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# Novel Nucleic Acid Stabilization and Enhancement Technologies for PCR Analysis

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## ABSTRACT

We have developed several novel technologies designed to improve upon life science research and biomedical diagnostics. One technology stabilizes and protects nucleic acid samples from degradation during storage at room temperature. We have also developed a highly effective PCR enhancer reagent that improves upon nucleic acid detection during PCR-based analysis. The objective of this study is to evaluate both of these technological developments and to assess their usefulness in applications in the research and diagnostics arenas. Sample integrity can be compromised as the result of multiple freeze-thaw cycles or prolonged exposure to increased temperatures using current storage procedures that rely on maintaining cold environments. The development of ambient temperature stabilization technologies would provide a useful alternative to current cold storage methods, and has particular application for sample shipments to core analysis facilities. We will also present data on enhancing PCR-based analysis (including RT-PCR and multiplex STR analysis) for compromised or limited sample types.

## INTRODUCTION

Currently, collection, transportation and storage of complex biological samples such as DNA, RNA, proteins, bacteria, blood, and other biological molecules requires maintaining cold environments to prevent or at least reduce the rate of degradation. Maintaining cold storage environments, even for small laboratories, requires multiple expensive refrigeration and freezer units, all of which consume energy and limited laboratory resources. Current methods of sample collection and transport back to the laboratory are also problematic, as shipping frozen samples on dry ice is expensive, with shipments ranging up to hundreds of dollars due to bulky containers and expedited delivery costs. Unfortunately, even under carefully monitored cold storage and shipment environments, repeated freeze-thaw cycles and fluctuating temperatures only serve to promote degradation and compromise results.

Despite all the precautions taken to maintain what is typically referred to as cold chain logistics, 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. There is also a critical need to develop technologies for simple and efficient collection, transport and storage of bacterial and blood samples that does not require cold chain logistics or complicated recovery protocols. For example, current methodologies for blood sample collection relying on paper-based technology requires lengthy protocols for recovery of genomic DNA at low yields. Until recently there were no products that stabilized complex biological samples at ambient temperatures as a means to eliminate the need for cold chain logistics and facilitate sample collection, transport and storage.

### From Nature to the Lab

Biomatrica has developed a high-performing biostabilization technology to prevent the degradation of biological materials at room temperature. SampleMatrix® is Biomatrica's novel platform technology that directly preserves and stabilizes biological samples at ambient and elevated temperatures. 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. Biomatrica's technology transfers the molecular principles of anhydrobiosis to a synthetic chemistry-based stabilization science that works by forming a thermo-stable barrier securely "shrink-wrapping" samples, such as nucleic acids and providing protection against degradation (Fig. 1).

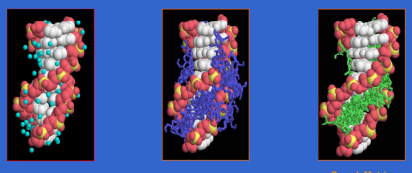


**Figure 1. Structural Prediction of SampleMatrix Interacting with Nucleic Acids.** Molecular modeling prediction of interactions of SampleMatrix with nucleic acid molecules. Nucleic acid shown in hydrated form (*hydrated state; left*). Trehalose disaccharides are predicted to interact with nucleic acid molecules through minor groove interactions based on hydrogen bonding (*Nature; middle*). SampleMatrix is predicted to form similar interaction patterns as trehalose (*SampleMatrix; right*).

### SampleMatrix Technology

SampleMatrix 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 30 µg of DNA, 10 µl of an overnight bacterial culture or ≤50 µl whole blood, depending on the specific SampleMatrix storage product. The steps involved in using SampleMatrix for storage of biological samples are outlined in Figure 2. By adding samples resuspended in water, buffer, growth media (*e.g.* LB media) or whole blood, SampleMatrix is rehydrated and mixes with the sample. Air-drying of the mixture results in a stabilizing glass that serves to protect the sample from degradation. Once completely dried, samples can be stored at room temperature or even elevated temperatures at relative humidity conditions ≤50%.

Sample recovery requires simple rehydration using water or a buffered solution. Furthermore, since the rehydration volume can be chosen between 10-100 µl, storage of samples in SampleMatrix 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. Most samples recovered following storage in SampleMatrix 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; additional purification is required to recover genomic DNA following storage of whole blood in SampleMatrix optimized for blood storage.


**Figure 2. Protocol for sample storage in SampleMatrix.** Samples are applied directly into SampleMatrix, dried, and then stored at ambient temperatures with ≤50% relative humidity. Sample recovery requires one-step rehydration, and the sample 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 collection, shipping and storage strategy to faciliate life science research and biomedical diagnostics. Studies were designed to evaluate the feasibility of using SampleMatrix for the ambient temperature storage of biological samples such as DNA, RNA, bacterial cells and blood, and their subsequent use in downstream applications. Recovered samples were used directly without further purifying to assess any interference or inhibition in downstream applications including electrophoresis, PCR, quantitative PCR, tranformation and genomic DNA purification procedures. A novel polymerase enhancer reagent was also evaluated for improved sensitivity and yield in PCR-based analysis, including end-point PCR and RT-PCR.

## MATERIALS AND METHODS

*Sample Preparation and Storage in DNA SampleMatrix:* Aliquots of DNA were applied to DNA SampleMatrix in the 1.7 ml standard microfuge tube format or 96-well plate format and allowed to dry overnight in a laminar flow hood before sealing and storage as described in each figure. Non-protected control samples (NP) were prepared by drying equivalent aliquots of DNA into an empty tube under identical conditions. Frozen control samples were kept at -20°C for identical time periods. Samples were rehydrated with water and used directly in various downstream applications without further purification. Aliquots (10 µl) of whole blood were applied directly into SampleMatrix formulated for storing blood and allowed to dry overnight in a laminar flow hood prior to long-term storage. Samples were rehydrated with 100 µl water and used directly for genomic DNA purification using the QIAamp DNA Blood Mini Kit. Identical samples were applied to Whatman FTA cards where described and stored dry for the same time periods. Frozen control blood samples were stored at -20°C until ready for use.

*Quantitative PCR analysis:* Aliquots of genomic DNA recovered following storage of blood were used as template for QPCR to determine recovery yields. TaqMan® reagents were used for the QPCR amplification using primers specific to the human β-actin gene.

*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 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 <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.

*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. 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 in a 25 µl final reaction volume. A 250 mM final concentration of the 18s rRNA probe was used (5' labeled with FAM and 3' labeled with TAMRA).

*Microarray analysis:* Total RNA was isolated from human fetal cartilage tissues as described in Krakow et al., (Mol Genet Metab. 2003. 79(1):34-42). Aliquots of total RNA were applied to RNAstable, dried overnight in a laminar flow hood and then stored for 1 day at room temperature. The quality and quantity of all the stored RNA samples were then analyzed using an Agilent 2100 bioanalyzer and NanoDrop® ND-1000 spectrophotometer. Fluorescent labeling of RNA and microarray analysis was performed as described in Krakow et al.

## RESULTS

### Long-term Storage of DNA

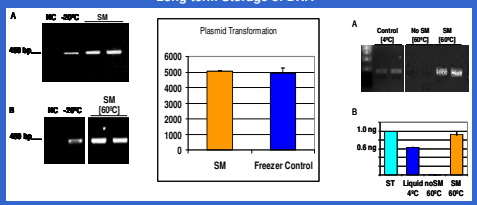


**Figure 1:** A 50 ng aliquot of pUC18 plasmid DNA stored in DNA SampleMatrix for (A) **2 years at ambient temperature** or (B) 10 months at 60°C under accelerated aging conditions **(equivalent to 11 years of room temperature storage)**, were analyzed by PCR using pUC18 specific primers. Control DNA (50 ng) was stored in at -20°C. NC: negative control.

**Figure 2:** Comparison of colony counts of bacteria transformed with pCU18 stored for **1.5 years at room temperature** in DNA SampleMatrix (SM) compared to control plasmid DNA stored at -20°C.

**Figure 3** Aliquots (20 ng) of human genomic DNA were stored in DNA SampleMatrix (SM) or left unprotected (No SM) and stored to 60°C for 10 months under accelerated aging conditions **(equivalent to 11 years of room temperature storage)**. An identical 20 ng aliquots of control DNA was stored at 4°C for 10 months. (A) PCR; (B) QPCR: 1 ng fraction of stored material, fresh DNA standard (ST- 1 ng).

### Long-term storage of RNA

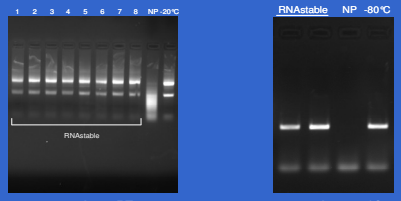


**Figure 4.** Long-term storage of total RNA. Aliquots of 1 µg 293T total RNA stabilized in RNAstable and stored dry at room temperature (*left*) or 50°C (*right*) with relative humidity <50% for 6 months. Samples were re-hydrated in DEPC-treated water and run on a 1.2% 1xTAE gel. *Left:* Lanes 1-8: 293T total RNA stored in RNAstable; NP; no protection control; positive control sample stored frozen. *Right:* Samples protected in RNAstable are shown in the two right lanes; unprotected (NP) sample is completely degraded; control sample stored at -80°C.

### Bioanalyzer and microarray analysis of RNA in RNAstable

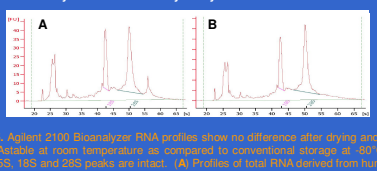


**Figure 5.** Agilent 2100 Bioanalyzer RNA profiles show no difference after drying and storage with RNAstable at room temperature as compared to conventional storage at -80°C. Note that the 5S, 18S and 28S peaks are intact. (**A**) Profiles of total RNA derived from human fetal cartilage samples (Krako *et al.*, Mol Genet Metab 2003. 79:34-42) stored at -80°C or (**B**) at room temperature protected in RNAstable for 1 day and analyzed using a bioanalyzer.

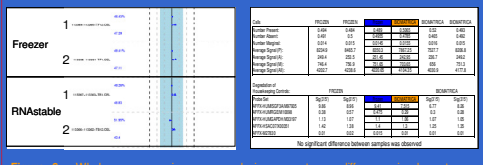


**Figure 6.** Whole genome microarray analysis suggests no difference in downstream biochemical applications or degradation when stored using RNAstable at room temperature. Quality control statistics using the 5'/3' ratios of actin and *GADPH* transcripts were plotted and indicate identical results between the two storage methods (*left*). The number of present and absent calls and the average signal intensities did not reveal any significant differences between samples stored frozen or those maintained at room temperature in RNAstable (*right*). *Microarray analysis performed by Dr. Vincent Funari, Cedars-Sinai Medical Center.*

### Room Temperature Storage of Blood Samples

SampleMatrix technology was used to develop a medium for the room temperature storage of whole blood in volumes up to 50 µl. Recovery of genomic DNA stored dry at room temperature protected in the matrix indicates improved sample protection and yield of recovery as compared to paper-based mediums (*e.g.* FTA-cards).

#### Improved Recovery of DNA Following Long-Term Storage of Blood

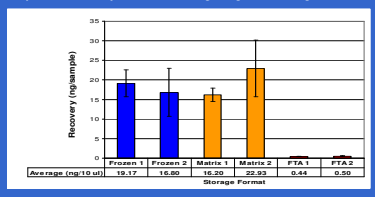


**Figure 9.** Aliquots of whole blood (10 µl) were stored dry in matrix or on FTA-cards for 6 months at room temperature. Control samples were stored at -20°C. Samples stored in the matrix were rehydrated and used directly without further purification for genomic DNA extraction with the QIAamp DNA Blood Mini Kit following manufacturer's protocol. Samples stored dry on FTA-cards were processed following instructions provided in the kit. QPCR analysis of recovered DNA indicates comparable levels of recovery following dry room temperature storage in the matrix as compared to frozen control samples, while recovery yields were significantly reduced following storage on FTA-cards.

#### Recovery of DNA From Blood After 6 Weeks Storage at Room Temperature

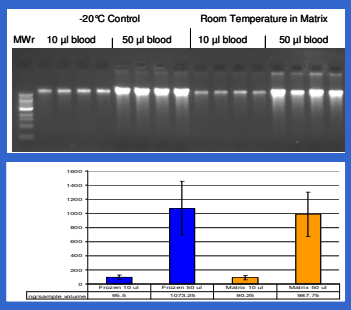


**Figure 10.** Aliquots of whole blood (10 µl and 50 µl) were stored frozen or applied into matrix and dried. Dried samples were then stored for 6 weeks at room temperature. Rehydrated samples were used directly for genomic DNA purification as described above. Aliquots were run on a 0.8% agarose gel and stained with ethidium bromide (*top*). DNA recovered from blood samples stored in the matrix were comparable to control samples. Aliquots of recovered DNA were also used as templates for QPCR analysis as described above. Results indicate comparable recovery levels (ng/sample volume) between samples protected in matrix at room temperature to frozen controls (*bottom*).

### Enhancing PCR-based Analysis

We have developed a novel PCR enhancer reagent for increasing sensitivity and yield of PCR-based reactions such as end-point PCR and RT-PCR. The enhancer (commercially available as PCRboost™) is used in place of water in the reaction and can be stored at room temperature*.

#### Improved Sensitivity and Yield

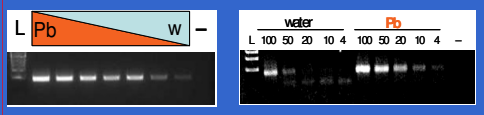


**Figure 8.** Addition of PCRboost improves sensitivity and yield during amplification of human genomic DNA templates. Aliquots (50 ng) of gDNA were amplified using human β-actin primers in decreasing PCRboost (Pb) and increasing water (w); (-) no template control; (L) ladder (*right*). Aliquots of 100, 50, 20, 10 or 4 ng of gDNA were used for PCR amplification in water or PCRboost (Pb); (-) control; (L) ladder (*left*).

#### Compatibility with Commercially Available *Taq* Polymerases

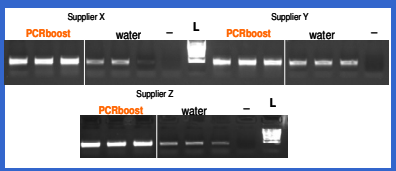


**Figure 9.** Aliquots (10 ng) of human gDNA were amplified with human β-actin primers in PCRboost or water using *Taq* polymerases from 3 different suppliers (X, Y and Z); (-) no *Taq* control; (L) ladder.

#### Enhancing RT-PCR Reactions

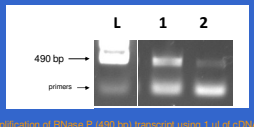


**Figure 10.** RT-PCR amplification of RNase P (490 bp) transcript using 1 µl of cDNA generated from 100 ng 293T total RNA using PCRboost (lane 1) or water (lane 2). Aliquots (10 µl) of PCR reaction were run on a 0.8% agarose gel stained with ethidium bromide.

**See "PCRboost: A Novel PCR Amplification Enhancer" application note for details on materials and reaction conditions.*

## CONCLUSIONS

- SampleMatrix technology allows for long-term stabilization of biological samples at ambient temperatures with ≤50% relative humidity and is secured even at elevated temperatures.
- Sample recovery requires a simple one-step rehydration – just add water.
- Recovered samples can be used directly without the need for further purification in downstream applications. Fluctuating and inconsistent temperatures during shipment will not damage samples stabilized dry in SampleMatrix.
- Sensitivity and yield are improved with addition of a novel PCR enhancer reagent that is compatible with several commercially available enzymes.
- Studies are currently underway to evaluate the compatibility of SampleMatrix for storing other types of biological samples including cells, tissues, viruses and assays. Long-term studies are ongoing for room temperature storage of blood in SampleMatrix.
- Results indicate that technology advances in sample preservation can prevent the degradation process of biological samples in life science research.