Analysis of One-Step and Two-Step Real-Time RT-PCR Using SuperScript III

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Real-time reverse transcription polymerase chain reaction (RT-PCR) is a commonly used technique to analyze gene expression. There has been little research conducted to test if Super-Script III quantitative one-step (reverse transcription carried out in the same tube as PCR) and two-step (reverse transcription carried out in a separate reaction) RT-PCR systems provide similar real-time results. In this study, real-time reactions were set up using the housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH), β_2-microglobulin (B2M), and RNA polymerase 2 subunit A (PolR2A). Reaction efficiencies were determined by generating standard curves using total RNA isolated from human skeletal muscle and brain. Reaction efficiencies ranged from $97.7 \pm 0.9\%$ to $99.4 \pm 1.8\%$ for one-step and $98.0 \pm 0.2\%$ to $102.6 \pm 1.3\%$ for two-step RT-PCR (R^2 values for all reactions ≥ 0.995). The sensitivities of one-step and two-step methods, as measured by cycle threshold values, were similar for GAPDH and B2M. However, for the lesser expressed PolR2A mRNA there was a 5 cycle lower threshold for one-step. In summary, both Super-Script III one-step and two-step methods yield reaction efficiencies close to 100% and produce similar, accurate, linear standard curves. However, using the one-step method with gene-specific priming may be more sensitive for quantification of certain genes such as PolR2A.

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Real-time, or quantitative reverse transcription polymerase chain reaction (RT-PCR), has become an increasingly popular technique for the analysis of gene expression.[1-3] However, there are two primary ways that real-time RT-PCR can be carried out. One method involves including the RT step into the same tube as the PCR reaction (one-step). The other method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR reaction (two-step).

There are advantages and disadvantages to both systems. The advantages to one-step real-time RT-PCR is that it is quicker to set up, less expensive to use, and involves less handling of samples, thereby reducing pipetting errors, contamination, and other sources of error. With the one-step method, gene-specific primers are used and both the RT and PCR occur in one reaction tube; therefore, other genes of interest can not be amplified for later analysis. The RNA from the original sample must be initially aliquoted for archival storage and future testing. The main advantage to two-step RT-PCR is that typically random hexamer or oligo dT primers are used in an RT reaction in a separate tube. This allows for the ability to convert all the messages in an RNA sample into cDNA, which would allow for archiving of samples and future testing of other genes.

Typically, scientists choose one-step or two-step methods based on these factors. However, there are other factors involved with each method that might produce differing real-time results. First, despite the luxury of being able to choose the method of priming (gene specific, random hexamer, or oligo dT), different methods of priming in the RT step have been shown to provide different sensitivities and efficiencies.[4-8] This may not only affect the run of the samples in real-time RT-PCR but also may affect the generation of the standard curve. In a review by Bustin and Nolan, results show that there is more linearity in a real-time run with template dilutions using gene-specific primers than using random hexamers.[1] Therefore, choosing priming meth-

ods in two-step RT-PCR such as oligo dT or random hexamers that would allow for ease of use in archiving, may lead to altered real-time results.

It has also been shown that the total amount of RNA in the reaction can affect RT efficiency.[1,5,9] This is especially of concern in one-step RT-PCR in generating the standard curve. If the RT enzyme has a different efficiency depending on the concentration of the RNA that is in each tube, then the cDNA being created will not be proportional or linear for each standard. This will effect the generation of PCR products and the generation of the standard curve (reaction efficiency will not be able to be accurately measured due to a low R^2 value in calculating the line of best fit). This would be less of a problem in the two-step method where cDNA is created first in one tube and then dilutions are generated from this tube.

There also may be a difference in stabilities of mRNA and cDNA. Typically it has been thought that DNA is more stable than mRNA and therefore cDNA is better to use and store. However, recently it has been demonstrated that mRNA may actually be more stable than the cDNA created after the RT step.[10] These differences in stability of cDNA and RNA may be a factor with use over long periods of time and with incubation times and temperatures used in one-step and two-step reactions.

These different factors involved with one-step and two-step methods may affect the results in a real-time run. While there has been research conducted on differences in priming strategies for real-time PCR,[1,4-8] there has been little research conducted directly comparing real-time one-step and two-step methods incorporating the different priming strategies. The few reports that have directly compared real-time one-step and two-step methods did not use the same real-time enzyme mixtures as our study (Invitrogen's SuperScript III quantitative RT-PCR kits) and obtained differing results.[6,7] Recently, Invitrogen developed SSIII which is a modified form of the MMLV-RT (Moloney murine leukemia virus-RT) which has increased thermal stability (45°C–60°C) and has gained popularity in use both in regular RT-PCR and real-time RT-PCR. There are some specific benefits to using this real-time system. The SSIII enzyme has been advertised to provide more full-length product than other RT enzymes, to reduce RNase H activity, and to be stable at increased temperatures allowing for higher RT incubation temperatures. In addition, the SSIII two-step kit utilizes a mixture of random hexamers and oligo dT in order to generate cDNA, and both one-step and two-step kits utilize Platinum Taq which is a hot-start enzyme for increased specificity.

In order to test one-step and two-step methods in various conditions, total RNA from two different tissue types were obtained (human skeletal muscle and brain) and three differently expressed genes were purposely chosen. The housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH), β_2-microglobulin (B2M), and RNA polymerase 2 subunit A (PolR2A) were selected for this study. These genes are universally expressed and have been used for real-time comparative gene expression studies. In addition, we specifically picked these genes because they differ in their relative expression level (GAPDH—high expression, B2M—high to intermediate expression, PolR2A—low expression).[11]

In real-time PCR reactions, generation of standard curves, calculation of reaction efficiency, and assessing the sensitivity of detection are critical to the accuracy of the results. Analysis of these factors in one-step and two-step kits using different tissue types and genes of variable expression levels is important information in selecting the proper real-time methodology. Therefore, the goal of this investigation was to determine if SSIII one-step and two-step systems had the same reaction efficiencies for differentially expressed genes and if one system was more sensitive than another.

MATERIALS AND METHODS

RNA, Primers and Probes, and Real-time Kits

Human skeletal muscle and whole brain total RNA were obtained from Ambion (Austin, Texas). The RNA comes in a 1 μg/μL concentration and the 260/280 absorbance ratio is between 1.7 and 2.1. Primers and probes were obtained from ABI (Foster City, California) TaqMan Gene Expression Assay catalog (GAPDH, Hs99999905_m1; B2M, Hs99999907_m1; PolR2A, Hs00172187_m1). These assays come in a 20× reaction mix, span an exon-exon junction, and are optimized to give close to 100% efficiency. The real-time RT-PCR reactions were carried out using SuperScript III Platinum One-Step Quantitative RT-PCR System and SuperScript III Platinum Two-Step Quantitative RT-PCR Kit made by Invitrogen (Carlsbad, California). Reactions were set up using an automated liquid handling system (CAS-1200, Corbett Robotics, Sydney, Australia) and real-time runs were performed on the Corbett-Research Rotor Gene 3000 (Sydney, Australia).

Protocols

Manufacturers' instructions were followed for setting up both the one-step and two-step reactions. Briefly, for one-step, the liquid handling system created a mastermix using 2× reaction mix, SSIII RT/Platinum

Taq Mix, magnesium (5 mM final concentration), and 20× primer/probe. Mastermix and sample were then added to each tube (20 μL total volume) and contents mixed. The RT step involved incubation at 55°C for 20 min. The PCR cycling conditions included an initial denaturation of 95°C for 3 min followed by 45 cycles of 95°C for 15 sec and 60°C for 45 sec. For the two-step method, the first strand cDNA synthesis was created in a total volume of 40 μL. The RT conditions were the same as one-step except that a 10-min incubation at 25°C was included at the start (due to the use of a mix of random hexamers/oligo dT primers) and a 5-min incubation at 85°C was included at the end (to terminate the RT reaction) as recommended by the manufacturer. For the PCR stage, the liquid handling system created a mastermix using 2× Platinum Quantitative PCR Supermix-UDG, magnesium (final concentration of 5 mM), and 20× primer/probe. Mastermix and cDNA sample were then added to each tube (20 μL total volume) and the contents mixed. The PCR cycling conditions were the same as for one-step except a 50°C incubation step (2 min) was included before the initial denaturation. For both one-step and two-step methods, control tubes for each gene that contained water instead of template RNA or cDNA were also run under the same conditions (no template controls).

In order to measure reaction efficiency a standard curve was generated using the standards of 1000, 100, 10, 1, and 0.1 ng of total starting RNA. The standards were generated by the automated liquid handling system using 10-fold serial dilutions made from either the starting RNA (for one-step) or from the same amount of starting RNA converted to cDNA (for two-step). Therefore, the same amount of starting RNA template was used for both one-step and two-step methods. For each reaction, the cycle threshold was determined as the cycle number at which the fluorescence value reached a threshold level. The threshold level was set above the background fluorescence in the exponential phase of the real-time curves.

Data Collection and Analysis

For each run, a standard curve was generated with log of the RNA concentration on the X-axis and cycle threshold on the Y-axis. A line of best fit was generated using the five concentration data points and the slope of this line as well as R^2 values were calculated. The slope of the line (m) was used to determine reaction efficiency with the following equation: $RE = [10^{(-1/m)}]/2 *100$. Using this equation, the reaction efficiency of an ideal PCR reaction where a doubling of the product occurs during each cycle would be 100%. Two runs were performed with each data point run in triplicate. Coefficient of variation (CV = standard deviation/mean *100) was calculated between runs as well as between the averages of one-step and two-step reactions.

The sensitivity of one-step versus two-step methods was compared using the cycle threshold values. The same threshold value was used in order to compare the cycle threshold values of the methods. For ease of comparison the 100-ng starting template amount was used for all comparisons of sensitivity.

RESULTS

Five dilutions of total RNA were tested with each gene and for each tissue type. A representative real-time reaction is shown in Fig. 1A with B2M. Each reaction was run in triplicate and these points were plotted on a graph of log of RNA concentration versus cycle threshold (Fig. 1B). The slope of this line was used to determine reaction efficiency. Table 1 demonstrates the reaction efficiency, R^2 values calculated from the line of best fit, and coefficient of variation comparing one-step and two-step reactions.

The standard curves that were generated in these runs produced linear results. The lowest R^2 value for the line of best fit for any experiment was 0.995. In general, all reaction efficiencies obtained were close to 100% and the efficiencies of one-step vs. two-step reactions were not appreciably different (Table 1). Comparing all genes tested, the one-step method obtained similar reaction efficiencies for all genes, while there was a slightly greater variability in reaction efficiency in the two-step method (with GAPDH being lower than B2M or PolR2A) (Table 1).

The variability between real-time runs was low. The largest reaction efficiency CV for one-step was 2.46% for skeletal muscle (B2M) and 2.03% for brain (B2M). The largest CV for two-step was 1.80% for skeletal muscle (PolR2A) and 1.24% for brain (PolR2A). In analyzing the CV for the data, there was slightly greater inter-assay variability in one-step reactions than two-step reactions.

The real-time reaction for the 100 μg template concentration was used to compare cycle threshold (CT) values and sensitivity between methods. CT values for all genes with each method are listed in Table 2. GAPDH and B2M CT values are similar comparing one-step and two-step reactions with the largest difference being 1 cycle using GAPDH in brain tissue. However, there was roughly a 5-cycle difference in both muscle and brain tissue with PolR2A with one-step being more sensitive. A 5-cycle difference indicates a 32-fold change in detection. This number is calculated by the
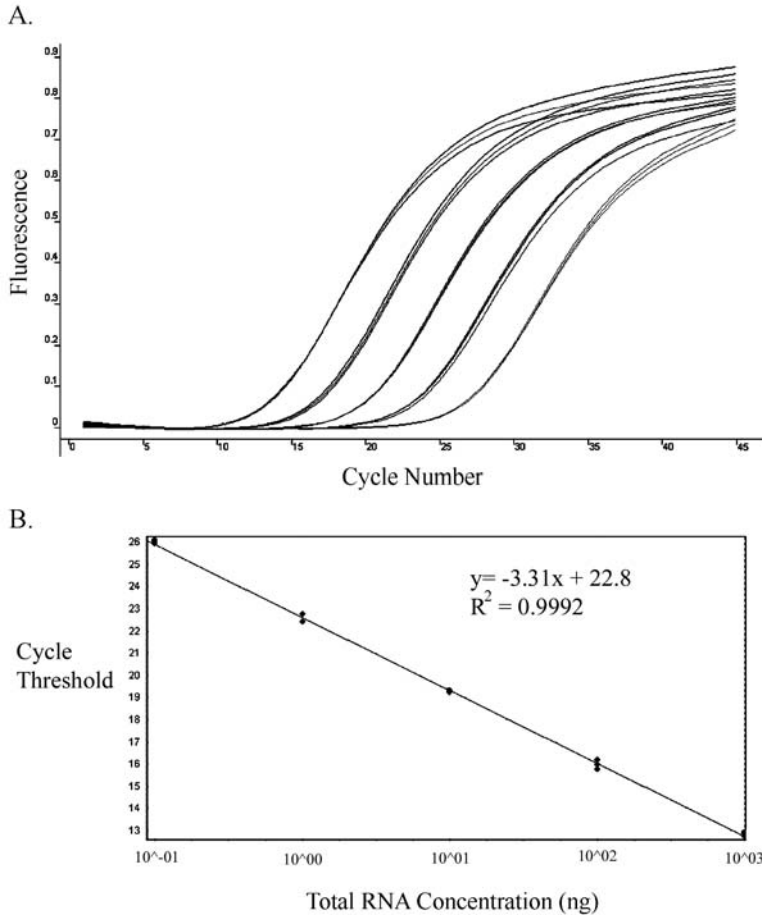
FIGURE 1

Real-time reaction run with β_2-microglobulin primer/probe and the SSIII one-step quantitative reverse transcription polymerase chain reaction kit. **A:** Real-time fluorescence using 1:10-fold dilutions of total RNA from skeletal muscle run in triplicate. **B:** The standard curve generated from the cycle thresholds of each of the dilutions and the equation of the line of best fit.

T A B L E 1

Reaction Efficiencies for One-Step and Two-Step Methods

Muscle	One-Step RE (%)	R2	Two-Step RE (%)	R2	CV (%)
Muscle					
GAPDH	99.4 ± 1.8	0.999	98.0 ± 0.7	0.999	1.0
B2M	99.0 ± 2.4	0.999	102.2 ± 0.3	0.999	2.2
PolR2A	99.2 ± 1.2	0.998	102.2 ± 1.8	0.996	2.1
Brain					
GAPDH	97.7 ± 0.9	0.999	98.0 ± 0.2	0.999	0.2
B2M	99.3 ± 2.0	0.998	101.8 ± 0.1	0.999	1.7
PolR2A	98.8 ± 1.1	0.997	102.6 ± 1.3	0.997	2.6

Reaction efficiency = RE = [10^(1/m)]/2 *100 where m = slope of the line of best fit.

Coefficient of variation = CV = standard deviation/mean *100.

TABLE 2

Cycle Threshold Values for One-Step and Two-Step Methods

	One-Step CT	Two-Step CT
Muscle		
GAPDH	12.8 ± 0.3	13.1 ± 0.2
B2M	15.3 ± 0.7	14.5 ± 0.2
PolR2A	21.1 ± 0.7	26.2 ± 0.2
Brain		
GAPDH	13.1 ± 0.3	14.1 ± 1.0
B2M	17.0 ± 0.1	16.6 ± 0.6
PolR2A	19.0 ± 0.5	24.1 ± 0.8

CT, Cycle threshold.

formula $2^{\Delta CT}$ where 2 is used assuming a doubling in every cycle or 100% efficiency in the reactions.

No template controls for GAPDH, B2M, and PolR2A were also run. There was no detectable increase in fluorescence for one-step or two-step runs with GAPDH or PolR2A after 45 cycles. There was an increase in fluorescence in the no template controls tubes for B2M in the one-step and two-step runs. These CT values were typically 5 cycles higher than the CT values for the smallest concentration of RNA (0.1 ng) that was used in the experiment.

DISCUSSION

The generation of standard curves and determination of reaction efficiencies of primer/probes is used in almost all real-time RT-PCR gene expression studies and is critical to obtaining accurate results. Both one-step and two-step methods are used in generating real-time data. However, it is still uncertain if differences in methodologies alter real-time RT-PCR results. Therefore, the purpose of this study was to determine the accuracy and consistency of results between SSIII one-step and two-step real-time RT-PCR methodologies.

There are several factors that may alter the real-time results between one-step and two-step real-time RT-PCR. A concern for the one-step reaction is that it has been shown that the reaction efficiency for RT enzymes varies with RNA concentration.[5,9] A concern for the two-step reaction is that the priming method may alter the linearity of the run.[1] If these were significant factors that alter the results using either the one-step or two-step SSIII methods, then standard curves and the line of best fit would not be able to be accurately generated. However, our results demon-

strate that the reaction efficiencies were close to 100% and the R^2 values were above 0.995 for both the one-step and two-step kits. Therefore, we conclude that these factors did not play a significant role in our study. Likewise, factors such as the tissue type and gene expression level also did not significantly alter the reaction efficiencies or R^2 values during the real-time runs. Comparable lines of best fit on the standard curve were obtained from RNA of two diverse tissue types (human muscle and brain tissue) as well as between differentially expressed genes.

While there was little difference in reaction efficiency and R^2 values between one-step and two-step methods, there was a difference in sensitivity of detection between methods with one of the genes tested. With the genes that were expressed in relatively high quantities (GAPDH and B2M) there was little difference in sensitivity between one-step and two-step methods. However, for the lower expressed PolR2A mRNA there was roughly a 5-cycle difference between one-step and two-step methods in both tissues studied with the one-step method being more sensitive. Assuming 100% efficiency and a 5-cycle difference, there was roughly a 32-fold difference in detection levels between one-step and two-step methods for this gene. Since the same PCR conditions and enzyme were used, the difference was probably due to the conditions in the RT reaction and was most likely related to a reduced priming efficiency in the two-step kit for this gene. It is possible that secondary structure in the PolR2A mRNA could have been responsible for the results. Random hexamers in the two-step method require a pre-incubation period at 25°C before the 55°C incubation. This incubation temperature allows the random hexamers to bind to the target and it is possible that secondary structure formation in the RNA may have hindered the binding of the primers or activity of the RT and thus reduced the production of cDNA. It is also possible that the low abundance of PolR2A mRNA may have been responsible for the results. Since the mRNA was in lower copy number, gene specific primers used in the one-step kit may have been more efficient at generating full-length cDNA than the random hexamers and oligo dT primers in the two-step kit. While several of these factors may play a role in the difference in sensitivity, our findings provide support for the use of the one-step method for increased sensitivity of detection of certain genes such as PolR2A.

A previous report has shown that the two-step method was more sensitive than the one-step RT-PCR reaction.[7] These authors used AMV RT (avian myeloblastosis virus reverse transcriptase) and Tfl DNA polymerase for the one-step and AMV RT and AmpliTaq Gold for the two-step. This work was pub-

lished in 1999 and since that time significant improvements in development of enzymes have been made in one-step reaction kits. In our work with the SSIII enzyme kits, the one-step and two-step kits were comparable and the one-step was even more sensitive for PolR2A.

A report published in 2004 by Peters et al. using an alpha-chain assay has compared the use of Invitrogen's ThermoScript (AMV-RT) one-step system using gene specific primers and ThermoScript two-step system using random hexamers.[6] In their study, the reaction efficiencies were similar in one-step and two-step methods and the sensitivity of the one-step with gene specific primers was slightly better than two-step which is similar to results from our study with SSIII one-step and two-step methods. Stahlberg et al. have tested SSIII versus several other RT enzymes (including ThermoScript) and found that SSIII gave the highest overall yields for six genes studied.[9] These data indicate that the SSIII kits may perform similarly to the ThermoScript kits in real-time reactions without the increased cost of using ThermoScript.

Interestingly, Peters et al. also tested the SuperScript II (MMLV-RT) two-step method and obtained a low reaction efficiency and sensitivity.[6] The authors concluded that AMV was more sensitive and had better efficiencies than MMLV. However, these results may be because of the lower incubation temperature for the RT step required with SuperScript II which may allow for increased RNA secondary structure.

Using the SSIII kits with the ABI TaqMan Gene Expression Assay primer/probes provided accurate real-time data in our experiments. In our experiment we found that these primer/probe sets worked effectively in Invitrogen SSIII real-time one-step and two-step kits. Therefore, these two components from different companies seem compatible for accurate real-time experimentation.

Employing the proper real-time kits, primer/probes, and methodology is critical to the development of a successful real-time experiment. Our results show that both one-step and two-step SSIII real-time RT-PCR methods in combination with the ABI primer/probes generate similar linear standard curves and have reaction efficiencies close to 100% that can be used to produce accurate real-time results. However, the sensitivity of the two methods may vary if random hexamers/oligo dT primers are used for the two-step. The one-step method with gene-specific primers appeared to be significantly more sensitive with a gene like PolR2A. The results of this study may help to provide more information to researchers in selecting the desirable method for real-time RT-PCR.

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