Long-term Stability of Total RNA in RNAstable® as Evaluated by Expression Microarray

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Abstract

Storage of labile RNA in laboratories is accomplished through ultra-low freezing of the nucleic acids. This, however, requires expensive freezers, convenient storage, reliable electrical power, and increased shipping costs, thereby making it a less viable option. Biomatrica (San Diego, CA) has created RNAstable®, a stabilization reagent that is used to store RNA in a dehydrated state at room temperature (RT) and to protect the RNA from degradation. Our objective was to investigate the functionality of RNA stored in RNAstable at extended time periods and at varying temperatures through use of Illumina® and Agilent RNA expression microarrays. We observed in Bioanalyzer electropherograms that total RNA extracted from 293 cells stored at RT in RNAstable for 4.5 and 11.5 months is similar in quality to RNA stored at -80°C. Illumina mRNA expression array QC metrics and gene expression patterns from RNAstable-protected RNA correlated well with those of freezer controls. In contrast, when RNA was stored at RT but without RNAstable, there were increased levels of degradation and a decrease in relatedness to both -80°C control and RNAstable-protected RNA. Significantly, when RNA was stored in RNAstable at 45°C for 4.5 months, equivalent to 22 months RT storage, RNA quality and expression microarray performance remained similar between RNAstable-protected RNA at RT and the -80°C controls. At 10.5 months, miRNA levels were compared among the storage conditions using miRNA expression arrays. Here too we found strong concordance between miRNA analytes when total RNA was stored in RNAstable or at -80°C. Further, Bioanalyzer electrophoresis of RNAstable-protected samples stored at RT for a relative total of 33 months or 50.5 months showed comparable integrity scores to those of -80°C controls. We conclude that use of RNAstable holds promise as an effective stabilization reagent for total RNA and should be useful in situations where shipping and storage options are resource limited.

Introduction

RNA is highly labile and prone to hydrolysis in the presence of alkaline solutions and degradation by ribonucleases. Typically, RNA is stored in RNase-free water and frozen at -80°C to prevent loss of RNA integrity, and thus requires the purchase and maintenance of large commercial freezers. Additionally, in the course of RNA processing and handling, exposure to freeze-thaw cycles, however brief, can compromise RNA quality. Shipping of RNA is also of concern, not only from the perspective of increased costs due to shipments requiring dry ice, but also because unexpected delays in transportation or disruption of packaging can thereby introduce RNA to elevated temperatures.

Several studies have described the effectiveness of commercially available products for protection of RNA prior to use in gene expression assays. However, these items were generally designed for tissue sample storage prior to nucleic acid extraction rather than the prevention of degradation of newly isolated RNA. Biomatrica has developed RNAstable, a synthetic matrix that enables the dry storage of RNA at room temperature (RT). RNAstable was developed based on the principles of anhydrobiosis, a natural biological mechanism utilized by some multicellular organisms that allows their survival in a dehydrated state. Once RNAstable is applied to an aqueous solution of RNA, the sample is desiccated by air-drying or use of a vacuum concentrator. A thermostable barrier is subsequently formed around the RNA effectively safeguarding RNA integrity for extended time periods. Samples are completely recovered for immediate use by rehydration. RNAstable thus offers an alternative technology for storing RNA at RT, bypassing the needs and concerns associated with cold storage and transport.

Maintenance of RNA integrity is critical in accurately assessing RNA expression in downstream PCR. Hernandez et al. performed microarray expression analyses on human liver RNA stored in RNAstable at RT or at -80°C for four weeks. Microarray quality control metrics showed nearly identical values for RNAstable-protected RNA or frozen controls. This study illustrated the suitability of RNAstable-protected RNA for use in microarray expression studies. Here we show data suggesting that RNAstable will preserve total RNA at extended storage times greater than 10-times previously reported for use in both mRNA and miRNA expression arrays.

Materials and Methods

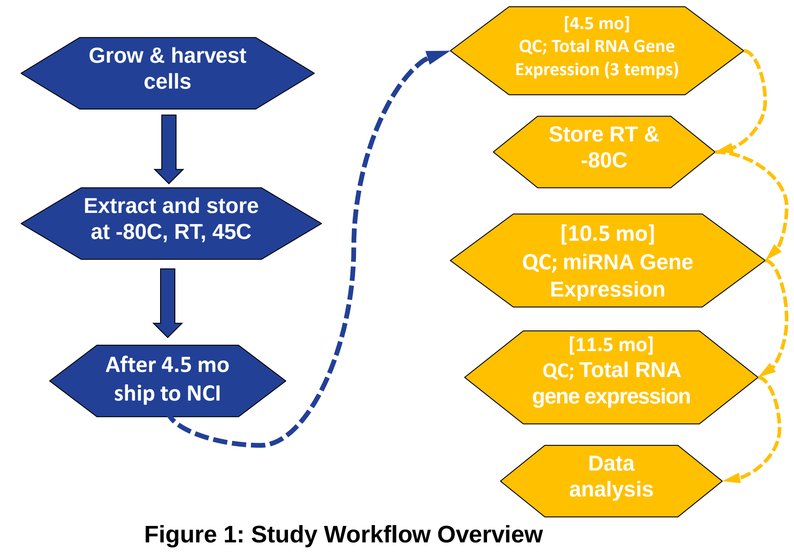

Figure 1: Study Workflow Overview

Results

Condition	RIN Scores	
	4.5 Months Storage	11.5 Months Storage
Control -80°C	9.80	9.80
RT with RNAstable	9.80	9.60
RT Unprotected	3.30	2.50
45°C with RNAstable	9.80	NA
45°C Unprotected	2.30	NA

Figure 2: Evaluation of integrity of mRNA stored in RNAstable
Replicates of extracted total RNA from 293 cells at the indicated storage condition were analyzed using the Bioanalyzer (Agilent Technologies). The samples were evaluated at 4.5 months, or 11.5 months. The 45°C 4.5 month storage is the equivalent of 22 months at RT . RIN values are an average across replicates for that sample group.

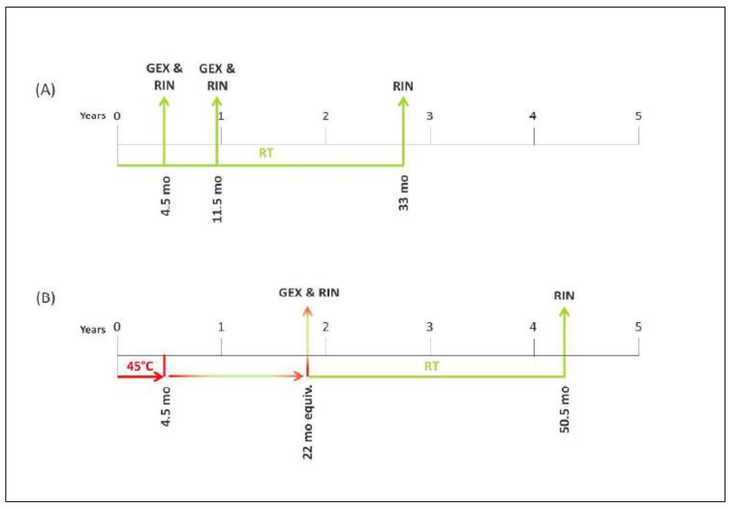

Figure 3: Timeline of sample storage and analysis in RNAstable.
Time = 0 is when total RNA were extracted and applied to RNAstable or left unprotected prior to shipping. (A) Aliquots of RNA stored in RNAstable at only RT (green) were rehydrated at the indicated time points for analysis by gene expression microarrays (GEX) and/or RNA quality on the Bioanalyzer (RIN). (B) To examine the effect of accelerated aging of RNA, samples were stored in RNAstable at 45°C (red) for 4.5 months, which is the equivalent of 22 months at RT, prior to GEX and RIN. Subsequently, remaining aliquots of RNA were stored at RT only (green) prior to RIN analysis.

	Comparison	Total targets ≥ 2-fold	Targets Increased ≥ 2-fold	Targets Decreased ≥ 2-fold
4.5 months Storage	RNAstable RT vs. -80°C	2 (0.01%)	1 (50.0%)	1 (50.0%)
	RNAstable 45°C vs. -80°C	2 (0.01%)	1 (50.0%)	1 (50.0%)
	Unprotected RT vs. -80°C	882 (3.60%)	0 (0.00%)	882 (100.0%)
	Unprotected 45°C vs. -80°C	3760 (15.3%)	87 (2.31%)	3673 (97.7%)
	Unprotected RT vs. RNAstable RT	661 (2.70%)	21 (3.18%)	640 (96.8%)
	Unprotected 45°C vs. RNAstable 45°C	3294 (13.4%)	165 (5.01%)	3129 (94.9%)

Figure 4:The effect of RNAstable on total RNA gene expression levels
RNA stored in RNAstable or unprotected is compared over time and evaluated for changes greater than two fold the original RNA level for single genes. Samples of RNA were stored for 4.5 months and 11.5 months at RT or 45°C and were analyzed on the Illumina Human Ref-8 v3 Expression Bead Chips or the Human HT-12 v4 BeadChips (Illumina, Inc., San Diego, CA). One-way ANOVA with paired contrasts were conducted and adjusted p-values less than 0.05 (Benjamini & Hochberg multiple sample comparison) were applied to the final gene list. The table only includes genes that have at least two fold expression level changes.

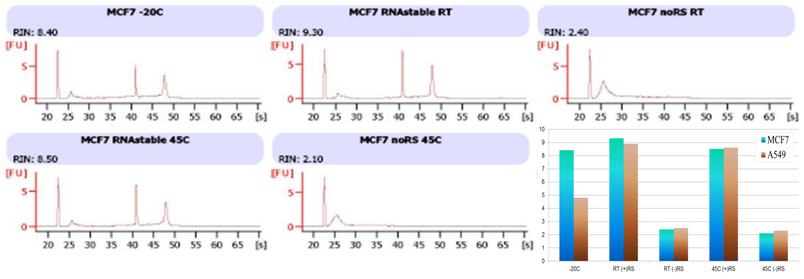

Figure 5: Validation of RNAstable in multiple cell types
Replicates of extracted total RNA from MCF7 were stored for 4.0 months at RT and analyzed using the Bioanalyzer (Agilent Technologies). RIN values are an average across replicates for that sample group.

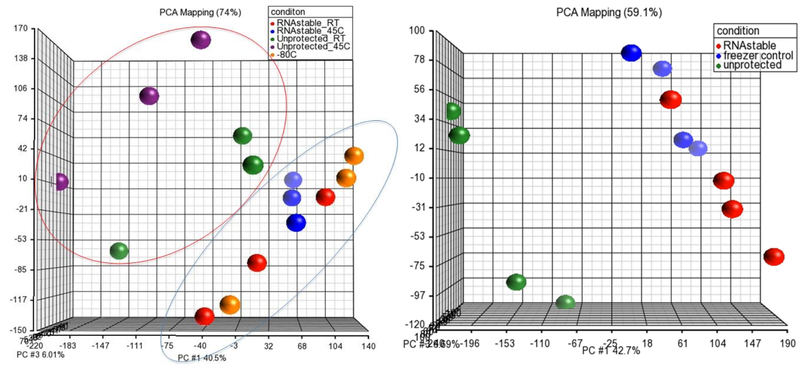

Figure 6: Principal component analysis (PCA) for mRNA gene expression variance
PCA was performed to show the level of variance in gene expression patterns among the different storage conditions. Consistent with the above data, PCA demonstrated that at both 4.5 months (A) and 11.5 months (B), frozen controls and RNAstable protected RNAs (RT and 45°C) clustered together. Unprotected RNA was separated from both -80°C and RNAstable protected samples, indicating a higher degree of variance and decrease in similarity.

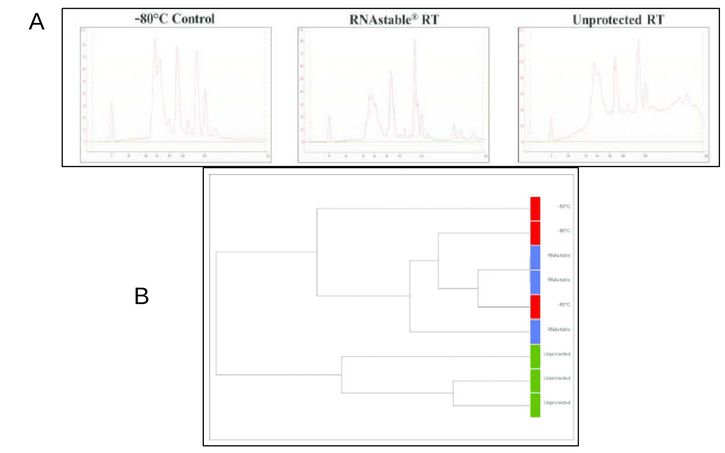

Figure 7: Evaluation of integrity of small RNAs stored in RNAstable
Bioanalyzer electropherograms are shown of the small RNA fraction from a sample of total RNA stored at -80°C, RT with RNAstable®,or RT without RNAstable. (A) Both protected samples show multiple small RNA species. (B) The degree of similarity in miRNA targets among the storage groups was assessed by hierarchical clustering and displayed as a dendrogram.

	Comparison	Correlation (r)
10.5 months storage	-80°C vs. -80°C	0.9633
	RNAstable RT vs. RNAstable RT	0.9759
	Unprotected RT vs. Unprotected RT	0.9478
	-80°C vs. RNAstable RT	0.9574
	Unprotected RT vs. -80°C	0.9325
	Unprotected RT vs. RNAstable RT	0.9228

Figure 8: The effect of RNAstable miRNA gene expression levels
RNA stored in RNAstable or unprotected were stored for 10.5 months at RT. The r correlation of miRNA expression profiles in the presence or absence of RNAstable are the mean Pearson correlation r values.

Conclusions

Collectively these data demonstrate that total RNA stored in RNAstable for extended time periods, whether at RT or at 45°C, is not dissimilar in quality to that of RNA stored in ultra-low temperatures (-80°C). As evidenced by the comparable RIN values between RNAstable-protected RNA and frozen controls, RNAstable prevented loss of RNA integrity at RT after 11.5 months of storage. Maintenance of RNA quality was observed at 4.5 months storage at the accelerating temperature of 45°C, indicating that RNAstable can protect RNA at RT for up to at least 22 months. When we lengthened RNA storage to 33 months for samples protected by RNAstable at RT, or to 50.5 months for samples originally in RNAstable at 45°C (for 4.5 months, then at RT), RIN values remained similar to frozen controls (RIN ≥9.3; data not shown). These findings implied that the behavior of RNA stored in RNAstable would be unaltered in microarray hybridization experiments as compared to RNA kept at the standard temperature of -80°C. Indeed, multiple quality control metrics including cRNA labeling efficiency, hybridization probe signal intensities, and correlation analyses all suggest that RNAstable-protected RNA is suitable for use in gene expression microarray analyses. Concordant with RIN scores, higher cRNA yields were generated from RNAstable-protected RNA in comparison to RNA stored in the absence of RNAstable. Additionally, due to the observed increase in degradation, overall hybridization signal intensities and probe detection rates showed a decreasing trend in unprotected RNA relative to frozen controls and RNAstable-stored RNA.