Innovative RNA preservation technologies for room temperature sample handling, storage and transport

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ABSTRACT

RNA integrity is a critical factor in obtaining meaningful gene expression data. Current methodologies rely on refrigeration of samples during shipment and storage. To address the critical need for alternative sample storage and transport technologies, we have continued to develop a room temperature RNA preservation technology that protects against degradation during dry storage. Our results demonstrate real-time stabilization of purified total RNA for >14 month at room temperature and even at 50°C for extended time periods. Recovered samples can be used directly in downstream applications including bioanalyzer and microarray analysis, cDNA synthesis, quantitation analysis (e.g. quantitative RT-PCR), reverse transcription, and gel analysis. Results will also be presented using the stabilization technology for the development of a reagent that permits convenient and efficient concentration of dilute, aqueous RNA samples obtained from limited sample types (e.g. needle biopsies). Picogram amounts (≤10 pg) of purified total RNA in up to 500 µl can be concentrated to only 10 µl with minimal sample loss as compared to conventional methods, (i.e. alcohol precipitation, SpeedVac®, microcolumns, etc.). RNA integrity is maintained during the concentrating process and the dried sample can be conveniently stored at room temperature for up to 1 week prior to rehydration and immediate use in downstream assays, including gene expression analysis and other molecular genetics applications. The development of novel RNA stabilization methods and reagents will have a significant impact on biomedical research by eliminating some of the detrimental variables associated with the handling, storage and transport of labile RNA specimens.

INTRODUCTION

From Nature to the Lab

SampleMatrix® is a novel platform technology that directly preserves and stabilizes biological samples at ambient and elevated temperatures and prevents their degradation. SampleMatrix 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 can protect their nucleic acids, proteins, membranes and cellular systems for survival and can be revived by simple rehydration. SampleMatrix technology transfers the molecular principles of anhydrobiosis to a synthetic chemistry-based stabilization science that works by forming a thermo-stable barrier to securely "shrink-wrap" samples such as nucleic acids, and provide protection against degradation (Fig. 1).

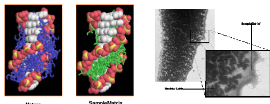

Figure 1. Structural Prediction of SampleMatrix interacting with Nucleic Acids. Molecular modeling prediction of interactions of SampleMatrix with nucleic acid molecules. Trehalose disacchrides are predicted to interact with nucleic acid molecules through minor groove interactions based on hydrogen bonding (Nature; *left).* SampleMatrix is predicted to form similar interaction patterns as trehalose (SampleMatrix; *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 storage medium on the bottom of tubes or in a multiwell plate format. Each sample storage tube or well contains enough storage medium to protect up to 100 µg of RNA. The steps involved in using RNAstable for purified total RNA storage are outlined in Figure 2. By adding aqueous RNA samples in either water or buffer, RNAstable is rehydrated and mixes with the sample. Through its natural affinity to RNA, RNAstable associates with the RNA in the liquid phase. Air-drying of the mixture results in a stabilizing glass that serves to protect RNA from degradation. Once completely dried, samples can be stored at room temperature and relative humidity conditions ≤50%. Sample recovery requires simple rehydration using water or a buffered solution. Recovered rehydrated samples can be used directly in downstream applications (such as reverse transcription, quantitative RT-PCR, cDNA synthesis, PCR, gel electrophoresis, bioanalyzer and microarray analysis) without inhibition or interference.

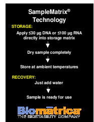

Figure 2. Protocol for RNA storage in RNAstable. Aqueous purified total RNA samples are applied directly into RNAstable, dried and then stored at ambient temperatures with ≤50% relative humidity. Sample recovery requires simple rehydration and the RNA is ready for use in downstream applications without the need for further purification.

A related product, RNAconcentrator™ is designed to concentrate dilute, aqueous RNA solutions from picogram amounts of starting material to only 10 µl. The unique stabilization medium allows for improved recovery as compared to traditional methods, as the thermo-stable properties of RNAconcentrator protect samples from further degradation. Samples are applied into the RNAconcentrator tube and dried down. The dried RNA sample can then be conveniently stored for up to 1 week at room temperature until ready for use. Concentrated RNA can be used directly in downstream applications without further purification, thus avoiding sample loss typically associated multiple wash steps and is particularly suitable for samples derived from limiting starting materials, such as stem cells, FFPE samples or needle biopsies.

MATERIALS AND METHODS

Sample Preparation and Storage in RNAstable: 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. Unprotected control samples (NP) were prepared by drying aliquots of purified total RNA into an empty tube under identical conditions. Samples were then stored at room temperature or at elevated temperatures for various times with relative humidity of <40%. 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.

Sample Concentration using RNAconcentrator: Aliquots of ~100 ng of purified total RNA in a 100 µl volume was concentrated using a SpeedVac into tubes with RNAconcentrator or into an empty tubes (SpeedVac), and also using ethanol precipitation (EtOH ppt). Concentrated samples were resuspended in 10 µl water and used as input template for reverse-transcription PCR (RT-PCR) for amplification of the human Hsp90 amplicon (2.3 kb). In another experiment, aliquots of 10 pg of purified total RNA in 100 µl volume was concentrated using RNAconcentraor or into an empty tubes (SpeedVac), and also using ethanol precipitation (EtOH ppt). Concentrated samples were rehydrated in 10 µl of water and 2.5 µl of each sample was used for quantitative RT-PCR analysis using TaqMan® One-Step RT-PCR reagents (18S ribosomal RNA).

cDNA Synthesis: 293T total RNA stabilized in RNAstable were stored at room temperature, 60ºC 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 transcripts. Aliquots of each reaction were run on 1.2% 1xTAE gels.

miRNA storage and analysis: Total RNA was purified from 293 cells and stored dry (500 ng) for 18 months at 50°C; control samples were stored at -80°C for the identical time period. Following storage, dried total RNA samples were rehydrated in 10 µl water to a final concentration of 50 ng/ µl. Control samples were thawed and also diluted. All samples were then serially diluted 10-fold to 5, 0.5 and 0.05 ng/µl and 5 µl of each dilution was used as template for reverse transcription using primers specific for hsa-miR-24 microRNA species with the mirVana™ miRNA Detection Kit (ABI;Foster City, CA). Detection of the highly expressed miRNA species is indicated by the successful amplification of a 90 bp amplicon.

RESULTS

Long-term Protection of RNA at Ambient Temperatures

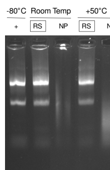

Figure 3. RNAstable protects RNA during long-term storage. Aliquots of purified 293 total RNA were stored dry for **14 months at room temperature or 50°C** either protected in RNAstable (RS) or non-protected (NP) in empty tubes. Control samples were stored at -80°C for the identical time period. The integrity of the RNA following storage was analyzed using agarose gel electrophoresis. Results indicate that RNAstable protects samples from degradation during long-term dry storage.

RNA Integrity Following 14 months Storage

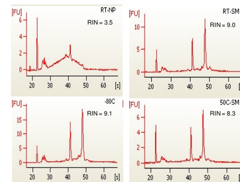

Figure 4. RNAstable Prevents Degradation. Following dry storage for 14 months in RNAstable, the integrity of recovered samples was assessed by bioanalyzer analysis using an aliquot of the rehydrated sample. Frozen control samples stored at -80°C and also non-protected samples stored at room temperature (NP) for the identical time period were also assessed. A comparison of resulting RNA Integrity Numbers indicates that RNAstable successfully prevents sample degradation during dry storage at ambient temperatures.

Protection of poly(A) mRNA After 14 months

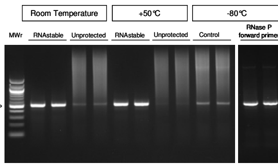

Figure 5. RNAstable protects poly(A) mRNA. Aliquots (500 ng) of total RNA purified from 293T cells was stored dry in RNAstable and kept at room temperature, +50°C, and -80°C for 14 months. Samples were rehydrated and used as templates in reverse transcription reactions including oligo d(T) primers. An aliquot of the cDNA reaction product was used to amplify the RnaseP amplicon (517 bp). Results indicate that RNAstable is protecting full-length poly(A) mRNA transcripts from degradation during long-term storage better than conventional freezer storage.

Improved Yield of Recovery

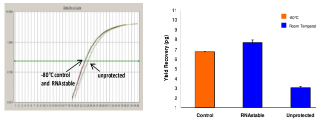

Figure 6. Quantitative RT-PCR analysis of samples recovered from long-term storage in RNAstable. (*left*) Aliquots of the RNA samples stored for 14 months were quantified using the 18S rRNA gene in a TaqMan One-Step RT-PCR reaction (ABI). Three samples were amplified for each storage condition and the results from the 9 reactions are overlaid on the graph. *(right)* Recovery levels (pg) for total RNA stored under different conditions.

Protection of microRNA after 18 months

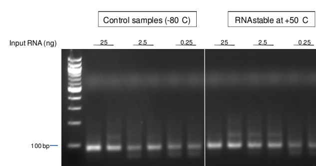

Figure 7. RNAstable protects microRNA in total RNA samples. Total RNA was purified from 293 cells and stored dry (500 ng) for **18 months at 50°C**; control samples were stored at -80°C for the identical time period. Following storage, dried total RNA samples were rehydrated in 10 µl water to a final concentration of 50 ng/ µl. Control samples were thawed and also diluted. All samples were then serially diluted 10-fold and used as template for reverse transcription using primers specific for hsa-miR-24 microRNA species with the *mir*Vana™ miRNA Detection Kit (ABI;Foster City, CA). Detection of the highly expressed miRNA species is indicated by the successful amplification of a 90 bp amplicon. Results indicate that RNAstable can protect microRNA species within total RNA samples even after long-term exposure to extremely elevated temperatures.

RNAconcentrator

The improved stability and recovery of RNA samples recovered from dry storage in RNAstable indicated that the technology could be adapted to concentrating highly dilute, aqueous RNA samples at room temperature. Based on RNAstable technology, RNAconcentrator is specifically formulated to concentrate dilute RNA solutions from picogram amounts of starting material to only 10 µl and is particularly amenable for samples derived from limiting starting material such as stem cells. The unique stabilization medium allows for improved recovery as compared to traditional methods, as the thermo-stable properties of RNAconcentrator protect samples from further degradation. Samples are applied into the RNAconcentrator tube and dried down. The dried RNA sample can then be conveniently stored for up to 1 week at room temperature until ready for use. Concentrated RNA can be used directly in downstream applications without further purification, thus avoiding sample loss typically associated multiple wash steps.

Concentration of Picogram Amounts of RNA

Comparison of Concentration Methods

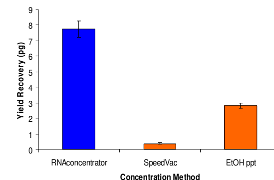

Figure 8. Improved recovery using RNAconcentrator. Aliquots of 10 pg of purified total RNA in 100 µl volume was concentrated using RNAconcentraor or into an empty tubes (SpeedVac), and also using ethanol precipitation (EtOH ppt). Concentrated samples were rehydrated in 10 µl of water and 2.5 µl of each sample was used for quantitative RT-PCR analysis using TaqMan reagents (18S ribosomal RNA). Results indicate significantly improved recovery of concentrated RNA using RNAconcentrator as compared to conventional methods.

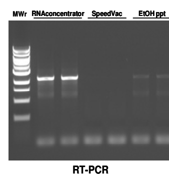

Figure 9. Amplification of concentrated RNA templates. Aliquots of ~100 ng of purified total RNA in a 100 µl volume was concentrated using a SpeedVac into tubes with RNAconcentrator or into an empty tubes (SpeedVac), and also using ethanol precipitation (EtOH ppt). Concentrated samples were resuspended in 10 µl water and used as input template for reverse-transcription PCR (RT-PCR) for amplification of the human Hsp90 amplicon (2.3 kb). Results indicate more abundant amplification from RNA templates concentrated with RNAconcentrator as compared to conventional methods.

CONCLUSIONS

- RNAstable technology protects precious and labile RNA samples (including poly(A) mRNA and microRNA species) from degradation during dry storage at ambient temperatures, and even at elevated temperatures often experienced during sample shipment.
- Sample recovery requires a simple one-step rehydration protocol.
- Recovered samples can be used immediately without further purification and are compatible with downstream applications.
- RNAconcentrator allows convenient room temperature concentration of picogram amounts of purified RNA while maintaining and preserving sample integrity. Starting material from as little as 10 pg RNA in 500 µl can be concentrated and rehydrated in small volumes of ≥10 µl water or buffer
- Results indicate that technology advances in RNA preservation can prevent the degradation process of biological samples used for life science research.