

Evaluation for Integrity of Extracted RNA by Reference Material of RNA

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Abstract

Physiological information that identified from RNA expression level in tissues or organs give us benefit in clinical diagnostics. After extracting RNA from blood, tissue, or organs, diagnostics were carried out by using technique for semi-quantitative estimation of specific mRNA, such as real-time PCR or DNA microarrays. Validation of RNA quality need to quantitative estimation of gene expression at the moment of sampling, because using degraded RNA will lead to incorrect results. In this study, we conducted a series of experiment to evaluate RNA integrity using the reference materials of RNA length of 1000 base pairs (refRNA1000), and compared with RIN. There are significant correlation between RIN and the amount of refRNA1000, in varying state of degradation. There are also significant correlations between the amount of refRNA1000 and endogenous gene such as *Actb* and *Rps18* in varying state of degradation in our study. The amount of endogenous gene reflects the extent of degradation more directly than that of RIN. To monitor the extent of degradation from the experimental starting point, we may refer the reference materials of refRNA1000. Not only for clinical diagnosis, but scientists always require the validity of experimental procedure, one of the candidate methods is using reference materials of RNA such as refRNA1000.

Keywords: RNA quality; RNA quantity; RNA diagnosis; Reference material

Introduction

Physiological information that identified from RNA expression level in tissues or organs give us benefit in clinical diagnostics. Thus, the diagnostic methods for evaluation of specific gene transcripts have been developed to indicate the presence or prognosis of disease, to predict or monitor the response to drug therapy, and to track disease kinetics [1-6]. Diagnostics were carried out by using technique for semi-quantitative estimation of specific mRNA, such as real-time reverse transcription PCR (RT-PCR) or DNA microarrays to samples prepared from blood, tissues or organs.

It should be noted that diagnostics are strongly due to the quality of mRNA. Intracellular RNA may be rapidly degraded *ex vivo* by specific and nonspecific endogenous nucleases. Contingent gene expressions also can be induced by sampling collections. Transportation procedures, storage conditions, purification methods and unknown factors significantly affect the quality of mRNA.

Validation of RNA quality need for experiments that try to quantitative estimation of gene expression at the moment of sampling, because using degraded RNA will lead to incorrect results. Commonly, the 28S/18S rRNA ratio is used to assess RNA quality, as the ratio close to 2 should be indicative of intact RNA in theory. Recently, Agilent Technologies introduced the RIN (an RNA integrity number) for assigning integrity value in order to RNA measurements [7]. The amount of endogenous gene reflects the extent of degradation more directly than that of RIN. It is difficult to determine the absolute amount of them in each experiment without standard of comparison. In order to guarantee measurement traceability, the reference materials of RNA were developed by National Institute of Advanced Industrial Science and Technology (AIST) and National Metrology Institute of Japan (NMIJ). The reference materials of RNA which has the length of 1000 base pairs (refRNA1000) were constructed synthetically. In this study, we conducted a series of experiment to evaluate RNA integrity using the refRNA1000, and compared with RIN.

Materials and Methods

RNA reference material

The synthetic constructed RNA has the length of 1000 base pairs, developed as candidate material of certificated reference materials (CRM) for spike-in microarray control RNA. The sequence of the refRNA1000 can be referred as Accession No. AB610945 in Genbank (<http://www.ncbi.nlm.nih.gov/nuccore/AB610945>). The solution of the refRNA1000 is produced 88.0 ng/ μ L. This refRNA1000 were added cells (5.0×10^5 cells) to be 20 pg. RNA reference materials with different sequences were supplied as ribonucleic acid solution for quantitative analysis as NMIJ CRM 6204 from NMIJ. The protocols for preparation and evaluation were described in Web pages (https://www.nmij.jp/service/C/crm/42/6204a_J.pdf). These CRM concentrations were determined by isotope-dilution mass spectrometry (ID-MS and inductively coupled plasma mass spectrometry (ICP-MS). RefRNA1000 to be degraded by RNase was confirmed previously.

Cell preparation

For monitoring degrading RNA situations, total RNA of the B16-BL6 mouse melanoma cell were used. The B16-BL6 melanoma is known to be highly invasive and spontaneously metastasizes [8]. This cell line is easily grown in culture media. These views are suitable for systems as diagnostic model using total RNA. The cell line was supplied by the Riken Cell Bank (Tsukuba, Japan) and cultured in PRMI1640 (GIBCO

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BRL, Life Technologies, Tokyo, Japan) containing 10% FBS (Moregate BioTech, QLD, Australia) in a 5% CO₂ humidified incubator at 37°C. Cells near confluence were gently separated into a single-to several-cell suspension with trypsin-EDTA, washed by PBS, and dispensed in 0.1 ml aliquots (5.0×10⁵ cells/tube) into plastic tubes. RNA reference material was added 20 pg/tube, and then stored at -80°C.

RNA degradation and extraction

Before RNA extraction, prepared tubes were incubated at 25°C for various times. After that, total RNA was extracted using RNeasy Mini Kit (Quiagen, Germany) following the manufacturer's guidelines. Total RNA were collected by 30 µL RNase-free water. The expected concentration of refRNA1000 is 0.67 pg/µL.

RNA evaluation – concentration and electrophoresis

RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For microcapillary electrophoresis, the Agilent 2100 bioanalyzer was used in conjunction with the RNA 6000 Nano LabChip kits. The RIN values were obtained automatically by algorithm based on a Bayesian learning that takes into consideration 8 features from the electrophoretic trace, including total RNA ratio.

RNA evaluation–real-time RT-PCR methods

RefRNA1000 were assessed by real-time RT-PCR with the MJ Mini Thermal Cycler (Bio-Rad) using primers as described in table 1. *Actb* and *Rps18* from cells RNA were also measured. The primers of these genes were designed to span intron to avoid detection of genomic DNA. iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) was used to reverse-transcribe and amplify the RNA template for 40 cycles, 25 µM 2xSYBR Green RT reaction mix, 0.2 µM each primer, 1µL iScript reverse transcriptase for one-step RT-PCR and 1 µL template. The endpoint used in the real-time RT-PCR quantification, C_p, is defined as the PCR cycle number that crossed an arbitrarily placed signal threshold. To determine semi-quantity of RNA, the calibration curve method was used to determine the relative amounts of RNA value.

Statistics

Pearson correlation coefficients were calculated to identify similarities among amount of refRNA1000, *Actb*, *Rps18*, and RIN value. The statistical significance of correlation coefficients was calculated.

Results

RNA yield

To characterize the degree of degradation, yield of RNA derived from cells were measured and microfluidics-based electrophoresis were performed after RNA extraction. RNA yield change is shown in figure 1. The yield of RNA decreases depending on degradation time. The isolation kit for this study use spin-column based on silica membranes technology, allows capture longer than 200 bases. So the nucleotide shorter than 200 bases result from degradation was lost during isolation.

RNA integrity

RNA extracted from cell line incubated at room temperature prior to extraction. After isolation of RNA, electrophoresis of the RNA was carried out using Agilent 2100 Bioanalyzer. Electropherogram profiles at different time of heat-mediated degradations are shown in Figure 2. Incubation time is indicated for each graph. In each figure, 18S and 28S rRNA peaks map to 42 sec and 50sec respectively.

Effect of degradation time on RIN values is shown in figure 3. The RIN values range from 10(intact) to 1(totally degraded) were calculated automatically by algorithm using Bayesian learning network taken into consideration 8 features from the electrophoretic trace. The RIN number decreases depending on degradation time.

Real -time RT-PCR

To evaluate semi-quantity of RNA, real time RT-PCR were performed. Figure 4 shows the result of real time RT-PCR with serial dilutions of refRNA1000. The standard curve of refRNA1000 was generated from 10 x serial dilutions. The derived standard curve showed that the correlation coefficient, r, is 0.998. The extraction efficiency of

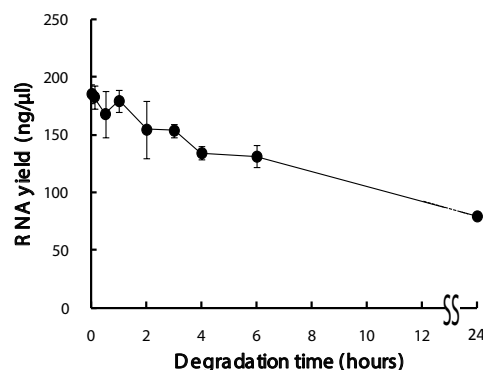


Figure 1: Effects of degradation time on RNA yield. RNA yield of each samples of different degradation times were measured. RNA extracted from cell line incubated at room temperature prior to extraction. Short fragments resulting from degradation were removed during extraction and RNA concentration decrease as degradation time.

| Gene | Forward primer | Reverse primer |
|--------------|-----------------------------|---------------------------------|
| RefRNA1000 | 5' TGGACCTCTGCGGCTATTTG 3' | 5' CGGTTGTATGGTGGC GGTTA 3' |
| <i>Actb</i> | 5' TCCACACCCGCCACAGTTC 3' | 5' CATTCCCACCATCACACCCTGG 3' |
| <i>Rps18</i> | 5' GATGGGCGGCGGAAATAGCCT 3' | 5' CGATGTCTGCTTTCCTCAACACCAC 3' |

Table 1: Primers used in the PCR. The nucleotide sequences of the PCR primers used to assay gene expression by real-time RT-PCR are shown.

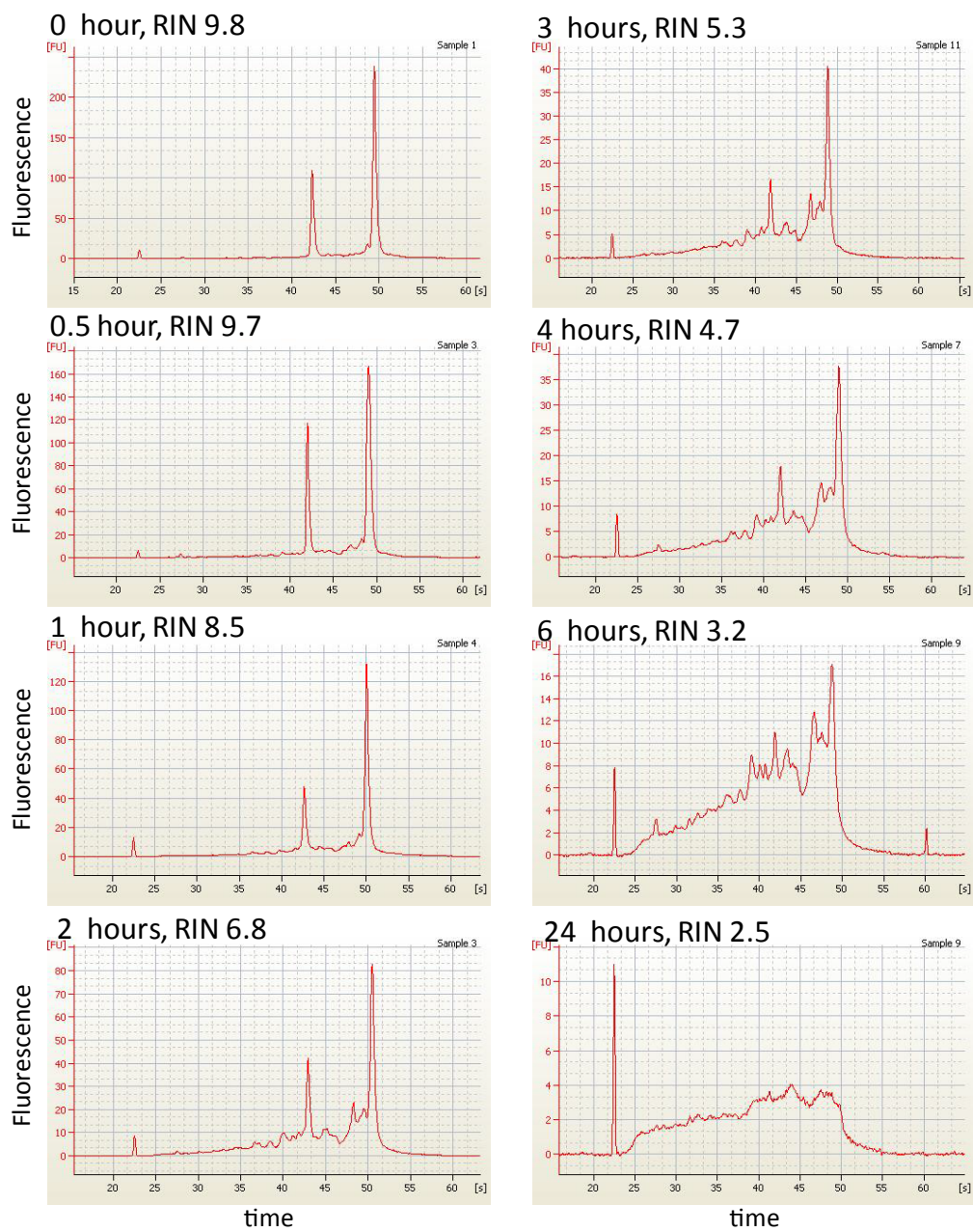


Figure 2: Electropherogram profiles at different time of heat-mediated degradations. RNA extracted from cell line incubated at room temperature prior to extraction. After extraction of RNA, electrophoresis of the RNA was carried out using Agilent 2100 Bioanalyzer. 18S and 28S rRNA peaks map to 42 sec and 50sec respectively. Incubation time is indicated for each graph.

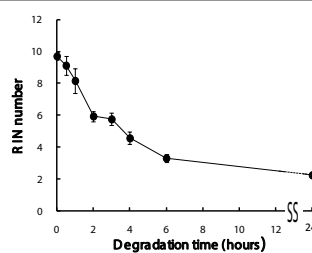


Figure 3: Effects of degradation time on RIN values. RIN numbers of each sample of different degradation times were measured. The RIN numbers were calculated automatically by algorithm using Bayesian learning network by Agilent 2100 Bioanalyzer.

refRNA1000 without cells results in the yield of extraction with 0.67 ± 0.15 (n=5).

Figure 5 shows relative values of refRNA1000, *Actb* and *Rps18* of degraded samples. The amounts of these RNA were calculated by standard curve and normalized by that of no degraded sample. The relative amounts decrease with degradation time, caused by dividing into short fragments or by alternation regardless of sequences.

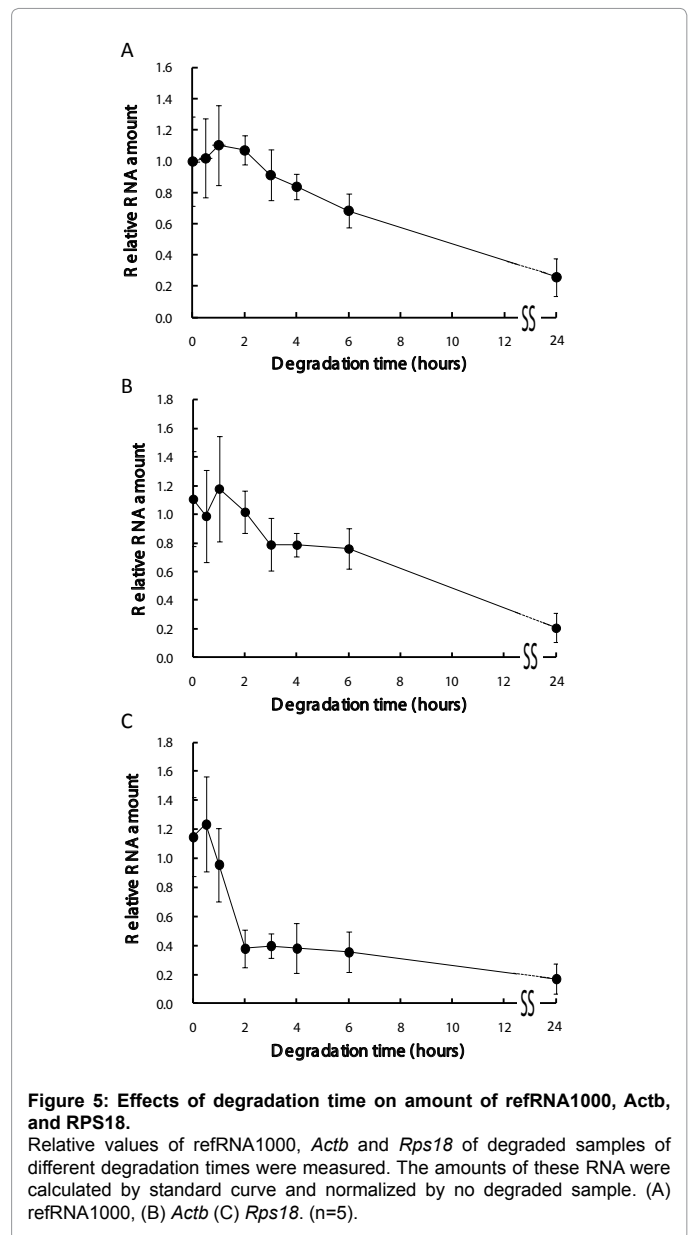
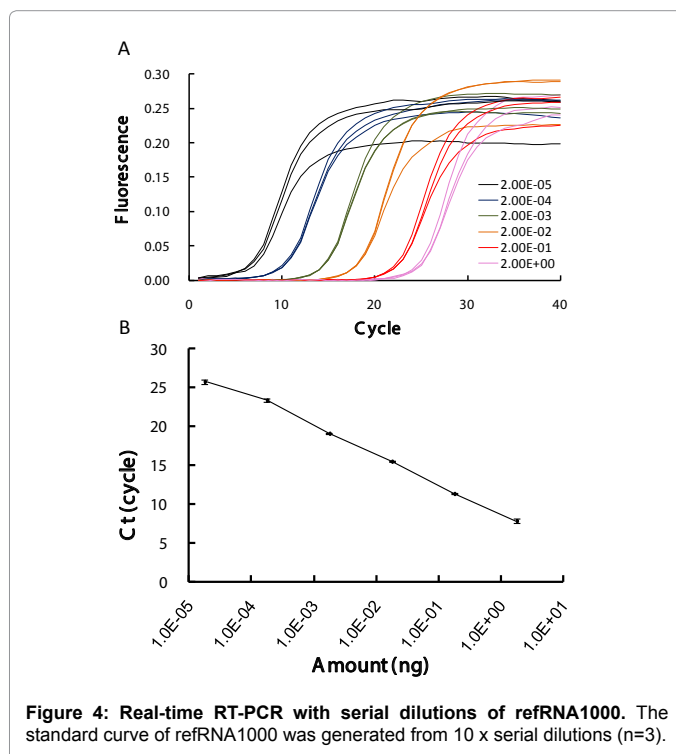
We examined correlation of pairwise correlation among refRNA100, *Actb*, *Rps18* and RIN. The correlation coefficients between refRNA1000 were 0.794, 0.546 and 0.635 for *Actb*, *Rps18*, RIN, and different were statistically significant ($p < 0.001$). The correlation coefficients between RIN were 0.640 and 0.857 for *Actb*, *Rps18*, and different were statistically significant ($p < 0.001$).

Discussion

RNA is useful to clinical diagnostics, because RNA contains an enormous amount of information on expression of messenger RNA and non coding functional RNAs which remain without being translated into proteins. But for evaluation of RNA expression levels, degradation is susceptible during storage or artificial manipulations such as cell separation and extraction. So it is important to evaluate RNA quality from the starting step to evaluating step.

Classically, RNA quality is evaluated by agarose gel electrophoresis stained with ethidium bromide. RNA considered of high quality when the ratio of 28S:18S bands is close to 2.0 or higher, but the ratio has been shown to be inconsistent. The performance of semi-quantitative estimation of mRNA using microarray or real-time RT-PCR studies were significantly influenced by RNA integrity [9,10]. Recently, two computer methods, the degradometer software and the RIN algorithm were developed [7,11].

RIN is the method for evaluation RNA integrity taking



characteristics of several regions of the recorded electropherogram into account, these regions are the marker region, 5S region, fast region, 18S fragment, inter region, 28S fragment and post region [7]. RIN was proposed instead of the 28S/18S ratio, as this ratio was shown to be unrelated to the amounts of housekeeping genes semi-quantified by real-time RT-PCR [12]. RIN is considered more reliable to evaluate RNA integrity based on the relation to the amount of housekeeping gene by real-time RT-PCR. The amount of endogenous gene reflects the extent of degradation more directly than that of RIN and can be carried out without expensive equipment.

To estimate the RNA integrity we propose to use refRNA1000, as there are significant correlation between RIN and the amount of refRNA1000, in varying state of degradation. There are also significant correlations between refRNA1000 and endogenous gene such as *Actb* and *Rps18* in varying state of degradation in our study. The results suggest for the benefit of refRNA1000 for evaluation of RNA integrity.

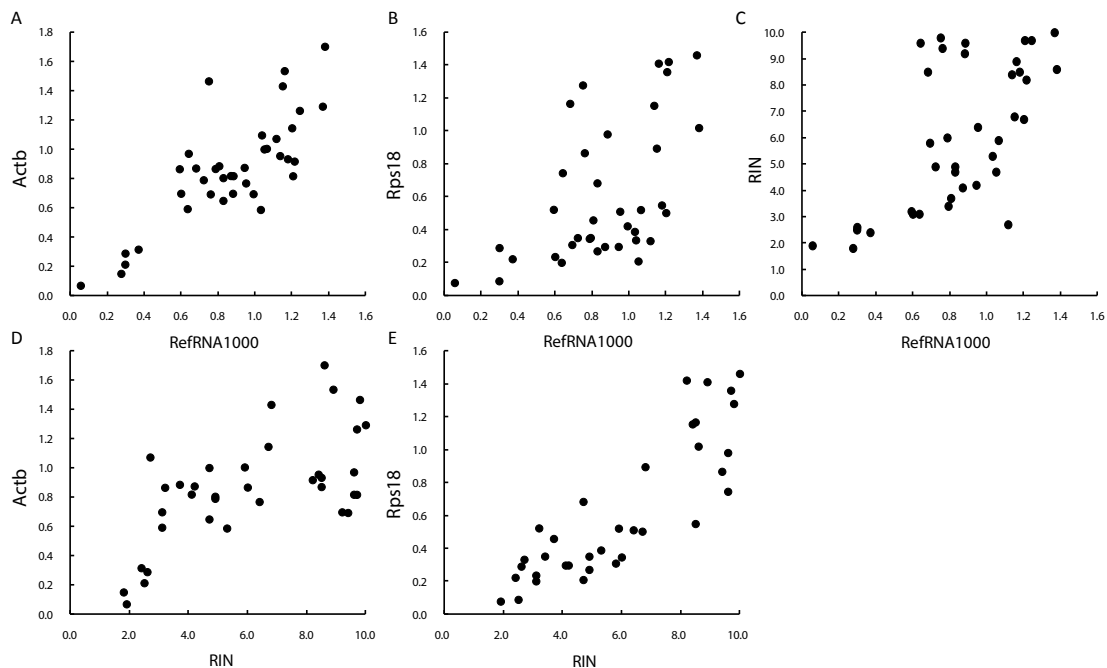


Figure 6: Scatter plot of degraded-related correlation coefficients. (A) RefRNA vs. *Actb*. (B) RefRNA vs. *Rps18*. (C) RefRNA vs. RIN. (D) RIN vs. *Actb*. (E) RIN vs. *Rps18*.

To monitor the extent of degradation, the extraction yield, the storage conditions and even the transportation procedures, we may refer the reference materials of refRNA1000 by poured refRNA1000s before extraction, before storage, before transportation, and just after sampling. Using ribonucleic acid solutions for quantitative analysis of NMIIJ CRM 6204 may evaluate the each procedures by different refRNA1000s at once. Not only for clinical diagnosis, but scientists always require the validity of experimental procedure, one of the candidate methods is using reference materials of RNA such as refRNA1000.

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