



ABSTRACT

We have developed a novel RNA stabilization technology designed to protect RNA samples from degradation stored dry at room temperature. The storage reagent (RNAstable™) is based on the natural principles of anhydrobiosis (meaning "life without water"), a biological mechanism employed by some multicellular organisms that enables their survival while dry for up to 120 years (Crowe et al., 1998, Ann. Rev. Physiol. 60:73-103). The objective of this study is to evaluate and develop an alternative handling, storage and shipping strategy to optimize RNA sample stabilization for use in life science research. Results demonstrate protection of total RNA samples (50-100 µg) purified from mammalian 293T cells stored dry in RNAstable for 36 months at room temperature as compared to unprotected control samples. Accelerated aging studies demonstrate stabilization of RNA for at least 2 years at room temperature. RNA samples stored at 50°C for 4 months were stabilized and showed no degradation as compared to freezer stored control samples; unprotected samples were completely degraded. Samples were recovered from storage in RNAstable by one-step re-hydration and used directly without further purification. Recovered RNA samples were used successfully as templates for first-strand synthesis for subsequent cDNA amplification, quantitative RT-PCR amplifications of the low copy number *RNase P* gene, and in bioanalyzer and microarray analysis. The development of RNA stabilization methods, tools and reagents will have a significant impact on life science research by eliminating some of the detrimental variables associated with RNA sample handling, storage and transport. Studies using RNA samples stored in RNAstable are currently being evaluated for other downstream applications.

INTRODUCTION

Each year, millions of biological samples are processed, distributed and stored worldwide. Currently, samples such as DNA, RNA, proteins, bacteria, viruses, tissues, and other biological molecules are stored cold to prevent, or at least reduce the rate of degradation. Even for small labs, maintaining these cold environments requires multiple expensive refrigeration and freezer units, all of which consume energy and limited laboratory budgets and storage space. Current methods of sample transport are also problematic, as shipping frozen samples on dry ice is expensive, with shipments ranging to hundreds of dollars due to bulky containers and expedited delivery costs. Unfortunately, even under carefully monitored cold storage environments, repeated freeze-thaw cycles and fluctuating temperatures only serve to promote degradation and compromise results.

Despite all the precautions taken to maintain a cold environment, the integrity of a sample is often compromised using current cold storage methodologies, particularly during long-term storage. For example, the average DNA sample lasts for about 10 years under cold storage conditions; unfortunately, this is not long enough if the sample itself is needed for future reference, as in the case of forensic samples or those used for diagnostics. Far more problematic are RNA samples, which are highly labile in nature with a tendency to degrade even under carefully controlled RNase-free conditions and cold storage. Even a short period of slightly elevated temperatures can compromise RNA integrity and detrimentally affect performance in downstream assays. Current methodologies are limited to storing RNA, either purified or in tissues, in cold environments; until recently there were no products that stabilized RNA at ambient temperatures.

From Nature to the Lab

Biomatrix has developed a high-performing biostabilization technology to prevent the degradation of biological materials at room temperature, eliminating the need for cold storage and shipping. RNAstable is Biomatrix's novel storage product that directly preserves and stabilizes RNA samples at ambient and elevated temperatures. RNAstable is based on the natural principles of anhydrobiosis (meaning "life without water"), a biological mechanism employed by some multicellular organisms that enables their survival while dry for up to 120 years. Anhydrobiotic organisms (such as tardigrades and brine shrimp) can protect their DNA, RNA, proteins, membranes and cellular systems for survival and can be revived by simple rehydration. Biomatrix's technology transfers the molecular principles of anhydrobiosis to a synthetic chemistry-based stabilization science that was used to develop RNAstable to prevent RNA degradation and stabilize the fragile molecule at ambient temperatures. RNAstable works by forming a thermo-stable barrier securely "shrink-wrapping" RNA samples and providing protection against degradation (Fig. 1).

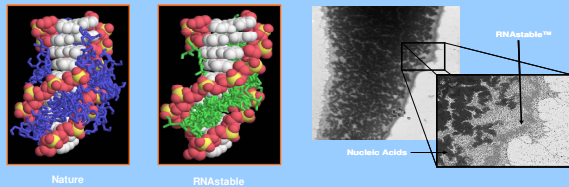


Figure 1. Structural Prediction of RNAstable interacting with Nucleic Acids. Molecular modeling prediction of interactions of RNAstable with nucleic acid molecules. Trehalose disaccharides are predicted to interact with nucleic acid molecules through minor groove interactions based on hydrogen bonding (Nature; left). RNAstable is predicted to form similar interaction patterns as trehalose (RNAstable; center). Electron microscopy shows the thermo-stable barrier that forms around nucleic acid molecules, which stabilizes and helps prevent degradation (right).

RNAstable Technology

RNAstable is supplied as a dried matrix on the bottom of tubes or in a 96-well plate format. Each sample storage tube or well contains enough matrix to protect up to 100 µg of RNA. The steps involved in using RNAstable for RNA storage are outlined in Figure 2. By adding RNA in either water or buffer, RNAstable is re-hydrated and mixes with the RNA. Through its natural affinity to RNA, RNAstable associates with the nucleic acid in the liquid phase. Air-drying of the mixture results in a stabilizing glass that serves to protect the RNA from degradation. Once completely dried, samples can be stored at room temperature and relative humidity conditions $\leq 50\%$ or stored in a moisture barrier container.

Sample recovery requires simple re-hydration using water or a buffered solution. Furthermore, since the re-hydration volume can be chosen between 10-100 µl, storage of RNA in RNAstable also provides an easy method for sample concentration, eliminating the need for time-consuming salt precipitations and sample loss due to multiple wash steps or micro-concentration columns. RNA samples recovered following storage in RNAstable can be used directly in downstream applications (such as reverse transcription, cDNA synthesis, PCR, gel electrophoresis, hybridization analysis, bioanalyzer and microarray analysis) without inhibition or interference.

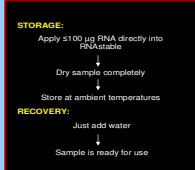


Figure 2. Protocol for RNA storage in RNAstable. RNA samples are applied directly into RNAstable, dried, and then stored at ambient temperatures. Sample recovery requires one-step rehydration, and the RNA is ready for use in downstream applications without the need for further purification.

Research Statement

The objective of this study is to evaluate and develop an alternative handling, storage and shipping strategy to facilitate biological research. Studies were designed to evaluate the feasibility of using RNAstable for the ambient temperature storage of RNA samples and subsequent use in downstream applications. Recovered RNA samples were used directly without further purifying to assess any interference or inhibition in downstream applications including electrophoresis, cDNA synthesis, RT-PCR, qPCR, bioanalyzer and microarray analysis.

Materials and Methods

Sample Preparation and Storage in RNAstable for cDNA synthesis: Aliquots of total RNA prepared from human 293T cells were applied to RNAstable in the 1.5 ml standard microfuge tube format and allowed to dry for 1.5 hours in a SpeedVac® without heat. An unprotected control sample (NP) was prepared by drying aliquots of total RNA into an empty tube under identical conditions. Samples were then stored for at room temperature or at elevated temperatures for various times with relative humidity of <50%. RNA was rehydrated by adding DEPC-treated water to a final concentration of 1 µg/µl for each sample. A 1 µg aliquot of each RNA sample was run on a 1.2% 1xTAE gel containing ethidium bromide. Freezer controls were kept at -20°C.

cDNA Synthesis: 293T total RNA stabilized in RNAstable were stored at room temperature, 60°C for 3 days or 50°C for 4 months with relative humidity of <50% prior to use as templates for first-strand synthesis. Each sample of total RNA (1 µg) was incubated with 300 ng of oligo dT at 65°C for 5 min. Samples were then cooled on ice for 10 min to allow annealing. Reverse transcription was performed using 50U of Stratascript™ Reverse Transcriptase and 40U of RNase Block. Samples were incubated at 42°C for 50 min to allow cDNA synthesis and then incubated at 70°C for 15 min to inactivate the RNase inhibitor. A 1 µl aliquot of first-strand synthesis product was then used as templates for amplification of the human β -actin, GAPDH or RNaseP transcripts. Aliquots of each reaction were run on 1.2% 1xTAE gels.

TaqMan® One-Step RT-PCR: Following storage for 3 months at 50°C or 80°C (control) RNA samples were rehydrated in 25 µl DEPC-treated water to a final concentration of 20 ng/µl for each sample. Serial dilutions were performed to a final concentration of 0.2 ng/µl. A 5 µl aliquot of each sample was used as template for expression of the 18s rRNA gene using TaqMan® One-Step RT-PCR (ABI) reagents. A final concentration of 400 mM was used for each forward and reverse primer in the reaction. A 250 mM final concentration of the 18s rRNA probe was used (5' labeled with FAM and 3' labeled with TAMRA). Reactions were prepared in a 25 µl final volume.

Microarray analysis: Total RNA was isolated from human fetal cartilage tissues as described in Krakow et al., (Mol Genet Metab. 2003; 79:1134-42). Total RNA was resuspended in DEPC-treated water and stored at -20°C until ready for use. Aliquots of total RNA were applied to RNAstable in the 1.5 ml standard microfuge tube format and allowed to dry overnight in a laminar flow hood and then stored for 14 days at room temperature. Control samples were stored for the same time period at 80°C. The quality and quantity of all the stored RNA samples were then analyzed using an Agilent 2100 bioanalyzer and NanoDrop® ND-1000 spectrophotometer, respectively. Fluorescent labeling of RNA and microarray analysis was performed as described in Krakow et al.

Results

RNA stabilization in RNAstable

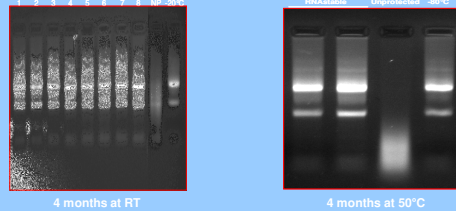


Figure 3. Aliquots of 1 µg 293T total RNA stabilized in RNAstable and stored dry at room temperature or 50°C with relative humidity <50% for 4 months. Samples were re-hydrated in DEPC-treated water and run on a 1.2% 1xTAE gel. Lanes 1-8: 293T total RNA stored in RNAstable; NP: no protection control; positive control sample stored frozen.

cDNA synthesis and RT-PCR using RNA stored in RNAstable

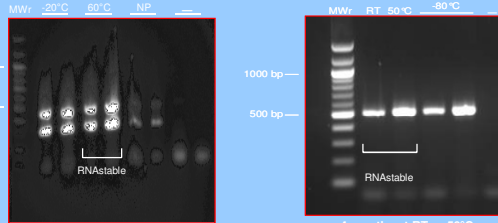


Figure 4. Aliquots of 1 µg of total RNA from 293T cells stabilized in RNAstable or unprotected sample were stored at 60°C for 3 days and used as templates for subsequent first-strand synthesis and amplification of the human β -actin (420 bp) and human GAPDH transcripts (312 bp) (left). Aliquots of 500 ng total RNA from 293T cells were stored in RNAstable for 4 months at room temperature or 50°C with relative humidity <50% and used as templates for first-strand synthesis and amplification of the RNaseP amplicon (517 bp) (right).

Compatibility of samples stored in RNAstable for qPCR analysis

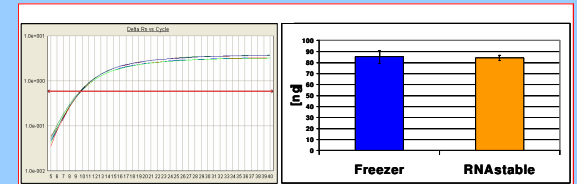


Figure 5. Aliquots of total RNA were stored at either -80°C or at 50°C either protected in RNAstable or unprotected for 3 months. An aliquot of the freezer stored RNA and the 50°C sample protected in RNAstable was quantified using the 18s rRNA gene in a One-step RT-PCR reaction (left). Three samples were amplified for each storage condition and the results from the 6 reactions are overlaid on the graph. Recovery levels (ng) for total RNA stored at -80°C compared to samples protected in RNAstable and stored at 50°C for 3 months (right).

Bioanalyzer and microarray analysis of RNA in RNAstable

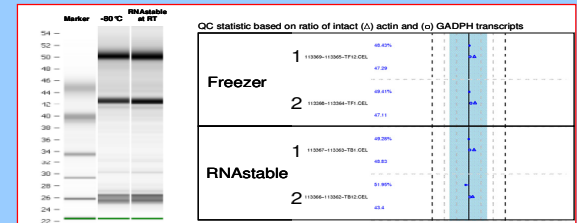


Figure 6. Profiles of total RNA derived from human fetal cartilage samples stored at -80°C or at room temperature (RT) protected in RNAstable for 14 days and analyzed using an Agilent 2100 bioanalyzer (left). Quality control statistics based on ratio of 5'3' actin and GAPDH transcripts indicate identical results between -80°C stored control sample and RNA protected in RNAstable at room temperature (right).

Cell	PROTON	PROTEIN	RNA	BIOMATRIX	BIOSTABILITY
Human Fibroblast	1,464	1,458	1,458	1,458	1,458
Human HeLa	1,461	1,457	1,457	1,457	1,457
Human Hepatoma	1,464	1,455	1,455	1,455	1,455
Human Spine PC	1,464	1,455	1,455	1,455	1,455
Human Spine MC	1,464	1,454	1,454	1,454	1,454
Human Spine AC	1,467	1,456	1,456	1,456	1,456

Degradable of	PROTON	PROTEIN	RNA	BIOMATRIX	BIOSTABILITY
RNA	1,461	1,457	1,457	1,457	1,457
Protein	1,464	1,455	1,455	1,455	1,455
Matrix	1,464	1,455	1,455	1,455	1,455
Stability	1,464	1,455	1,455	1,455	1,455

No significant difference between samples was observed.

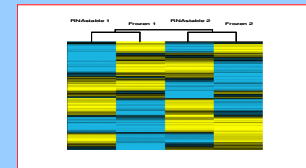


Figure 7. Microarray analysis was performed using the Affymetrix HG-U133 Plus 2.0 array that allows for the complete coverage of the Human Genome U133 set plus 6,500 additional genes for analysis of over 47,000 transcripts (Krakow et al.). Statistical analysis including data normalization and transformation was performed using the BioConductor R package. No significant difference was observed between control or RNAstable stored samples (left). Analysis indicates identical clustering between frozen control samples and those stored at room temperature in RNAstable (right).

Results

- RNAstable allows for long-term stabilization of RNA samples at ambient temperatures with sample recovery by rehydration.
- Recovered RNA can be used directly without the need for further purification in downstream applications. Fluctuating and inconsistent temperatures during shipment will not damage RNA stabilized dry in RNAstable.
- Sample stability is secured even at elevated temperatures of 50-60°C.
- Studies are currently underway to evaluate the compatibility of RNAstable for storing other types of RNA including microRNA, siRNA, plant total RNA and ribozymes, and use in other downstream assays.
- Results indicate that technology advances in sample preservation can prevent the degradation process of biological samples in life science research.
- Products are currently being developed for stabilizing proteins, complex samples and cells.

Acknowledgements

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