT reaction should increase the efficiency of the RT reaction by specifically priming only target mRNA species. To test this hypothesis, duplicate RT reactions were performed on a set of serially diluted runoff transcripts, identical except for the addition of the specific primer (T12) or the random hexamers. Experiments were repeated with and without non-target background RNA (Figure 3). Following real-time PCR, Ct values were determined as a measure of the level of target cDNA produced in the RT step. RT reactions primed with either the specific primers or the random hexamers generated sufficient target cDNA for detection in the PCR assay for all transcript dilutions tested (500 ag to 500 fg).

Random hexamers yielded better results as compared to the specific T12 primer in the absence of any neutral background RNA; however the difference is slight as indicated by the nearly identical linear relationships. When the neutral background RNA was added to the RT reactions, the random hexamers clearly out-performed the specific T12 RT primer. While the results here are contrary to the proposed hypothesis, similar experiments with several other leukemia translocations (data not shown) had demonstrated that specific primers were preferential to random hexamers. It is likely that the design of the T12 primer, when used as an RT primer, could be improved and potentially out-perform the random hexamers. Despite this, primer T12's performance was sufficient and as such was used in all subsequent RT reactions.

Effect of RNA concentration on RT efficiency.

Hiding runoff RNA transcripts in non-target background RNA more closely resembles real-life deployment of the RT-PCR assay. For example, looking for leukemic fusion

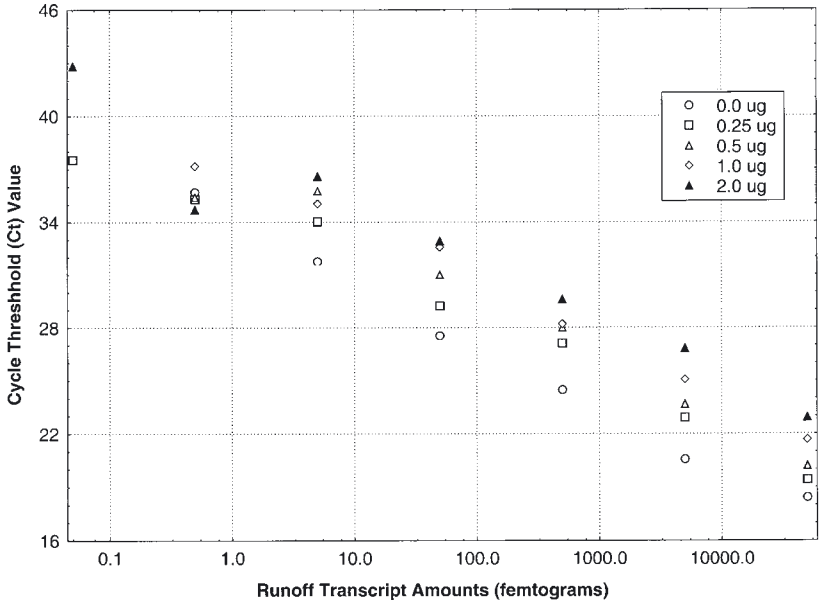

Figure 4. The effects of background RNA concentration on RT-PCR sensitivity. Five different concentrations (as noted by symbols) of background RNA were mixed with runoff RNA transcripts (across 7 orders or magnitude) in 20μl RT reaction volumes. Completed RT reactions were assayed by real-time PCR. Cycle threshold values were plotted for each RT reaction. Lower Ct values indicate PCR reactions that contained more detectable cDNA and thus more efficient RT reactions. At the lower end (0.5 and 0.05 fg) the reactions are less reproducible and often fail (likely as there are no cDNA molecules to provide template).

transcripts will always be carried out in a pool of non-target cellular RNA. However, the efficiency of the reverse transcriptase reaction is negatively affected by the concentration of background RNA (Curry *et al.*, 2002). Optimal levels of total cellular RNA that can be assayed and that still permit the efficient production and detection of target cDNA can be determined from the following data. With increasing concentrations of background RNA, Ct values increase indicating that lesser amounts of cDNA are being produced (Figure 4). Thus, as the background of non-target RNA increases, the efficiency of the RT enzyme to produce target cDNA decreases. When very small amounts of RNA template exist for reverse transcription, this effect, although still present is now less pronounced (as noted by the compression of the Ct values at the 0.5 fg range). In such cases, the efficiency of the RT enzyme is thus more equal between different levels of background RNA. During RT-PCR analysis, it is desirable to screen as much RNA as possible per RT reaction, particularly when searching for rare RNA species. However, optimization of maximal amounts which can be screened while maintaining optimal resolution should be determined. The use of a specific primer with a high density of RNA present may also result in increased mis-priming, thus the RT temperatures may need to be increased to increase the specificity. The remaining experiments utilized a background RNA amount of 0.5 μg per 20 μl RT reaction volume at the temperatures previously specified.

To eliminate the concern that the background RNA might have some effect directly on the real-time PCR reactions, duplicate PCR reactions with set amounts of cDNA template were reacted with and without background RNA. No difference in mean Ct values from these experiments was detected, indicating that background RNA has no direct inhibitory effect on the real-time PCR reactions.

One might also consider using mRNA extracted from whole RNA in order to increase the RT-PCR's resolution, by permitting more of the potential target RNA to be concentrated into a smaller RT reaction volume. While this might be efficient for quantifying mRNA species that are relatively abundant, species that are relatively rare might be lost during the mRNA purification process. In addition the mRNA extraction process involves considerable expense.

Effect of RT reaction loading volumes on PCR Ct values.

To economize on PCR reagents while optimizing detection sensitivity during RT-PCR assays, maximal amounts of the RT reaction need to be loaded into the PCR reaction. However, components of the RT reaction, most notably the components of the RT buffer, might be deleterious to the PCR progression. Effects of RT reaction loading were determined by mixing a PCR reaction (20μl final volume) containing a set amount of DNA template (linearized plasmid 20, 120, or 620 ag/reaction) with varying amounts of a sham RT reaction (containing 50 ng/ul colon RNA as background, but with no detectable template).

Contrary to what was initially expected, increasing loads of the sham RT reaction into the PCR were related to decreasing Ct values and therefore increasing detection sensitivity (Figure 5, Plot D). This is rather puzzling as the PCR template amount is uniform through all these PCRs. This effect was significant for the 120 and 620 ag template levels (R^2=0.5, df=37, $p < 10^{-6}$ and R^2=0.26, df=38, p=0.0007 respectively) but not the smaller 20 ag template reactions. With this lower template amount, considerable variation in Ct values was observed, and indeed a number of the reactions failed to produce a fluorogenic reaction, as they likely had no template (20 ag is approximately 5 plasmid molecules, and some PCR tubes may actually have no template at this level).

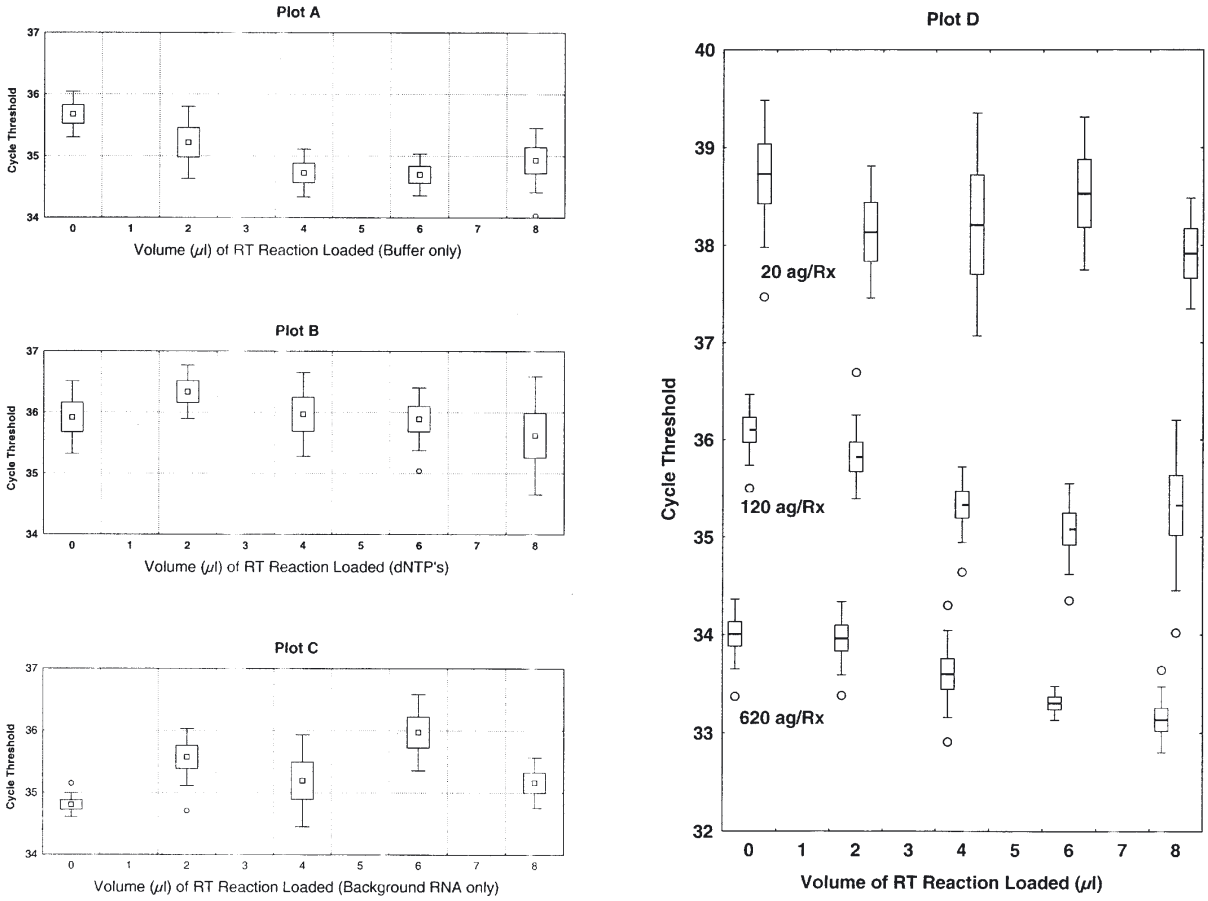

Figure 5. A demonstration that increasing volumes of a sham (template-less) RT reaction result in decreasing Ct values. A plasmid template was delivered independently of the RT reaction loading. Mean Ct values indicated by a bar, boxes are mean +/- standard errors, and whiskers are means +/- standard deviations. Outliers are represented by circles. Plot D contains data obtained from three different template levels; top = 20ag/reaction, middle 120ag/reaction, bottom 620ag/reaction. Plots A, B, and C demonstrate the effect of each of the three RT reactions components.

As this RT loading effect was contrary to expectations, the component of the RT reaction responsible was sought. To this end, three types of sham RT reactions were prepared; one with only the 10X buffer (Stratagene), another contained only the dNTPs, and the last one had only background RNA. PCRs were spiked with 200ag/reaction of the plasmid template. Figure 5 plots A, B, and C depict the results. Neither the presence of background RNA nor dNTPs had any significant effect on the Ct values. However the increasing volumes of the sham RT reaction with just the RT buffer significantly decreased the Ct value (linear regression, R^2=0.27, df=28, p=0.004). Noting that the TaqMan™ universal master mix system was designed to work with a broad range of PCR primers, it is probable that with the specific primer set used here, the RT buffer has the effect of favoring the PCR reaction. This could occur through modification of the pH or perhaps modification of the $MgCl_2$ and or KCl concentrations of the reaction. Thus, this effect may not be noted for all assays. However, it is clear that large volumes of RT reactions (up to 8 μl or more) can be successfully loaded into the PCR without significant deleterious effects, and in some cases may improve the PCR sensitivity.

Discussion

Real-time RT-PCR offers exceptional resolution capabilities for detecting and quantifying specific RNA species. A variety of factors influence the results, in terms of Ct values, that directly relate to initial PCR (cDNA) template amounts. PCR reaction volumes can be greatly reduced from the recommended size of 50 μl and significant cost savings realized. The use of electronic pipettes to handle volumes is preferable to manual pipettes which incur a significant amount of variation. The decision to use specific or random primers to generate cDNA must be determined by needs, but careful design of a specific primer is essential to ensure maximal RT efficiencies. Previous studies had demonstrated that the use of random primers may cause an 19-fold over estimation of mRNA copy number as compared to a specific primer (Zhang and Byrne 1999). While the data here do not demonstrate such a large fold difference between the random and specific primers, the random primers did generate a difference of approximately four Ct values, but only in the presence of the non-targeted background RNA. For the accurate determination of a

specific mRNA copy number, the use of a longer specific primer is likely warranted.

The presence of non-target background RNA significantly impacts the efficiency of the RT reaction as revealed through the real-time PCR results, and care must be taken to balance resolution requirements against maximizing the amount of RNA screened in the RT reaction.

Experimental Procedures

A cloned *AML1-MTG8* cDNA fusion gene was a generous gift of Dr. Ching-Ping Tseng from Chang Gung University (Taiwan). This plasmid was used to generate mRNA runoff transcripts using the T7-MEGAshortscript™ kit (Ambion) as directed. Contaminating plasmid DNA was significantly reduced by additional DNase I (Roche) digestion steps and purification of the RNA runoff transcript on Qiagen's RNA spin columns. After these additional steps, contaminating plasmid DNA could not be detected in 1 ng of the runoff transcript RNA using the real-time PCR assay (described below).

Reverse transcription of the *AML1-MTG8* fusion mRNA was performed in a 20 μl reaction cocktail. RNA runoff transcripts were mixed with 4 nM specific MGT8 (Tobal and Liu Yin 1998) primer (T12, 5'-AGGCTGTAGGAGAATGG) (MWG Biotech Inc.) or 120 ng of random hexamers (GibcoBRL). RNA background, when employed, consisted of human colon total RNA (Stratagene). RNAs (runoff template and background) and primer mixtures (10μl) were initially heated to 65°C for five minutes and then allowed to cool to room temperature. A 10 μl reverse transcriptase cocktail (1X RT buffer (as supplied by RT enzyme manufacturer)), 100μM dNTPs (Roche), 4 Units of Prime RNase Inhibitor™ (Eppendorf), and 25 units RT enzyme (Stratagene) was the added to the RNA/primer mixtures and reacted at 42°C for 50 minutes followed by 95°C for 5 minutes.

Real-time PCR was used to detect full-length cDNAs. Real-time primers and TaqMan™ probe were as described by (Marcucci *et al.* 1998). One correction to the reverse primer was made (5'-ATC CAC AGG TGA GTC TGG CATT) which removed a single adenine residue after the underscored adenine. Optimal probe and primer concentrations were determined by following the manufacturer's directions (Applied Biosystems). Real-time PCRs consisted of 5μl of the reacted RT cocktail, 10μl of TaqMan™ Universal Master Mix reagent (2X)(Applied Biosystems), 900 nM of both the forward and reverse primers, and 90 nM of the TaqMan™ probe (MWG Biotech Inc.) in a final volume of 20μl. When required, the reaction volume was scaled up or down while maintaining the same ratio of components. Reactions were run on an ABI Prism 7700 real-time PCR machine (Applied Biosystems) using the standard cycling conditions but with 45 rather then 40 cycles. Data was collected and analyzed with the Sequence Detector software v1.7 (Applied Biosystems).

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