

RESEARCH ARTICLE

Effect of Temperature Towards RNA Concentration: Quantitative Investigation with SpectrophotometerFeby Fariska Savira,¹ Novi Silvia Hardiany^{2*}¹Medical Science Program, ²Department of Biochemistry and Molecular Biology, FM Universitas Indonesia

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Abstract

Ribonucleic acid (RNA) is a thermodynamically unstable molecule. The way RNA samples are preserved is critical to maintain maximum yield and quality, therefore it is useful for molecular analysis such as real time-PCR. There are many contradictions and variations regarding the temperature for RNA storage. The aim of this study is to find the ideal temperature to store RNA among -80°C, -20°C and 4°C by determining changes in RNA concentration over two weeks of storage time. The liver of eight rats was divided into three groups, weighing from 25-26 µg. Samples were homogenized, isolated and stored in -80°C, -20°C and 4°C freezers. Absorbance was measured with spectrophotometry at 260 and 280 nm to determine the concentration of RNA. There was no significant difference in the concentration of RNA samples stored in all temperatures after two weeks, both experimentally and statistically (Kruskal-Wallis, -80°C p=0.949; -20°C p=0.885; 4°C p=0.935). In conclusion, RNA can be stored in -80°C, -20°C and 4°C for two weeks without quantity reduction. Longer duration of study and RNA quality analysis is recommended to check for RNA degradation.

Keywords: RNA, temperature, concentration, storage, spectrophotometry

**Efek Suhu terhadap Konsentrasi RNA:
Investigasi Kuantitatif dengan Spektrofotometer****Abstrak**

Ribonucleic acid (RNA) adalah molekul yang tidak stabil secara termodinamik. Cara penyimpanan RNA sangat kritis untuk menjaga kuantitas dan kualitasnya agar dapat digunakan untuk analisis molekuler seperti real time-PCR. Tujuan penelitian ini adalah mengetahui suhu ideal penyimpanan RNA di antara -80°C, -20°C dan 4°C dengan melihat perubahan pada konsentrasi RNA selama dua minggu masa penyimpanan. Delapan hati tikus dibagi menjadi tiga kelompok dengan berat sampel 25-26 µg. Sampel hati dihomogenisasi dan diisolasi, lalu disimpan pada suhu -80°C, -20°C and 4°C. Absorbansi diukur dengan spektrofotometri pada gelombang cahaya 260 dan 280 nm untuk mendapatkan konsentrasi RNA. Tidak ditemukan perbedaan bermakna antara konsentrasi RNA dengan ketiga suhu penyimpanan selama dua minggu, baik secara eksperimental maupun secara statistik (Kruskal-Wallis, -80°C p=0,949; -20°C p=0,885; 4°C p=0,935). Disimpulkan bahwa RNA dapat disimpan pada suhu -80°C, -20°C dan 4°C selama dua minggu tanpa perubahan kuantitas. Durasi percobaan sebaiknya diperpanjang dan analisis kualitas RNA dapat dilakukan untuk melihat apakah ada degradasi terhadap RNA.

Kata kunci: RNA, suhu, konsentrasi, penyimpanan, spektrofotometri

Introduction

RNA is essential in the normal functioning in eukaryotes and prokaryotes.^{1,2} Isolation of RNA for molecular studies is a common procedure. The expression of certain gene is determined by measuring the RNA (usually mRNA) expressions through molecular assays such as real time-PCR.^{3,4} The advancing knowledge on the molecular basis of malignancies has led many scientists to come up with gene therapy as the curative option for cancer.^{3,5} Investigating the assembly and release of viral particles onto cell surface as well as viral RNA genome organization, may be fundamental in finding therapies for incurable viral disease such as those caused by HIV.³

Many factors lead to the usability of RNA samples in generating a reproducible result, thus the ability to store RNA at ease, while maintaining its highest quality and quantity, is essential.⁶ Ribonuclease activity, which can be found in almost all living organisms, are quite stable and easily contaminate RNA samples to cause degradation.⁷ RNA is an unstable material, being less thermodynamically stable compared to DNA due to 2'-OH group present on the ribose ring, which advocate hydrophilic attack directed at the 5'-3' phosphodiester bond. Moreover, higher temperatures and pH elevation have been shown to promote RNA hydrolysis.^{6,7}

The statement among various studies and even RNA kit producers in regards to the ideal temperature for RNA storage has been inconsistent. While many consider -80°C as the gold standard, some deduce that storing in -20°C and 4°C fridge is sufficient.⁸⁻¹⁰ In the Laboratory of Biochemistry and Molecular Biology of Faculty of Medicine Universitas Indonesia, we assume that RNA is best preserved at ultra-low temperatures, thus -80°C freezer has been continually used to preserve RNA samples. Due to the lack of data regarding the reliability of -80°C as the ideal temperature for RNA storage, this study is aimed to establish whether -80°C is actually the most suitable temperature or storage in -20°C and 4°C is adequate, so the laboratory may apply the result onto daily practices with confidence.

Methods

This experimental study was conducted at the Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia. The research took approximately four months and was conducted from November 2014 until February

2015. Twenty-four RNA samples were isolated from the tissue of rat liver using the Geneaid Total RNA Mini Kit (Geneaid, Taiwan) according to the protocol provided in the kit. Tissues (25mg) from the eight different frozen rat livers (stored for ± 1 month in -80°C) were transferred into 24 of 1.5 ml microcentrifuge tubes. Cells of the liver tissue were lysed with RB buffer and β -mercaptoethanol by centrifugation using DNase and RNase-free micropestle until the solution become homogenous and transferred through the filter column. Ethanol (70%) diluted in RNase and DNase free ddH₂O was added and lysates were filtered through RB column onto collection tube to facilitate RNA binding. DNA contaminant was removed by mixing DNase into the reaction buffer, which was loaded onto the centre of RB column matrix. Samples were then washed with W1 buffer and wash buffer provided in the kit. Purified aqueous RNA was suspended in RNase-free water.

The freshly isolated RNA samples were quantified by spectrophotometry using the varioskan flash multimode reader (Thermo Fisher Scientific, USA). All of the RNA samples were vortexed for a short duration (± 10 seconds). Sample holders were cleaned with aquadest and scratch-free tissue (KimWipes) before use, removing any dust. RNA samples were loaded onto sample holders, with RNase free water used as dilution factor (1:1 ratio). Absorbance was read at 260 and 280 nm. Readings on photometric, blank subtraction (both 260 and 280 nm) along with concentration and purity were collected. Afterwards, 8 samples of purified RNA were kept in 4°C fridge, and another 2 batches of 8 were placed in -20°C and -80°C freezers, respectively (total 24 samples).

For the following two weeks, absorbance from each group of samples stored in different temperatures was read on the same day every week. All of the thawing process was done as quickly as possible on ice, and the samples were kept immediately into the relevant storage temperatures (-80°C, -20°C and 4°C refrigerator) to avoid degradation.

All the statistical analyses were done using the SPSS software with parametrical anova test for parametric data and Kruskal-Wallis test for non-parametric data.

Results

Eight different frozen rat liver that had been previously stored for ± 1 month in -80°C were weighed off and separated into three different

groups for each temperature (-80°, -20°, and 4°C) and was re-frozen for a month in -80°C. RNA was extracted using total RNA mini kit (Geneaid) and quantified by spectrophotometer as the initial measurement. Samples were then stored at -80°C, -20°C, and 4°C, each with 8 different samples. On the same day for the next two weeks, the absorption was re-measured and the concentration and purity was calculated.

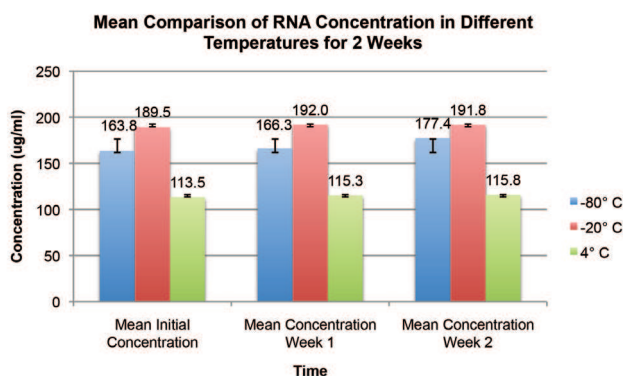


Figure 1. Mean Comparison of RNA Concentration in -80° -20° and 4° C for 2 Weeks

The change in RNA concentration from the initial measurements and two weeks of storage was negligible (Figure 1). Samples stored in 4°C showed a slight fluctuation of $\pm 2 \mu\text{g/ml}$ after a week and stayed relatively the same on the second week. Similarly, mean RNA concentration in -20°C was $\pm 3 \mu\text{g/ml}$ higher on the first week and decreased around $\pm 1 \mu\text{g/ml}$ on week 2. The mean concentration of RNA samples kept at -80°C raised by $\pm 3 \mu\text{g/ml}$ on the first week and increased by $\pm 11 \mu\text{g/ml}$ on the week after.

Shapiro-Wilk test showed that the data did not have normal distribution. We proceeded with non-parametric Kruskal-Wallis test to analyze the sample and the result indicated that there was no significant difference between the storage temperature and RNA concentration (-80°C $p=0.949$; -20°C $p=0.885$; 4°C $p=0.935$).

Discussion

There was no significant difference in concentration of RNA amongst the three different temperatures (-80°C, -20°C, and 4°C) over two weeks of storage time both statistically (Kruskal-Wallis, -80°C $p=0.949$; -20°C $p=0.885$; 4°C $p=0.935$) and experimentally (based on raw data). Initially, we hypothesized that -80°C was the ideal temperature and the concentration would stay

constant overtime. However, the concentration of samples stored in -20°C and 4°C also stayed relatively constant. We concluded that there was no correlation between RNA concentration and -80°C, -20°C, and 4°C as the temperature of storage. In addition There was no significant difference between the concentrations of RNA with short-term storage time (in this case, 2 weeks) in any of the three temperatures. Therefore, RNA may be stored in -80°C, -20°C and 4°C for two weeks with no quantity reduction.

Detection of RNA quantity by means of spectrophotometer runs on the basis of UV light absorption by the conjugated double bonds at the aromatic rings of nitrogenous bases, at the wavelength of 260nm.¹¹ Hydrolysis of the 3',5'-phosphodiester bond takes place due to the presence of the 2'-hydroxyl group that acts as nucleophile, leading to displacement of the phosphate group which further leads to cleavage of RNA backbone.⁶ We speculate that quantity of RNA may decrease upon complete degradation, as the detection of RNA by spectrophotometer relies on the UV absorption by the nitrogenous base that might be lost during RNA degradation process. Previous studies have shown that RNA remained intact after being left for 4 hours on ice.¹¹ The length of time to leave our RNA samples on ice was relatively short, thus RNA degradation is unlikely. Similarly, findings on RNA analysis from avian influenza virus (AIV) samples showed that negligible degradation was observed in storage temperature of -20°C and 4°C after 2 weeks of storage.¹²

Previously, a study in 2006 demonstrated that concentration of fresh RNA samples stored at -20°C decrease after just one day of storage and a month for samples stored at -80°C before any notable reduction of RNA yield was detected.¹² Degradation was observed after a week of storage in -20°C, and 3 months for samples in -80°C.¹³ The disparity of results compared to the data we obtained may arise due to the quality of the kit for RNA isolation used in this study, the length of time in between freeze-thaw cycle, and possibly other procedural issues. Another study found that RNA being stored with low concentration correlates with decreased integrity.¹⁴ Comparison of RNA with concentration of 250ng/ul and 25ng/ul was made and a statistically significant degradation was seen in lower concentration.¹⁴ However, the result was observed after 4 years of storage duration, whereas we focused on the effect of short-term storage on RNA concentration, so comparison cannot be made. Nonetheless, initial

concentration of RNA being stored may have a role in preventing early degradation.

RNA can be easily degraded by spontaneous cleavage of the phosphodiester bond, attacking the hydroxyl group through trans-esterification catalyzed by acid or base.¹⁵ Ribonucleases are also present nearly in all cells and can easily contaminate and degrade RNA that has been purified. However, it is possible that the quality of RNA may be downgraded overtime in any of the three temperatures even if the concentration stays reasonably constant. It is not possible to check for RNA quality with the use of spectrophotometer, as it is a tool for quantification.¹⁶ RNA quality can be determined by electrophoresis and exposure under UV light with bromide staining.¹⁶ RNA samples are put in alkaline buffer to deprotonate phosphodiester backbones fully and to have negative charge, then loaded to the gel by the cathode end to run towards the anode end upon electrical current activation. Result should show two bands of rRNAs, with small sized rRNA being half the size of large rRNA. mRNAs may appear as smear as it typifies population with heterogeneous size. At times, tRNA bands may be seen at the bottom of the gel.^{17,18} Degraded RNA on electrophoresis is characterized by 28S and 18S bands that are smaller than the expected sizes, or the presence of smears of these bands. tRNAs and 5.8S rRNAs that are smaller in size may be seen co-migrating at the gel edge.^{18,19} The appearance of smears with blunt rRNA bands and the loss of 2:1 ratio seen in good quality RNA indicate partially degraded RNA.²⁰ If the RNA has already been completely degraded, then extremely low molecular weight smear may be observed. Protein-contaminated RNAs have the tendency to aggregate at the top of the gel near the well.^{21,22}

Conclusion

There are no marked changes in the concentration of RNA when stored in three different temperatures (-80°C, -20°C and 4°C) over two weeks of storage duration, therefore RNA may be stored in any of these temperatures without reduction in quantity. However, there is no indication if the RNA is usable for molecular analysis. It is recommended that if the length of this study should be extended to at least one month for the three different temperatures (-80°C, -20°C and 4°C) and RNA quality analysis (e.g. through the use of electrophoresis) may be incorporated to check for the integrity of RNA samples.

References

1. Patton JG, Franklin JL, Weaver AM, Vickers K, Zhang B, Coffey RJ et al. Biogenesis, delivery, and function of extracellular RNA. *J Extracell Vesicles*. 2015;4:27494.
2. Murray RK, Bender DA, Botham KM, Kennelly PJ, Rodwell VW, Weil PA, editors. *RNA Synthesis, Processing, & Modification*. Harper's illustrated biochemistry. 29th ed. USA: McGraw-Hill; 2012.p.627-34.
3. Bobbin ML, Burnett JC, Rossi JJ. RNA interference approaches for treatment of HIV-1 infection. *Genome Med*. 2015;7(1):50.
4. Hernandez GE, Mondala TS, Head SR. Assessing a novel room temperature RNA storage medium for compatibility in microarray gene expression analysis. *Biotechniques*. 2009; 47(2): 667–70.
5. Geisler S, Collier J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat Rev Mol Cell Biol*. 2013;14(11):699-712.
6. Berg JM, Tymoczko JL, Stryer L. *RNA Synthesis & Splicing*. Biochemistry. 7th ed. New York: W.H. Freeman; 2010. p.1158-73.
7. Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. *J Biomed Biotechnol*. 2009; 574398.
8. Hernandez GE, Mondala TS, Head SR. Assessing a novel room temperature RNA storage medium for compatibility in microarray gene expression analysis. *Biotechniques*. 2009;47(2):667–70.
9. Sewart S, Barraclough R, Rudland PS, West CR, Barraclough DL. Molecular analysis of a collection of clinical specimens stored at 4 °C as an alternative to snap-freezing. *Int J Oncol*. 2009;35(2):381-6.
10. Baleriola C, Johal H, Jacka B, Chaverot S, Bowden S, Lacey S et al. Stability of hepatitis C virus, HIV, and hepatitis B virus nucleic acids in plasma samples after long-term storage at -20°C and -70°C. *J Clin Microbiol*. 2011;49(9):3163-7.
11. Sun H, Sun R, Zhang X, Liu Y, Hao M, Wang Y et al. Effect of duration of ex vivo ischemia time and storage period on RNA quality in biobanked human renal cell carcinoma tissue. *Ann Surg Oncol*. 2016;23(1):297-304.
12. Fereidouni SR, Flobig A, Starick E, Harder TC. Effect of swab matrix, storage time and temperature on detection of avian influenza virus RNA in swab samples. *Avian Dis*. 2012;56(4 Suppl): 955-8.
13. Seelenfreund E, Robinson WA, Amato CM, Tan A-C, Kim J, Robinson SE. Long-term storage of dry versus frozen RNA for next generation molecular studies. *PLoS One*. 2014;9(11): e111872.
14. Oliveri EHR, Franco Lde A, Pereira RG, Mota LD, Campos AH, Carraro DM. Biobanking practice: RNA storage at low concentration affects integrity. *Biopreserv Biobank* . 2014;12(1):46-52.

15. Kasahara T, Miyazaki T, Nitta H, Ono A, Miyahishima T, Nagao T et al. Evaluation of methods for duration of preservation of RNA quality in rat liver used for transcriptome analysis. *J Toxicol Sci.*2006;31(5):509-19.
16. Cseke LJ, Kirakosyan A, Kaufman PB, Westfall MV. Isolation and Purification of RNA. *Handbook of molecular and cellular methods in biology and medicine.* 3rd ed. USA: CRC Press; 2011.p.178-81.
17. Kieleczawa J. Assessing DNA & RNA following purification. *DNA sequencing II: optimizing preparation and clean up.* Massachusetts: Jones & Barlett Learning; 2006.
18. Avison M. Isolation and Analysis of RNA. Measuring gene expression. New York: Taylor & Francis Publishing; 2008. p.72-3.
19. Russell S, Meadows LA, Russell RR. Sample Collection and Labeling Microarray technology in practice. California: Academic Press; 2008.p.74-5.
20. Verlag C. Materials and methods. Effects of salt stress on ecophysiological and molecular characteristics of populous *Euphratica olic.*, *Populus X canescens (Aiton) Sm* and *Arabidopsis thaliana L.* Gottingen: Cuvillier Verlag; 2008.p.20-2.
21. Grody WW, Nakamura RM, Kiechle FL, Strom C. General procedures. *Molecular diagnostics: techniques and applications for the clinical laboratory.* Massachusetts: Academic Press; 2009.p.566-7.
22. Meurant G. Electrophoresis of RNA. *RNA methodologies: a laboratory guide for isolation and characterization.* California: Academic Press; 2012.p.141-3.