Short Technical Reports

Low Efficiency of the Moloney Murine Leukemia Virus Reverse Transcriptase during Reverse Transcription of Rare t(8;21) Fusion Gene Transcripts

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ABSTRACT

The resolving power of RT-PCR is limited by the efficiency of RNA-to-cDNA conversion. Methods to determine this efficiency, using a real-time PCR assay for quantifying AML1-MTG 8 *[t(8;21)] fusion gene transcripts, are described. The efficiency is shown to be directly proportional to RNA template levels. The Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme's conversion efficiency was calculated to be approximately 20%. The efficiency was even lower (6%) when target templates were rare (single molecules) in the RT reactions. Levels of nonspecific or background RNA present in the RT reaction reduced the reverse transcriptase's conversion efficiency. This background effect was particularly pronounced when the specific template was present in rare amounts.*

INTRODUCTION

Reverse transcription (RT) of RNA to cDNA combined with real-time PCR is widely employed for a variety of molecular assays. Assays to detect fusion gene transcripts commonly found in leukemia and lymphomas are such an example (2,8). These assays permit the detection of minimal residual disease in chemotherapy-treated patients and can be used as biomarkers for the return of the disease (1,4,6) or for the occurrence of novel therapy-related translocations (3). With few exceptions, the detection of cells bearing translocation-generated fusion transcripts represents the extreme limits of this technology's resolution. The initial RT step, which converts the fusion gene's mRNA transcript into a single strand of cDNA, is fundamental to the resolution of these assays. Currently, little is known about the efficiency of the reverse transcriptase enzyme commonly used to perform this initial conversion: a modified Moloney murine leukemia virus (MMLV) reverse transcriptase.

Commercially available MMLV reverse transcriptases have been modified to remove the inherent RNAse H activity and thus improve the potential cDNA yields. Commercial manufacturers suggest improved yields with this modified enzyme but fail to provide any indication of actual conversion efficiencies. Here we describe simple, real-time RT-PCR methodologies to determine the specific conversion efficiency of MMLV reverse transcriptase. One assay relies on a limiting dilution step of a reacted RT cocktail before detection with real-time PCR and the ability of that PCR to detect a single full-length cDNA. The other approach relates known input RNA quantities to detected output cDNAs. Both of these two different approaches were taken, as the first permitted the efficiency to be accurately determined when relatively small amounts of target RNA are available for conversion, whereas the second approach was effective for much larger amounts of RNA template.

A leukemic fusion gene is generated by a translocation [t(8;21)] occurring between chromosomes 8 [acute myeloid leukemia 1 (*AML1*)] and 21 [myeloid leukemia gene on chromosome 8 (*MTG8*)]. This fusion gene was chosen because a real-time RT protocol had previously been described (5) and is currently being utilized in house. The assay is highly sensitive, and previous reports purport to detect three copies of cDNA (5).

Effects of a non-target background RNA on the efficiency of the RT reaction, and thus the assay's ability to detect rare RNA transcripts (*AML1-MTG8*) within a neutral background, is described. Whereas the data presented here are specific to the t(8:21) fusion gene RT-PCR, the results are likely indicative of the reverse transcriptase's potential efficiency.

MATERIALS AND METHODS

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, Austin, TX, USA) as directed. Residual plasmid DNA was significantly reduced by additional DNase I (Roche Applied Science, Basel, Switzerland) digestion steps and purification of the RNA runoff transcript on RNeasy® spin columns (Qiagen, Valencia, CA, USA). After these additional steps, contaminating plasmid DNA could not be detected in 1 ng of the RNA runoff transcripts by the real-time PCR assay.

RT of the RNA runoff transcripts was performed in a 20-µL reaction. Transcripts were mixed with 4 nM specific MGT8 (7) primer (T12, 5′-AGGCTGTAGGAGAATGG-3′) (MWG Biotech, High Point, NC, USA). RNA background, when employed, consisted of human colon total RNA (Stratagene, La Jolla, CA, USA). This background RNA was repeatedly used as a negative control and never demonstrated the presence of the fusion gene transcripts. RNA (runoff transcripts and background) and primer mixtures (10 µL) were annealed by an initial heating to 65ºC for 5 min and then cooled to room temperature. A 10-µL RT cocktail [1× RT buffer (Stratagene), 100 µM dNTPs (Roche Applied Science), 4 U Prime RNase Inhibitor™ (Eppendorf, Hamburg, Germany), 25 U reverse transcriptase (Stratagene)] was then added to the annealed RNA/primer mixtures and reacted at 42ºC for 50 min, followed by 95ºC for 5 min.

Real-time PCR was used to detect full-length cDNAs (molecules that extend from the T7 primer past the furthest PCR primer, <690 bp). Real-time primers and TaqMan™ probe were as described by Marcucci et al. (5). One correction to the *AML1-MTG 8* reverse primer was made (5′-ATCCACAGGTGAGTCTGGCATT-3′) that removed a single adenine residue after the underscored adenine. An initial range finding RT-PCR experiment utilized four separate RT reactions with RNA runoff transcript template diluted over four orders of magnitude. Completed 20-µL RT reactions were diluted with an equal amount of water, and 5 µL were then

Table 1. Efficiency of Stratascript™ Reverse Transcriptase (Stratagene) in Converting RNA Runoff Transcript into Full-Length, PCR-Detectable cDNA

Background RNA	Runoff RNA Molecules	Positive Reactions	Total Reactions	C_t of Positive Reactions ± SD	Efficiency
None	70	12	48	39.5 ± 0.7	19.7%
1 μg/reaction	70	4	48	40.6 ± 0.5	6.0%

Efficiency percentage was calculated using the Poisson [-ln (negative reactions/total reactions)/possible number of cDNA molecules)]. Each of the 48 reactions had the potential to contain 1.458 (70/48) cDNAs, assuming 100% conversion efficiency. The C_t values indicate that the positive reactions contained just one detectable cDNA, thereby permitting the use of the Poisson calculation.

added to a 200-μL real-time PCR (100 μL TaqMan Master Mix reagent (2×) (Applied Biosystems, Foster City, CA, USA), 6 nM both forward and reverse primers, and 0.6 nM TaqMan probe (MWG Biotech). This total reaction was then distributed into 20 10-μL reactions and run on the ABI PRISM® 7700 real-time PCR machine (Applied Biosystems) using the standard cycling conditions but with 45 rather then 40 cycles. Positive reactions were then scored. The RT reactions that yielded less than 20 positive reactions, and thus being within range for limiting dilution analysis, were then further analyzed. Part of the remaining diluted RT reaction (20 μL) was added to 950 μL real-time PCR cocktail, the mixture distributed across 96 separate PCR tubes, and then reacted. Positive wells were scored, and the reverse transcriptase's efficiency was determined.

Statistical analysis was performed using Statistica®, a commercially available statistical software package (Statsoft, Tulsa, OK, USA). Differences between proportions and means were tested using a Student's *t* test. Poisson analysis was used to determine efficiencies for the limiting dilutions and is described below. Log-linear regression was performed to relate input RNA values to output cDNA values.

RESULTS

Single-Molecule PCR Templates Sufficient to Generate a Real-Time PCR Signal

To demonstrate that a single template will generate a real-time PCR signal, a *Sac*I-linearized plasmid was serially diluted down to the attogram range. Figure 1 shows the results of these real-time PCRs (10 μL) seeded with 0, 0.5, 1, 5, or 10 plasmid molecules. Real-time PCR offers quantifying abilities, as demonstrated through the cycle threshold (C_t) values that vary in proportion to the starting template amounts. Thus, single-molecule templates in this experiment yielded C_t values of around 39.5, whereas five-molecule template reactions had C_t values of 37.5. Of course, one must appreciate that it is technically impossible to place precisely one, two, or three molecules into a reaction, but an average distribution around those numbers was obtainable. Single plasmid molecules are thus detectable by the real-time PCR assay.

Non-Target Background RNA Does Not Affect PCR Results

There was concern that the non-target background RNA used in the RT reaction might have some influence on the PCR results. To demonstrate that this background RNA has no effect, a single RT reaction was prepared without background RNA, and the cDNA was used as template. Real-time PCRs containing the cDNA were split into eight replicates that contained background RNA and eight replicates that did not. Several different amounts of cDNA template were used, and the mean C_t values were calculated. Background RNA had no effect on the mean

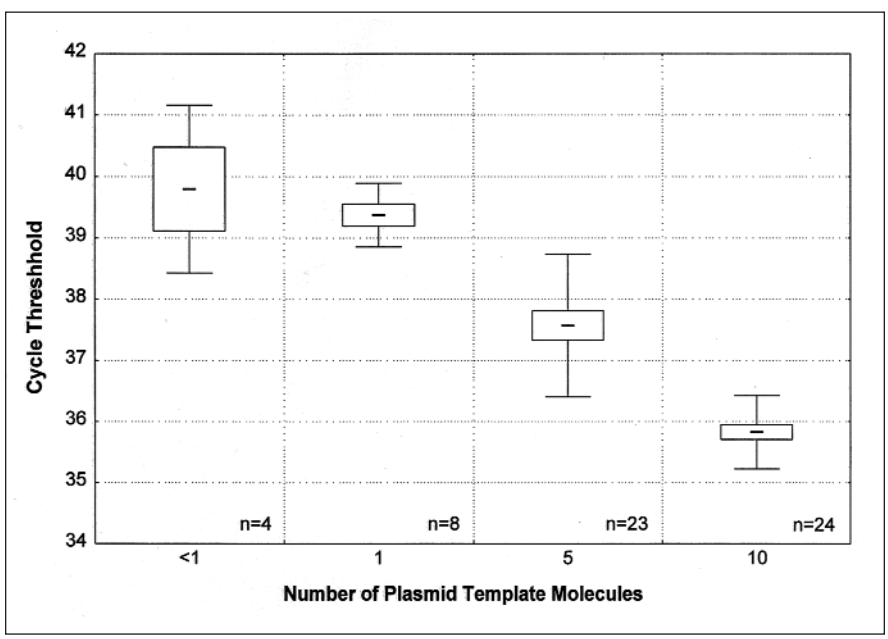

Figure 1. Mean C_t values, standard deviations, and error obtained from single and multiple numbers of initial template molecules. Plasmid bearing the *AML1-MTG8* fusion gene is approximately 4100 bp (2.5×10^6 g/mol), and 100 ag consists of 25 individual molecules. PCRs (10 μL) were seeded with 0, 0.5, 1, 5, or 10 plasmid molecules in replicates of 24, and data from positive reactions were averaged. Means are indicated by bars with standard errors (boxes) and deviations (whiskers).

Table 2. Calculation of Stratascript Reverse Transcriptase Efficiency Starting with 500 ag (1400 Molecules) Runoff RNA Transcripts

Background RNA	No. cDNA molecules/ C_t range	Mean C_t ± SD	Positive reactions	Total cDNA molecules	Total cDNA molecules of possible 700
No Background	3/≤37.5	36.9 ± 0.5	4	12	
Overall C_t	2	38.8 ± 0.6	43	86	135
39.3 ± 1.03	>37.5 to 39.5				19.3% efficiency
	1/≥39.5	40.2 ± 0.4	37	37	
With Background	3/≤37.5	37.5	1	3	
1 µg					
RNA/reaction	2	38.8 ± 0.5	26	52	89
	>37.5 to 39.5				12.7% efficiency
Overall C_t	1/≥39.5	40.5 ± 0.8	34	34	
39.7 ± 1.23					

The C_t ranges of the one, two, and three cDNA molecule reactions were abstracted from Table 1. The efficiencies are calculated from the total number of cDNA molecules reverse-transcribed from a possible 700 RNA molecules (as half of the original reaction was limited by dilution). The Poisson calculation was not used, as the widely ranged C_t values indicated that there were more than one cDNA molecules in the positive reaction. A simple fraction calculation was sufficient to determine the reverse transcriptase efficiencies from this data. Negative reactions are not shown, but there were 10 (no background) and 35 (background).

C_t values obtained (data not shown). Therefore, inclusion of background RNA in RT reactions does not have any influence on the PCR results.

Determination of RT mRNA Conversion Efficiency

Whereas manufacturers have typically determined rates at which isotopes are incorporated into precipitated cDNAs, the approaches taken here attempt to determine an actual conversion efficiency of RNA template into PCR-detectable (full-length) cDNA molecules. Determining the actual efficiency of the reverse transcriptase's conversion of RNA template into cDNA is complicated by several technical difficulties. One significant challenge is in quantifying the number of reverse-transcribed cDNA molecules. Real-time PCR is ideally suited to overcome this challenge. Another technical difficulty is determining the conversion efficiencies for RT reactions where the RNA template is at very low levels (1–100 RNA molecules). Again, real-time PCR meets this challenge with its extremely broad dynamic range for detection.

Two different limiting dilution approaches were undertaken in an attempt to determine accurately the efficiency of the reverse transcriptase during the RT of rare target RNA into cDNA. The first approach utilized RT reactions containing hundreds of RNA runoff transcripts templates that were reacted, and the cDNA products were then diluted over many PCR tubes. In this way, cDNA molecules generated in the RT step are limited by dilution such that only one template on average will be present in each PCR. Thus, a positive PCR indicates a single cDNA template. For this approach to accurately access the RT enzyme efficiency, the dilution must be sufficient to limit just one or less cDNA molecules per reaction. This can be assessed by the C_t values generated. A total of 96 PCRs were used to screen two separate RT reactions performed with

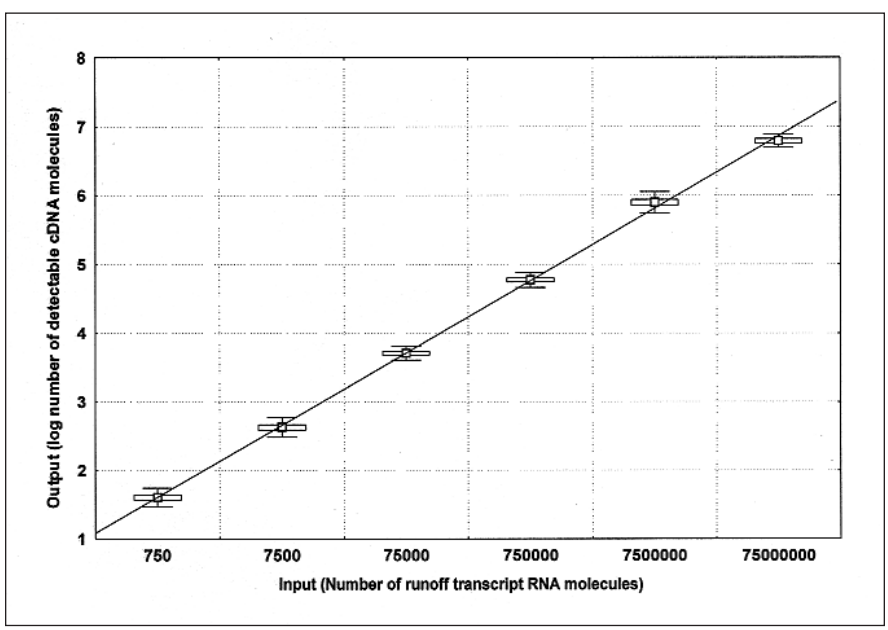

Figure 2. Efficiency of the Stratascript reverse transcriptase in converting RNA runoff transcripts into detectable full-length cDNA.

and without background RNA (1 µg). Each RT reaction contained 50 ag runoff transcript RNA, which is approximately 140 copies. Half of the completed RT reaction was limited over 48 PCR tubes and assayed. Table 1 shows the results of this survey. The mean C_t values are identical to those values for single templates (Figure 1); thus, the positive reactions contained just one single detectable cDNA molecule. Of the possible 140 runoff RNA molecules available for cDNA conversion, just a fraction was converted. A simple calculation using the Poisson distribution for limiting dilution analysis was then used to determine the conversions efficiencies (Table 1). The presence of background RNA had a considerable effect and lowered the RT efficiencies by nearly 3-fold ($P = 0.04$). This method is particularly good at determining the enzyme efficiency when a relatively small number of RNA templates are available for conversion, as single full-length cDNA products can be scored.

The second approach taken was to convert a relatively small number of RNA template molecules and then again to limit those cDNA molecules by dilution, but also to use the quantification power afforded by real-time PCR. When an RT reaction containing 700 RNA runoff transcripts was diluted across 96 PCR tubes, 84 positive reactions (without background RNA) and 61 positive reactions with background RNA were detected (Table 2). However, in these experiments, the range of C_t values is much greater, between 36.9 and 41.5, indicating that some of the positive reactions contained more than one cDNA molecule as PCR template. In this case, the positive tubes can be subdivided into tubes that contain one or more cDNA templates on the basis of the C_t value generated and the total number of cDNA molecules determined. With this procedure, the RT reaction efficiency was 12.7% and 19.3% with and without background RNA, respectively. Again, the effect of the background significantly impedes the efficiency of the reverse transcriptase in reverse-transcribing the RNA runoff transcripts ($P < 0.002$).

While these two scoring methods are useful at determining the RT efficiency when only a relatively small number of target RNA is available, they would obviously fail with higher template amounts. To further explore this issue, the RT efficiency was sought using a bulk approach. With this approach, the power of real-time PCR to quantify PCR templates is fully brought to bear. A standard curve of C_t values was generated for known amounts of plasmid bearing the fusion gene. RT reactions containing known amounts of runoff transcript (750–750 000 copies) were also screened alongside the plasmid standard curve (1–10 000 copies). The sequence detector software permits unknown values to be obtained through the use of the standard curve C_t values. Each standard curve point was repeated four times, and each runoff transcript was repeated in three or four different RT reactions with or without the addition of background RNA (1 µg/reaction).

For each RT reaction, the amount of input template (mRNA, the runoff transcript) was known, and the C_t values obtained permitted the number of output molecules (cDNA, full-length and amplifiable) to be determined from the standard curve. There was little or no difference between linear regressions derived from experiments with or without background RNA (likelihood ratio test, $P = 0.88$). For this reason, the two data sets are combined. Figure 2 plots the number of input mRNA molecules against the number of detected output molecules ($y = -0.839 + 1.037\times$, $R^2 = 0.98$, $P < 10^{-6}$). A clearly linear relationship exists between RNA input and the detected cDNA output. Over the range of relevant input concentrations (100–100 000) the log-linear regression implies about a 20% efficiency (17%–22%). With this bulk approach, the efficiency of the RT reaction is in the range of the previous methodological calculations. However, the inhibitory effect of the background RNA observed with the previous methods was not seen with this bulk approach. This is likely because, in the bulk approach, higher amounts of template are contained within the background RNA, while the previous methods employed relatively rare template in the same amount of background. With the smaller numbers of template, correct priming of the RNA template is likely to be hindered by background RNA.

DISCUSSION

Efficiency of the MMLV reverse transcriptase is influenced by several factors, and the resolution of the assay described here is clearly limited by the conversion efficiencies at the RT step. The effects of background RNA are noticeable only when relatively small numbers of RNA template are available for conversion to cDNA. This effect is absent when much larger amounts of RNA template are available for RT. However, the overall efficiency of the reverse transcriptase was determined to be only approximately 20%, which is to say that only 20% of a given RNA template will be converted into PCR-detectable cDNA. Conversion efficiency was considerably lower (6%) when RNA templates in the RT reactions were exceedingly rare. With efficiencies this low, one could expect that less than 1 out of 10 RT reactions would yield a suitable PCR-amplifiable cDNA for detection. At such rare levels, the assay will be unable to confidently detect rare transcripts. The obvious solution would be to increase the number of RT reactions so as to dramatically increase the quantity of starting RNA in which the target template is rare.

It should be noted that the quality of the RNA runoff transcript will directly affect these kinds of experiments, and care should be taken to produce high-quality RNA. The reverse transcriptase efficiency should be considered when employing this widely used enzyme. Assays to determine mRNA expression will be directly affected by this limited enzyme efficiency, and heed should be taken when preparing and interpreting standard curves used to determine RNA expression levels. Methods to empirically determine the efficiency of reverse transcriptases have been described, and their usefulness in determining which enzyme to employ in RT-PCR assays is demonstrated.

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