

# Greatly Improved Real-Time RT-PCR from Small Quantities of Degraded RNA Samples

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## Introduction

Real-time or quantitative RT-PCR (qRT-PCR) using RNA isolated from small populations of cells can be challenging, due to lack of sensitivity and reproducibility. In addition, only a limited number of reactions can be performed, due to the small amount of RNA template available. These problems are compounded when the RNA sample is compromised or degraded, e.g., samples from laser-capture microdissection, paraffin-embedded tissue, or cell-sorting procedures. The new MessageBOOSTER™ Whole-Transcriptome cDNA Synthesis Kit for qPCR uses rapid and easy RNA amplification and cDNA synthesis procedures that greatly increase the sensitivity, reproducibility, and number of qRT-PCR reactions that can be obtained from a small sample of RNA. In addition, the kit includes a unique population of mixed-sequence primers to prime cDNA synthesis from sites throughout the transcriptome. Using these primers virtually eliminates the 3' sequence bias that is often associated with oligo(dT)-based RNA amplification methods.

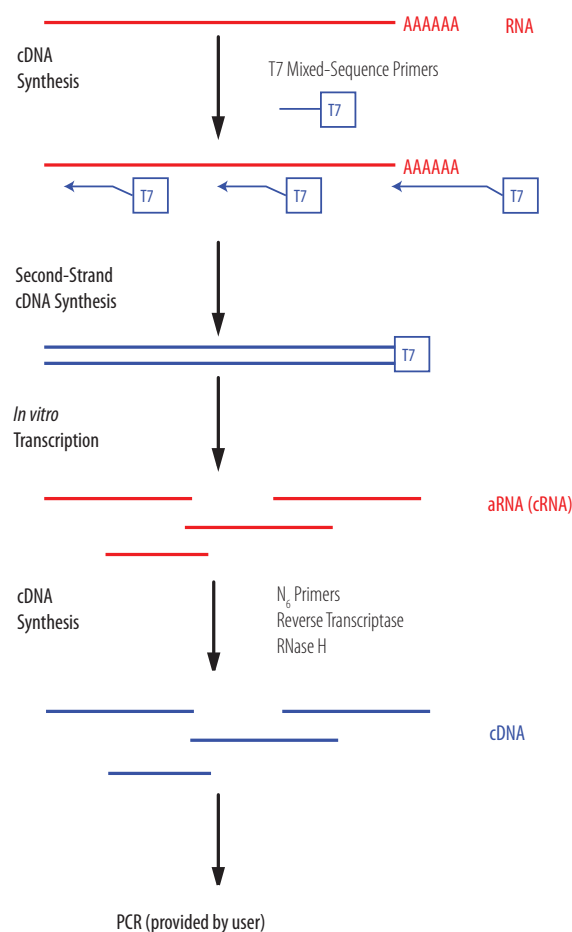
In this report, we demonstrate that a MessageBOOSTER Whole-Transcriptome Kit reaction produces amplified cDNA from as little as 10 ng of partially degraded RNA, and 100 ng of severely degraded RNA. The amplified cDNA has a greatly improved 3'/5' ratio (reduced 3' bias) compared to that produced by a standard oligo(dT)-primed RNA amplification procedure.

## Methods

A MessageBOOSTER Whole-Transcriptome Kit reaction amplifies the RNA from as little as 10 ng of compromised RNA sample to produce large amounts of aRNA (antisense RNA; also called cRNA). The final step in the procedure converts the amplified aRNA to cDNA that is ready for end-point PCR or qPCR. An overview of the MessageBOOSTER Whole-Transcriptome Kit procedure is shown in Fig. 1. Briefly, the process involves the following steps:

1. The RNA in a total RNA sample is reverse-transcribed into first-strand cDNA. The cDNA synthesis is primed using the proprietary MessageBOOSTER Whole-Transcriptome Primers, each containing a phage T7 transcription promoter sequence at its 5' end.
2. The cDNA is converted into dsDNA that contains a T7 transcription promoter in an orientation that will produce aRNA.
3. High yields of aRNA are produced in a rapid *in vitro* transcription reaction using the dsDNA template.
4. The aRNA produced is reverse-transcribed into cDNA. Diluted aliquots of the cDNA can be used without further purification for end-point or real-time PCR, or the cDNA can be stored for archival purposes.

Samples representing increasing levels of degradation were prepared from HeLa cells by incubating intact total RNA in the presence of 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, and 16 mM MgCl<sub>2</sub> for 5 minutes at 75°C (partially degraded RNA), or at 94°C (severely degraded RNA) (Table 1, Fig. 2). cDNA was



**Fig. 1. Overview of the procedure for the MessageBOOSTER™ Whole-Transcriptome cDNA Synthesis Kit for qPCR.** The kit procedure can be completed in about 8 hours.

**Table 1.** Input RNA samples for cDNA synthesis.

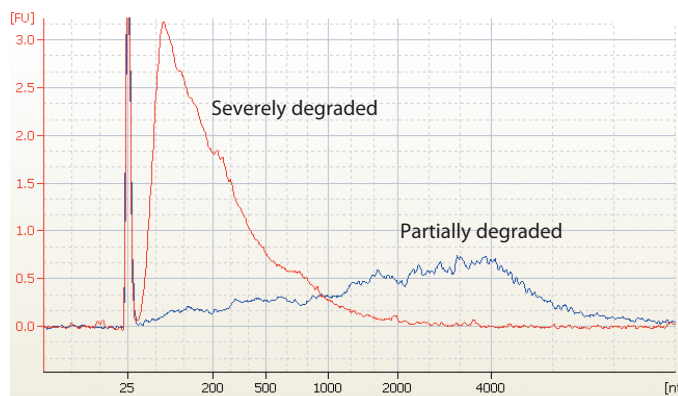
RNA Sample	Size of RNA Fragments	Amount of Input Total RNA for a MessageBOOSTER™ Whole-Transcriptome Kit Reaction
Partially degraded RNA	0.5-4.0 kb	≥10 ng
Severely degraded RNA	0.1-1.0 kb	≥100 ng

produced from specified amounts of each RNA sample using either the MessageBOOSTER Whole-Transcriptome cDNA Synthesis Kit or an oligo(dT)-primed RNA amplification and cDNA synthesis method.

Two sets of PCR primers were obtained to detect each of 10 human transcripts. One primer set was designed to anneal near the 3' end of its respective cDNA and the other set near the 5' end of the cDNA. An example is shown in Fig. 3.

The calculated 3'/5' ratios indicates nearly identical representation of the 5' and 3' ends of the actin sequence in the cDNA produced by the MessageBOOSTER Whole-Transcriptome Kit.

Diluted aliquots of the cDNA produced by each of the RNA amplification reactions were used for qPCR, using the TAQXpedite™ GREEN Real-Time PCR Kit (EPICENTRE), and the 3'- and 5'-PCR primer pairs for each of the 10 transcripts. A standard curve for each set of primers was generated using serial dilutions of unamplified human reference cDNA to ensure a PCR efficiency of 90%-110% using these primers (data not shown). The cycle threshold ( $C_T$ ) values obtained with each primer pair were used to calculate the 3'/5' ratio for each cDNA produced by each of the RNA amplification procedures using the equation  $3'/5' \text{ ratio} = 2^{(C_T^{5'} - C_T^{3'})}$ . For example, if  $C_T$  values of 5' and 3' PCR were 24.5 and 24.45 respectively, the 3'/5' ratio was calculated as:  $2^{(24.5 - 24.45)} = 2^{0.05} = 1.04$ .



**Fig. 2.** Bioanalyzer electropherograms of partially degraded (blue), and severely degraded (red) total RNA. Approximately 100 ng of partially degraded RNA, and 200 ng of severely degraded RNA was loaded onto an RNA Nano chip, and analyzed using the Eukaryote Total RNA Assay parameters.

## Results

### Compromised RNA Samples

qPCR graphs for the 3' and 5' amplicons for each of the 10 transcripts were produced and the 3'/5' ratio for each transcript was calculated as described previously. A 3'/5' ratio of 1.0 indicates that 3' and 5' sequences of the original RNA transcript are equally represented in its amplified cDNA and, therefore, the amplification introduces no bias. Further, a 3'/5' ratio close to 1.0 correlates with better coverage of the entire transcriptome, enabling studies (e.g., exon mapping and splice-site identification) to be made on any region of a transcript.

**Table 2.** Comparison of 3'/5' ratios from degraded RNA samples. The 3'/5' ratios of amplified cDNA produced by both methods were determined as described in the text. A ratio of ∞ indicates that the 5' PCR amplicon was not detected. Transcript size and transcript abundance are shown in parentheses.

RNA Quality	cDNA Synthesis Method	Actin (1,792; high)	GAPDH (1,310; high)	TFRC (5,010; medium)	ZNF (2,878; low)	HPRT (1,331; low)	TUBA (1,706; low)	PDE3A (4,124; medium)	ENSA (2,512; low)	HMBS (1,380; low)	GUSB (2,245; medium)
Partially degraded	Oligo(dT)	416	64	128	5.7	9.8	832	34.3	73.5	22.6	955
Severely degraded	Oligo(dT)	1,260	274	446	∞	∞	∞	∞	45.3	∞	∞
Partially degraded	MessageBOOSTER Whole-Transcriptome Kit	2.3	0.63	1.3	1.7	1.3	12.1	1.7	3.5	1.4	4.3
Severely degraded	MessageBOOSTER Whole-Transcriptome Kit	9.2	1.4	2.1	4.9	0.3	1.9	6.1	13.9	0.3	7.5

**Table 3.** The number of qPCR amplifications from cDNA produced by the MessageBOOSTER™ Whole-Transcriptome Kit.

RNA Quality	Input Total RNA	No. of qPCR Amplifications	
		Low-Abundance Transcripts	High-Abundance Transcripts
Partially degraded	10 ng	>10	>500
Severely degraded	100 ng	>10	>500

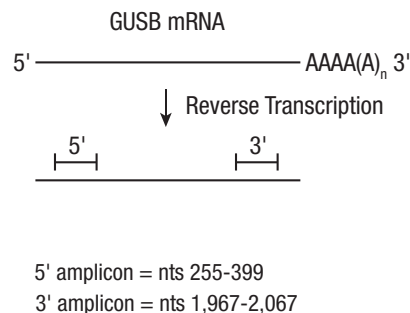
Fig. 4 shows the qPCR graphs for the 3'- and 5'-end amplicons from amplified actin cDNA produced from degraded RNA by the MessageBOOSTER Whole-Transcriptome Kit reaction, and by the oligo(dT)-primed RNA amplification and cDNA synthesis procedure. The graphs clearly show that the  $C_T$  values of the 3' and 5' amplicons from the cDNA produced using the MessageBOOSTER Whole-Transcriptome Kit are nearly identical. The calculated 3'/5' ratios indicates nearly identical representation of the 5' and 3' ends of the actin sequence in the cDNA produced by the MessageBOOSTER Whole-Transcriptome Kit. In contrast,  $C_T$  values of the 3'- and 5'-amplicons from cDNA produced by the oligo(dT)-primed reaction are vastly different.

Our results show that, even with severely degraded RNA, sufficient cDNA is produced for qPCR analysis of low-abundance transcripts.

Table 2 compares 3'/5' ratios of amplified cDNA produced from degraded RNA samples for all 10 of the transcripts evaluated. In all cases, the amplified cDNA produced by a MessageBOOSTER Whole-Transcriptome Kit reaction showed significantly improved 3'/5' ratio compared to 3'/5' ratios of amplified cDNA produced by oligo(dT)-primed RNA amplification and cDNA synthesis.

#### RT-PCR from Small Quantities of Degraded Samples

The number of qPCR amplifications that can be performed using amplified cDNA produced by a MessageBOOSTER Whole-Transcriptome Kit reaction was found to be dependent on i) the quality of the total RNA sample; ii) the amount of input RNA; and iii) the abundance of the transcript(s) of interest. Typically, the amplified cDNA is diluted 1:2 or more with distilled water, depending on the amount of input RNA template. A 1- $\mu$ l aliquot of the diluted cDNA is used per 25- $\mu$ l qPCR amplification. Table 3 reports the number of qPCR amplifications that can be obtained after using the MessageBOOSTER Whole-Transcriptome Kit to synthesize cDNA. Our results show that, even with severely degraded RNA, sufficient cDNA is produced for qPCR analysis of low-abundance transcripts, although more input RNA is required compared to partially degraded samples. Input RNA amounts higher than those indicated in the table will yield correspondingly more qPCR amplifications.



**Fig. 3.** Design of PCR primer pairs. For each of 10 HeLa transcripts (an example is shown for GUSB), primers were designed to anneal within 300 bases of the 5' or 3' ends of their respective cDNA, except for ZNF (785 bases from the 5' end, due to the high GC content at the extreme 5' end).

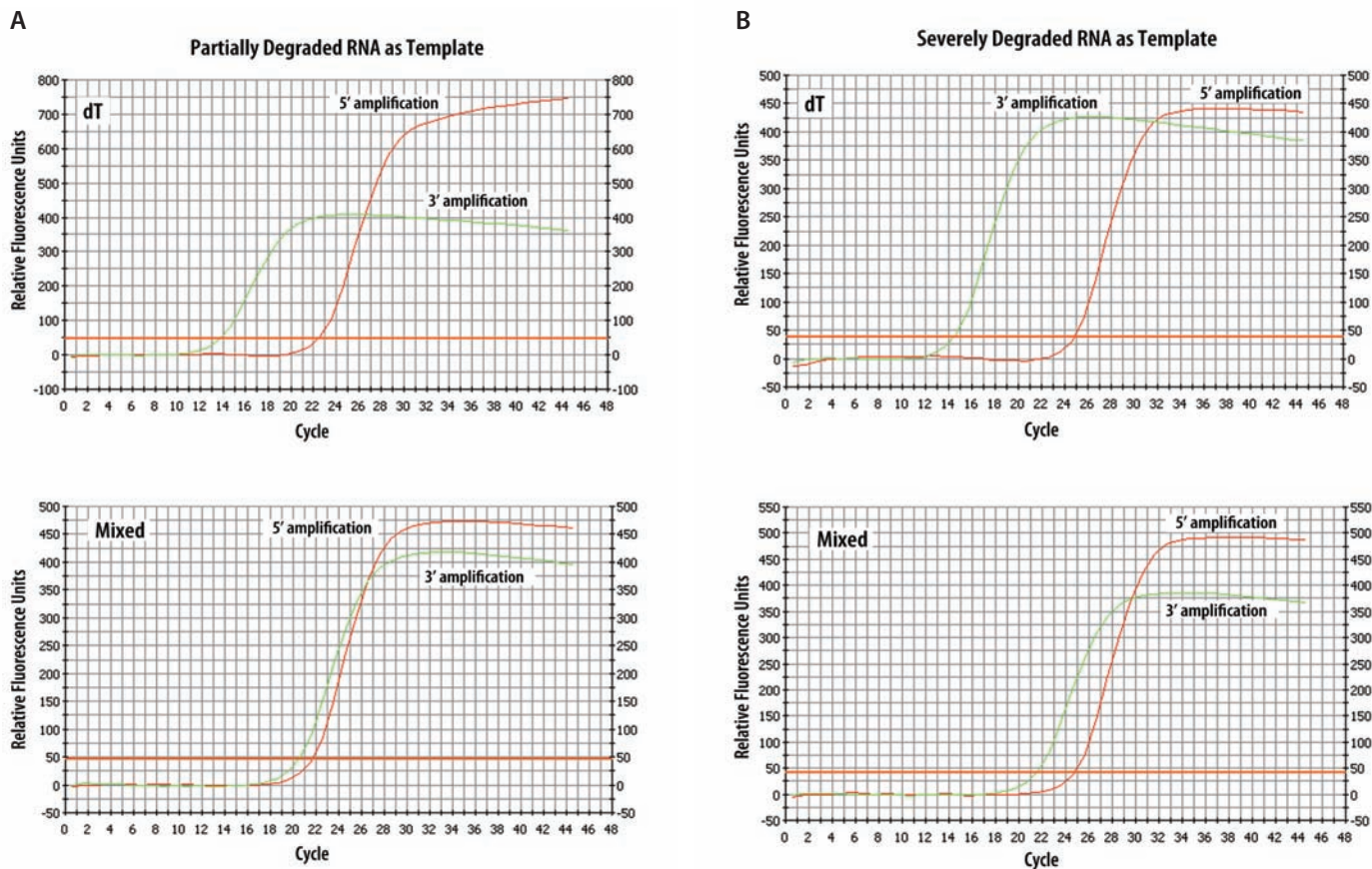
#### Conclusions

The MessageBOOSTER Whole-Transcriptome cDNA Synthesis Kit for qPCR produces amplified cDNA with greatly improved 3'/5' ratios from degraded total RNA samples, as compared to a standard oligo(dT)-based method. A single reaction produces enough amplified cDNA for hundreds of qPCR amplifications from as little as 10 ng of input degraded RNA. Using the kit to produce amplified cDNA from small quantities of degraded RNA samples will enable the user to:

1. Design PCR primers to any region of the transcript(s) of interest.
2. Identify exons and alternative splice sites that may be present in any region of a transcript.
3. Generate large amounts of cDNA for hundreds of qPCR amplifications.
4. Generate large amounts of cDNA for archival purposes.

#### Ordering Information

MessageBOOSTER™ Whole-Transcriptome cDNA Synthesis Kit for qPCR  
MBWT80510 10 Reactions



**Fig. 4.** Analysis of 3'/5' ratios of amplified cDNA. qPCR graphs of 5' (red) and 3' (green) amplicons from actin cDNA produced using the MessageBOOSTER™ Whole-Transcriptome Kit reaction and from oligo(dT)-primed RNA amplification and cDNA synthesis. Data are shown for input RNA that was partially degraded (Panel A) and severely degraded (Panel B). The 3'/5' ratio of actin cDNA produced by the MessageBOOSTER Whole-Transcriptome Kit reaction and the oligo(dT)-priming method, respectively, were calculated as follows: Panel A, 2.3 and 416; Panel B, 9.2 and 1,260.

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