

RNA Quality Control Using External Standard RNA

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Abstract

In this paper, we propose a new evaluation method using external standard RNA for quality control of the extracted RNA. RNA Integrity Number and UV absorption are generally used as a basis for RNA quality control; however, these methods do not always reflect the quality of mRNA. While standard RNA is supposedly designed on the basis of mRNA, it has the potential to be used to evaluate the quality of the mRNA. In this study, we took into consideration the three essential factors, viz., yield of mRNA, inhibition to DNA polymerase, and degradation of mRNA for determining the RNA quality using standard RNA. It would be possible to know yield of mRNA and inhibition of the enzyme reaction by adding standard RNA before RNA extraction and looking at standard RNA loss. Degradation was evaluated by comparing the differences in the 3' and 5' regions of the RNA. In our study, it was demonstrated that in the crude extract of *Saccharomyces cerevisiae*, degradation was comparatively higher at the 3' end of RNA than at the 5' end. Hence, the degree of RNA degradation can be evaluated by comparing the ratio of degradation from the 3' and 5' end.

Key words: RNA degradation, RNA quality control, Standard RNA

Introduction

Expression levels of functional mRNA denote the essential functions that are required to retain life under various conditions. Thus, the degree of mRNA expression is widely employed to understand the cellular conditions (Wang et al. 2009; Qin et al. 2016). Therefore, northern blotting, RT-qPCR, and DNA microarray were employed to quantify mRNA. Furthermore, RNA sequencing using a next-generation DNA sequencer and Digital RT-PCR is essential to study “omics”. Technologies focusing on mRNA are steadily developing and offer higher sensitivity, whereas techniques for determining the quality of extracted mRNA are still not at par. To analyze functional mRNA, it is essential to establish a more precise RNA quality control method (Kashofer et al. 2013; Li et al. 2015).

In this study, we validated a new RNA quality control method using an external standard. We used the RNA Solutions by Qualitative Analysis (AIST, Japan) as the external standard RNA. Standard RNAs have the potential to be used to evaluate mRNA directly because standard RNAs are designed based on human mRNA

(Tong et al. 2006). Standard RNA has already been used to evaluate RNA yield (Takahashi et al. 2013). However, in this study we focused not only on yield but also RNA degradation. Standard RNAs are available in five different types, viz., three each of 533-nt and two each of 1033-nt, and are designed in a way that they share low homology sequences with natural sequences. Furthermore, they have the potential to be used to directly evaluate certain factors simultaneously. For this study, we evaluated the yield of mRNA from cells, inhibition by contaminants, i.e. unknown cellular component such as DNA polymerase, and degradation of mRNA by using standard RNA.

RNA yield is a factor denoting the quality, but it is usually ignored as a base for evaluation of quality control. Low yield of RNA suggest that the extraction procedure is not appropriate or that cellular disintegration causes RNA damage (Kashofer et al. 2013). We evaluated the final yield of total RNA and not the efficiency of RNA extraction. Comparison of RNA expression from different samples with different efficiencies of RNA yield may affect the final outcome. Therefore, we evaluated the final yield of RNA and the efficiency

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of yield during RNA extraction. In this study, RNA was extracted from *Saccharomyces cerevisiae* and *Escherichia coli* by the hot phenol method (Sambrook et al. 1989). Standard RNA was added to both RNA extraction procedures and the efficiency of yield was evaluated for RNA extraction by measuring the amount of standard RNA in the RNA-extracted solution.

The prepared RNA samples are not always free of contaminants since they can be contaminated with proteins and polysaccharides; these contaminants can lead to unwanted enzymatic reactions that inhibit RNA extraction and, hence, denote false positive results (Pionzio and McCord 2014). A260/A230 and A260/A280 ratios are generally used as a base for determination of RNA quality (Sambrook et al. 1989; Manchester 1996). Absorbance at 230 nm usually denotes contamination by organic solvents or TE. Absorbance at 260 nm denotes mass of nucleic acids; RNA and DNA. Absorbance at 280 nm denotes contamination by proteins or DDT. Contamination, measured by the A260/A280 ratio, is considered to inhibit reaction of enzymes (e.g., reverse transcriptase and DNA polymerase). A260/A280 and A260/A230 ratios are widely accepted for evaluating the contamination in the prepared RNA samples. However, this ratio does not directly evaluate inhibition. Thus, standard RNA can be used to evaluate the degree of unknown contamination that may inhibit enzyme reaction. In this study, we employed real-time qPCR to quantify the final value of standard RNA and to determine the quantity of inhibition in RNA-extraction solutions of *S. cerevisiae* and *E. coli*.

Evaluation of mRNA degradation is not easily accepted by organizations that are responsible to record the values obtained from all prepared mRNA samples. RNA Integrity Number (RIN) value and 18S/28S ribosomal ratio are the most used parameters for determining mRNA quality control (Imbeaud et al. 2005). This method considers two kinds of rRNA as internal standards. The extracted total RNA is subjected to capillary gel electrophoresis, and an electropherogram is constructed from the fluorescence for determining retention time. By comparing two peaks of rRNA and with the ideal peak, RNA quality is evaluated. However, because this method evaluates rRNA that comprises a major portion of the total RNA, it does not always reflect the quality of mRNA, which comprises a small portion of the total RNA (Feng et al. 2015). While standard RNA is designed on the basis of mRNA, it has the potential to evaluate mRNA quality. In this study, we evaluated mRNA degradation using standard RNA. We also validated the difference in degradation of RNA structure from the 3' and 5' ends.

This study, hence, helps in establishing a method using external standard RNA that can be used to directly evaluate the extracted mRNA quality.

Experimental

Materials and Methods

Standard RNAs. We used RNA solutions for qualitative analysis (AIST, Japan) as external standard RNA. This standard RNA is available in five different types: 500-A, 500-B, and 500-C are of 533 nt each and 1000-A and 1000-B are of 1033 nt each. Concentration of the solution was 33.4, 32.2, 32.1, 68.2, and 64.1 ng/ μ l, respectively. 10^{-3} diluted standard RNAs were used in this experiment. Standard RNA 500-B has low complementary sequences and tends to form a single strand. Standard RNA 500-C has high complementary sequences and tends to form double strands. The secondary structures of both the standard RNA were predicted using the software "CentroidFold" (<http://www.ncrna.org/centroidfold>).

Strain and growth conditions. We used *E. coli* strain JM109 (TakaraBio, Japan) [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* (*rK- mK+*), *e14-* (*mcrA-*), *supE44*, *relA1*, Δ (*lac-proAB*)/*F'*(*traD36*, *proAB+*, *lac Iq*, *lacZ* Δ M15)] and *S. cerevisiae* strain S288C (NBRC 1136, Japan) [*MAT α* *SUC2 mal mel gal2 CUP1* (*cir+*)] for RNA extraction. *E. coli* was cultured in lysogeny broth (LB), comprising of 1% tryptone, 0.5% yeast extract, and 1% NaCl, and incubated at 37°C in an incubator-shaker with the rotation speed set at 120 rpm. *S. cerevisiae* was cultured in yeast peptone dextrose (YPD) broth comprising of 2% peptone, 1% yeast extract, and 2% glucose, and incubated at 30°C in an incubator-shaker with the rotation speed set as 120 rpm. *E. coli* and *S. cerevisiae* were isolated from the 1 ml culture suspensions by centrifugation at 15 100 \times g at 4°C for 1 min when the OD of the broth at 600 nm was 0.6, and the supernatant was discarded. *E. coli* and *S. cerevisiae* cells were washed by distilled water and collected. These samples were then used for RNA extraction.

RNA extraction by hot phenol method and evaluation of RNA yield. *E. coli* and *S. cerevisiae* were suspended in sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). To prepare suspensions, 10 μ l of 10^{-3} dilution of standard RNA 1000-A, 12.5 μ l of 10% sodium dodecyl sulfate (SDS), and 300 μ l of phenol were added, and the suspensions were incubated at 65°C for 5 min. The suspensions were then conjugated for 10 sec and incubated at 65°C for 10 sec; this step was repeated 10 times. Next, suspensions were centrifuged at 15 100 \times g, at 25°C for 5 min. Supernatants were transferred to new tubes, and 10 μ l of 10^{-3} dilution of standard RNA 1000-B was added. To these supernatant mixtures, 300 μ l of chloroform was added, and the samples were conjugated and centrifuged at 15 100 \times g at 25°C for 5 min. Then, 100 μ l of supernatants were transferred to new tubes; 10 μ l

of 10^{-3} dilution of standard RNA 500-A, 11 μ l of 3 M sodium acetate, and 287 μ l of 99.5% ethanol were added to the suspension. Suspensions were then incubated at -20°C for 4 h, centrifuged at $17,400 \times g$ at 4°C for 30 min, and then supernatants were discarded. Next, 500 μ l of cold 70% ethanol was added to the precipitate and centrifuged at $17,400 \times g$ at 4°C for 1 min. The supernatants were separated. Precipitates were dissolved in 100 μ l of nuclease-free water. These suspensions were designated as "RNA extraction."

Real-time qPCR for evaluation of RNA yield and inhibition of enzymes. To evaluate yield of RNA, each standard RNA in all RNA extractions was quantified by one step real-time qPCR using One Step SYBR[®] PrimeScript[™] RT-PCR Kit II Perfect Real Time (Takara-Bio, Japan). Table I lists the primers used for real-time qPCR, which targets standard RNA 1000-A, 1000-B and 500-A. To evaluate inhibition of the enzyme, we also quantified samples which same amount of standard RNAs was added to RNA extraction.

Calculation of the inhibitory effect. To evaluate the inhibitory effect caused by contaminants, we re-added standard RNA at the same concentrations to the extracted RNA solution. Concentration of RNA in this sample (A) and in the extracted RNA solution (B) were measured by RT-qPCR, and the differences between both values were calculated. These differences denoted the concentration of standard RNA to be re-added to the extracted RNA solution to quantify results, and we could determine the inhibitory effect that affected the yield of mRNA in the RNA-extracted solution. Inhibitory effect was calculated by the following formula:

Inhibitory effect (%) = $100 - [(A - B) \div C] \times 100$, where A denotes the concentration of standard RNA in the RNA-extracted solution in RT-qPCR, B denotes the concentration of standard RNA in the RNA-extracted solution, and C denotes the concentration of the added standard RNA.

Preparation of *E. coli* and *S. cerevisiae* crude extract. *E. coli* was isolated from the 1-ml culture sus-

pension by centrifugation at $15\,100 \times g$ at 4°C for 1 min when the OD of the broth at 600 nm was 0.6. Then, 300 μ l of PBS was added, and the suspension was subjected to sonication. The sonicated suspension was centrifuged at $15\,100 \times g$ at 4°C for 10 min, and supernatant was collected. The collected supernatant was diluted to the concentration of 10^{-1} , and the concentration of protein was calculated to be equal to 0.4 mg/ml. The protein concentration was measured by ultraviolet absorption spectrometry.

S. cerevisiae was isolated from the 10-ml culture suspension by centrifugation at $2\,430 \times g$ at 4°C for 10 min when the OD of the broth at 600 nm was 0.6. Next, 300 μ l of PBS, acid-washed glass beads in the size range of 425–600 μm (Sigma, USA) were added to the suspension. The suspension was conjugated for 10 sec and incubated on ice for 10 sec; this process was repeated 10 times. The suspension was centrifuged at $15\,100 \times g$ at 4°C for 5 min, and supernatant was collected. The protein concentration was calculated to be equal to 36.7 mg/ml. These conditions were selected after several pre-experiments..

RNA degradation. Five microliters *E. coli* and *S. cerevisiae* crude extract was added to 20 μ l of 10^{-3} dilutions of standard RNA 500-B and 500-C, respectively. Standard RNA-added *E. coli* crude extract was degraded for 0, 7.5, 15, 30, 60, and 120 min at 37°C . Standard RNA-added *S. cerevisiae* crude extract was degraded for 0, 10, 20, 60, and 240 min at 30°C . After degradation, 10 μ l of 10^{-3} dilution of standard RNA 500-A was added to the solution, and the solution was purified by RNaeasy (Promega, USA). Standard RNAs were eluted by 50 μ l of nuclease-free water.

Real time qPCR for evaluation of RNA degradation. Standard RNAs that were degraded by *E. coli* or *S. cerevisiae* crude extracts were transcribed using ReverTra Ace[®] qPCR RT Master Mix (Toyobo, Japan). All cDNAs were quantified using Power SYBR[®] Green PCR Master Mix (ThermoFisher, USA). The PCR targets were at the 5' end, middle, and the 3' end using specifically

Table I
Primers used in the RT-qPCR.

Target	Forward Primer	Reverse Primer
1000-A	5'-CAACCGGTGTGATCAGGACA-3'	5'-AGGACAGTCCGCATAAGCAC-3'
1000-B	5'-TACCAGCGCTTCTGTACGAC-3'	5'-GAGCTGTATCCGTGCCGTAA-3'
500-A	5'-TCGACGGCCTAATACGTGTC-3'	5'-CGTGAATCTCGGAGCGGTAA-3'
500-B 3'end	5'-GGGTAGCGATTTAACGACTCG-3'	5'-CAGAGCCTGCCTTATCGTGA-3'
500-B middle	5'-CCGAACGCTACGTGACGATA-3'	5'-ATCTACATGTTCCGTGCGCA-3'
500-B 5'end	5'-AGACTAAATCTCGGCGTCGG-3'	5'-TAGATAGGGTCCGCATGACG-3'
500-C 3'end	5'-GCACGACCGAATTATGCACC-3'	5'-AACCACTGACGTGAGCGATT-3'
500-C middle	5'-TAGACGCGCCTTACTCCTCT-3'	5'-TAGTGGAGCTCGCGGATTTG-3'
500-C 5'end	5'-GGACTAAACGCACTGAATACCG-3'	5'-ATCGCCCGTACTATCCGGTA-3'

designed primers (Table I). To eliminate manual error in purification, standard RNA 500-A was also quantified using a primer (Table I). Calibration curve was calculated by quantifying undegraded 10^{-1} and 10^{-2} dilutions of standard RNA solution. Survival rates of each region were calculated from real-time qPCR results and corrected by quantifying standard RNA 500-A.

Results

Industrially available standard mRNA was used for quality control of prepared total RNA from *S. cerevisiae* and *E. coli*. We evaluated the yield, inhibitory effect, and degree of degradation using the standard RNA.

Before RNA extraction, standard RNA of known concentration was added into the sample tubes. We evaluated the mRNA yield, by tracing the amount of added standard RNA during the experiment. We added standard RNA during all key steps in the protocol. Total RNA was extracted from *S. cerevisiae* and *E. coli* using the hot phenol method (Sambrook et al. 1989). The hot phenol protocol proceeds in three main steps. The first step is cell disruption using phenol and SDS, the second step is purification using chloroform, and the third step is ethanol precipitation. We added different standard RNA before all three steps. Standard RNA 1000-A was added to all suspensions before reconstituting the samples, standard RNA 1000-B was added before purification using chloroform, and 500-A was added before ethanol precipitation. We measured the residual standard RNA by real-time qPCR and calculated the RNA yield.

Fig. 1 shows the yield of standard RNA in each RNA extraction step. In RNA extraction from *S. cerevisiae*,

Table II
Inhibition of RT-qPCR by RNA extract solutions from *S. cerevisiae* and *E. coli* were evaluated using standard RNAs (%).

Species	1000-A	1000-B	500-A
<i>S. cerevisiae</i>	-1.1 ± 4.8	50.4 ± 4.0	0.3 ± 3.1
<i>E. coli</i>	7.0 ± 3.2	44.6 ± 3.0	27.7 ± 2.8

the yield of standard RNA at the cell disruption step was approximately 35%, approximately 60% after purification using chloroform, and approximately 100% after ethanol precipitation. For RNA extraction from *E. coli*, the yield of standard RNA at the cell disruption step was approximately 5%, approximately 60% after purification using chloroform, and approximately 100% after ethanol precipitation, similarly to that observed for *S. cerevisiae*.

To evaluate the inhibitory effect caused by contaminants, we re-added standard RNA at the same concentrations to the extracted RNA solutions and measured. Table II shows the inhibitory effect of RNA extract solution from *S. cerevisiae* and *E. coli* by RT-qPCR. Values closer to 0 denote lower inhibitory effects for RT-qPCR, whereas values closer to 100 denote higher inhibitory effect. Inhibitory effect of RNA-extract solution from *S. cerevisiae* using standard RNA 1000-A and 500-A was almost 0% and that with 1000-B was approximately 50%. For *E. coli*, inhibitory effect using standard RNA 1000-A, 1000-B, and 500-A was approximately 10%, 50%, and 30%, respectively. Standard RNA 1000-B showed higher inhibition effect for RT-qPCR than 1000-A and 500-A for both organisms.

We evaluated RNA degradation using standard RNAs. Standard RNA 500-B and 500-C were degraded

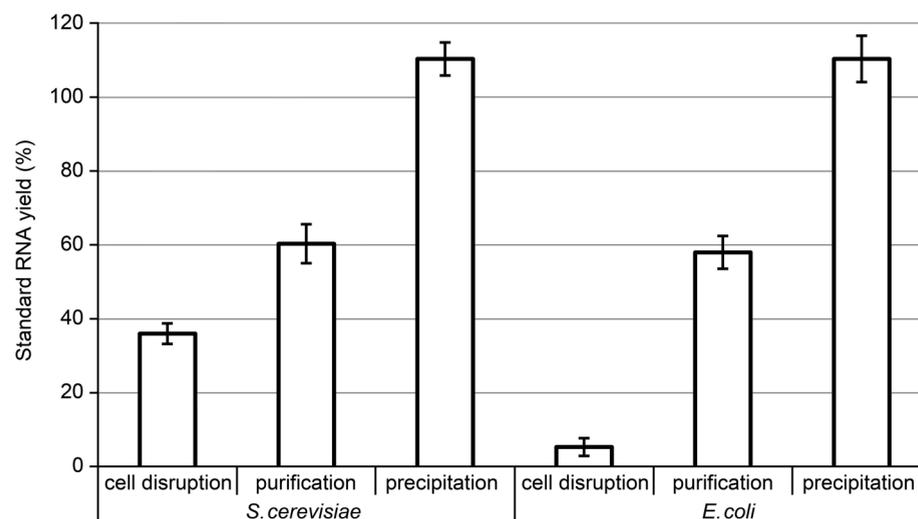


Fig. 1. RNA yields from *S. cerevisiae* and *E. coli* with hot phenol RNA extraction method using standard RNAs. Standard RNA yields were calculated based on the RT-qPCR result. Cell disruption (using phenol and SDS) was evaluated using standard RNA 1000-A. Purification (using chloroform) was evaluated using standard RNA 1000-B. Precipitation (using ethanol) was evaluated using standard RNA 500-A.

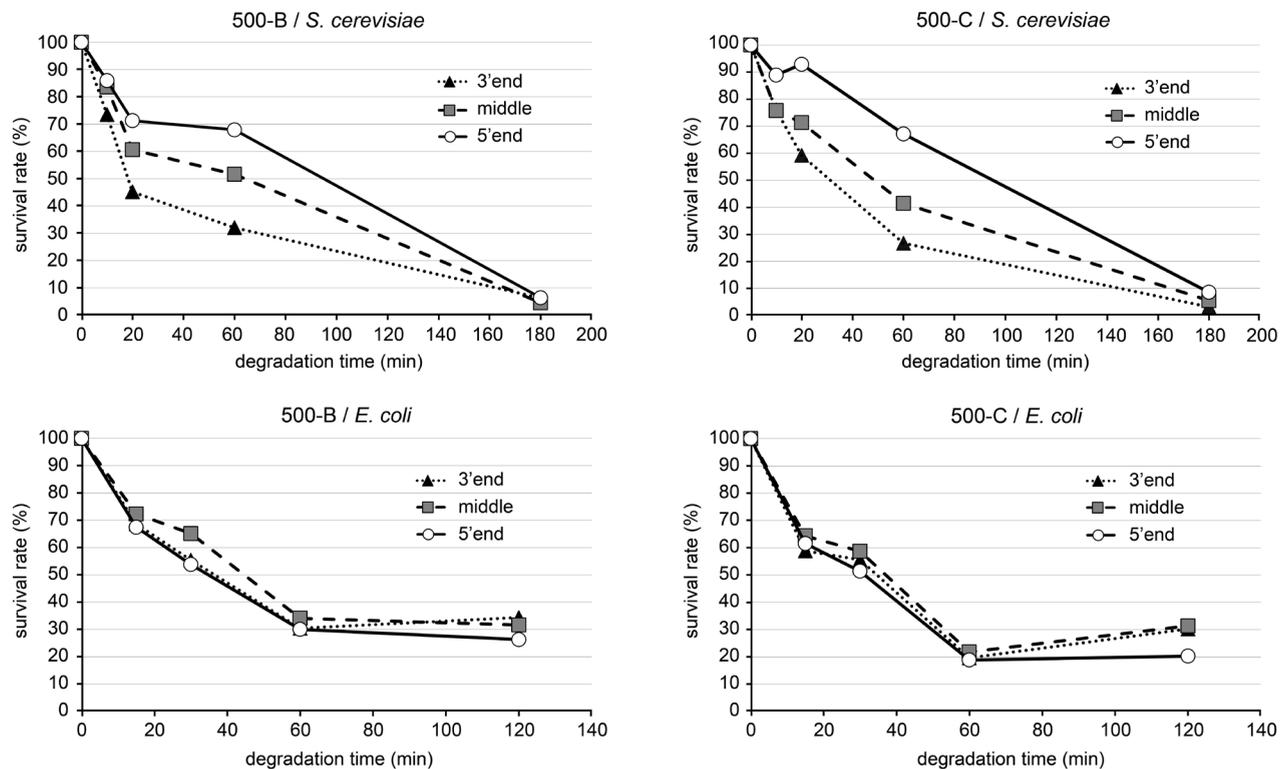


Fig. 2. Degradation of standard RNAs 500-B or 500-C by *S. cerevisiae* or *E. coli* crude extract.

by *E. coli* or *S. cerevisiae* crude extract, and the survival rate was evaluated by RT-qPCR; RNA was quantified by measuring PCR products that amplified the targeted RNA region. We quantified three regions (5' end, middle portion, and 3' end) using RT-qPCR.

Fig. 2 shows the survival rate of each region on both standard RNAs over time. We discovered that RNA degradation by *S. cerevisiae* crude extract was apparently biased toward the 3' end and was easier to degrade it than the 5' end. *E. coli* exhibited no bias. To conform the accuracy, we measured the survival rate again in three samples when the survival rates of each 3' end region were about 50%. Fig. 3 shows the quantified survival rate for triplicates of each region on both standard RNAs degraded by *S. cerevisiae* crude extract for 20 min and by *E. coli* crude extract for 30 min. In *S. cerevisiae* crude extract, the 3' end was degraded easily, and the 5' end was barely degraded in both standard RNA. For degradation by *E. coli* crude extract, no difference was

observed in the degree of degradation of both ends region in both standard RNA.

We attempted to evaluate the standard RNA quality of samples degraded by *S. cerevisiae* crude extract. Table III shows degradation of standard RNA 500-B and 500-C by *S. cerevisiae* crude extract and the ratio of 3' end to 5' end survival rate. Correlation was observed between the total degradation value and the ratio of 3' end to 5' end, until RNA was degraded by approximately 50%. This value of degradation should not be used for further analysis, and, thus, the ratio of 3' end to 5' end can be used as the value for determination of the quality of RNA. Moreover, the 3' end is more prone to degradation than the 5' end, as demonstrated by quantifying RNA degradation by measurement of the 3' end of standard RNA.

We demonstrated that RNA can be easier degraded at the 3' end than the 5' end by *S. cerevisiae* crude extract, indicating that 3' to 5' exotype RNase activity

Table III
Survival of 3' end and 5' end and the ratio of 3' end to 5' end regions of standard RNAs after degradation with *S. cerevisiae* RNA crude extract.

Standard RNA	500-B					500-C				
	Degradation time (min)	0	10	20	60	180	0	10	20	60
3' end (survival %)	100	73.5	45.2	32.1	6.3	100	76	59.2	26.7	3.2
5' end (survival %)	100	85.9	71.2	67.9	6.4	100	88.9	92.9	67.2	8.6
3' end /5' end (ratio)	1	0.86	0.63	0.47	0.99	1	0.86	0.64	0.4	0.37

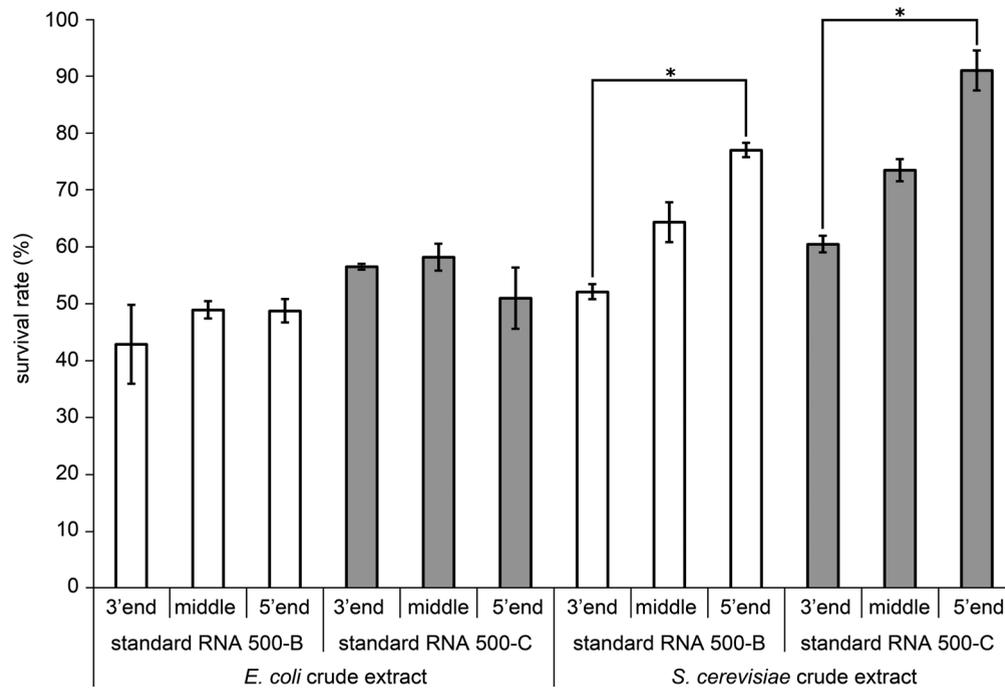


Fig. 3. The different degree of RNA degradations depends on the RNA regions in *S. cerevisiae* crude extract. Standard RNAs were degraded by *E. coli* crude extract at 30 min and by *S. cerevisiae* crude extract at 20 min. *, $p < 0.001$, $n = 3$, t-test.

is stronger in *S. cerevisiae*. Furthermore, single-stranded RNA can be more easily degraded than double-stranded RNA by *S. cerevisiae* RNase, as observed in this experiment from the degree of degradation of standard RNA 500-B that was higher than that of standard RNA 500-C (Fig. 2). Standard RNA 500-B tends to form a single strand, whereas standard RNA 500-C tends to form a double strand. We showed that the degree of RNA degradation by *S. cerevisiae* RNase is different for each RNA region and structure, suggesting that the prepared RNA were one-dimensional structures due to degradation.

Discussion

We calculated the yield of mRNA in each step for both *S. cerevisiae* and *E. coli*. We found that the final yield of standard RNA 1000-A from *S. cerevisiae* was approximately 35%, whereas that from *E. coli* was approximately 5%. Hot phenol was added after reconstituting the cell suspensions by thawing, which could have led to degradation of standard RNA. Lower yield of RNA from *E. coli* can be due to the higher activity of RNase, or RNase may have not affected the cells. We confirmed that ethanol precipitation is a method that benefits the final yield.

This information could fast-track the development of advanced preparation procedures. The use of standard RNA has the potential to evaluate and normalize the dif-

ferences in yield of RNA from different organisms and to elucidate the reason behind low yields from specific organisms. The mRNA yield is usually not considered for determining the quality as the yield can be easily increased by increasing the volume of biological sample used to extract mRNA. Biological resources are often limited, and clinical samples and the samples obtained from the crime site are often in minute quantities, so the cost for performing forensic analysis is not economical (Georgiadis et al. 2015). Intestinal jejunum and skin are representative organs that present difficulty in mRNA extraction (Berglund et al. 2007; Heumüller-Klug et al. 2015). Thus, standard RNA may contribute toward advancing the quality of mRNA yield from these organs.

The extracted RNA may contain impurities that cause inhibitory effect on enzymatic reactions, leading to false positive results (Kashofer et al. 2013). We attempted to evaluate inhibition for real-time qPCR using standard RNA but result was incorrect. The possible reason for this result may be that the sequences of standard RNA were affected by RT-qPCR. Standard RNA is an external standard, and because the exact concentration is known, we may directly evaluate inhibitory effect. The direct evaluation of inhibitory effect disagrees with the lack of manual skill in this experiment. Furthermore, it should be noted that standard RNA may exert an inhibitory effect by interaction with secondary structures and other nucleic acids (Bustin et al. 2009).

We evaluated RNA degradation using standard RNAs. Standard RNAs were degraded by *E. coli* or

S. cerevisiae crude extract, and quantified by measuring PCR products that amplified the targeted RNA region. However, because the degree of degradation was different for each RNA region owing to cells condition and RNA structure, the quantified RNA measurement by RT-qPCR may not be a fool-proof method. RNA degradation in the cell differs among different species (Cannistraro and Kennell 1991). It has been demonstrated that bacteria, e.g., *E. coli* and *Bacillus subtilis*, degrade RNA by primarily employing endo-type RNases (Lehnik-Habrink et al. 2012; Hui et al 2014), whereas eukaryotic cells, e.g., *S. cerevisiae*, degrade RNA by employing exo-type RNases in exosomes (Szczesny et al. 2012). Therefore, if the extracted RNAs were degraded by RNase, particularly in eukaryotic cells, there is a chance that the degree of degradation was different for each RNA region.

To confirm one-dimensional degradation and to evaluate RNA degradation, standard RNAs are suitable. In conclusion, we propose the following quality control method for RNA degradation: first, standard RNA must be added during the procedures of RNA isolation; secondly, RNA degradation must be evaluated by comparing the structure and the ratio of the 5' end and 3' end of standard RNA. Furthermore, the quality of prepared RNA may be evaluated by measuring the 3' end of the standard RNA as it is more prone to degradation than the 5' end. Hence, RNA degradation quality control can be evaluated by comparing the 5' end and the 3' end of standard RNA for samples that exhibit biased RNA degradation. Further studies are required in other types of cells, including blood cells, visceral cells, and cultured cells.

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