# Ambient temperature stabilization of nucleic acids in human blood and saliva

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## Abstract

**Biospecimen stabilization during collection and transport is of critical importance in the fields of biomedical research and molecular diagnostics. The emergence of personalized medicine has placed an even greater emphasis on the significance of protecting the quality and reliability of clinical samples. The current methods for blood and saliva collection depend heavily on cold-chain logistics and are fraught with potential complications to specimen integrity that can result in inconsistencies in sample analysis. Nucleic acids in biological specimens are threatened by nucleolytic attack, oxidative damage and hydrolysis. Such agents can severely disrupt sequencing, genotyping and expression profile analyses. In this report we describe the development of chemical stabilization formulations that address this problem of sampling induced inconsistencies in human blood and saliva. These formulations preserve nucleic acids in these biospecimens even under extreme environmental shipping conditions and remove the need for cold-chain logistics during sample collection, transport or storage. Here we show that the genomic DNA in blood collected in DNAgard® Blood Tubes is stabilized during extreme temperature fluctuations ranging from -20°C to +45°C. The effectiveness of the stabilization formulation is demonstrated by genotyping of DNA recovered from blood after 5.5 months of storage at room temperature. The analogous DNA stabilization technology for saliva specimens, DNAgard Saliva, preserves sample integrity for more than six years at ambient temperature. We also compare freezer storage with the room temperature stabilization formulation RNAgard Blood and the market leader in ambient temperature blood RNA stabilization. Our results demonstrate that RNAgard Blood is a valuable alternative to cold-storage for preserving RNA expression profiles for at least 7 days at ambient temperature.**

## Materials and Methods

**DNA stabilization in whole blood:**

Human whole blood was collected in VACUETTE® DNAgard® Blood Tubes, $K_2$-EDTA Vacutainers™ (Becton Dickinson), or in a competitor's blood DNA collection tube and subjected to a temperature cycle (figure 1) or stored at room temperature (figure 2). Control samples in $K_2$-EDTA Vacutainers were stored frozen at -20°C. DNA was isolated using QIAamp Blood Maxi kits (QIAGEN) or by the competitor's specified method. Long range PCR analysis was conducted with 250 ng of input DNA and primers to amplify a 22 kbp amplicon of the tPa gene using the Manual PCR Extender System (5 PRIME). Genotyping was performed on the Human CytoSNP-12 SNP Genotyping Array (Illumina).

**DNA Stabilization in Saliva:**

Sample collection: Individual donors were asked to fast for at least a 30 min period. Donors were required to wash their mouths with water to remove any food remaining 10 minutes before starting saliva collection . Saliva collection was completed within 15 minutes.
The collected saliva was immediately mixed with DNAgard saliva (DGS) in 4:3, v/v, saliva to DGS ratio. DNA was isolated using the column based QIAamp DNA mini kit (QIAGEN), Gentra-Puregene (QIAGENE), Organic (pheonol/chloroform) extraction and a competitor method. DNA integrity was analyzed on a 0.8% agarose gel and 10% of the eluted DNA was loaded to the gel. DNA yield was determined by fluorescence using the Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen).

**RNA stabilization in whole blood:**

Human whole blood from a single donor was collected in $K_2$-EDTA-treated vacutainers and aliquots stored with either no formulation (NP) or Biomatrica's stabilization formulation (RNAgard Blood), and stored at room temperature. Control samples were stored at -80°C. Blood was also collected in a competitor's tubes and stored at room temperature. RNA was isolated at specified times using the RiboPure Blood Kit (Ambion) or the competitor's system. Reverse transcription and cDNAs amplification was performed with the iScript Reverse Transcription Supermix (Biorad) using specified primer sets. Fold-change in gene expression was calculated relative to expression at the time at collection ("time 0",) using the ΔΔCq method of relative quantification. For Illumina Human HT-12 gene expression array, RNA was extracted and quantified as above, processed for microarray analysis and analyzed according to the manufacturer's instructions.

## Results I – genomic DNA stabilization in blood

A.

Temperature Cycle

| Temperature | Time |
|---|---|
| Room temperature | 2 days |
| 37°C | 2 days |
| Room temperature | 2.5 days |
| -20°C | 1 day |
| 45°C | 1 day |

B.

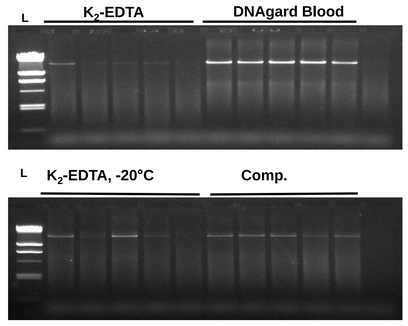



**Figure 1. Genomic DNA stability in whole blood samples subjected to extreme temperature fluctuations.** Blood from 5 donors collected in VACUETTE DNAgard Blood Tubes, $K_2$-EDTA Vacutainers and in a competitor's blood collection tubes were subjected to an 8 day shipping simulation **(A)**. A separate set of blood samples collected in $K_2$-EDTA Vacutainers were stored at -20°C as controls. DNA purified from blood stored in the four conditions for each of the five donors was analyzed by long-range PCR amplification of a 22 kbp region of the tPa gene **(B)**. Equal input DNA was used for each amplification reaction. L = lambda DNA-HindIII digest (New England Biolabs). Also shown are representative (-) Taq and (-) DNA template control samples.

A.

Call Rates

| Donor | DNAgard Blood | Frozen |
|---|---|---|
| 1 | 0.9979994 | 0.9975027 |
| 2 | 0.9927651 | 0.9931350 |
| 3 | 0.9970026 | 0.9984962 |
| 4 | 0.9982392 | 0.9985784 |

B.

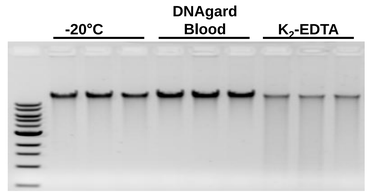
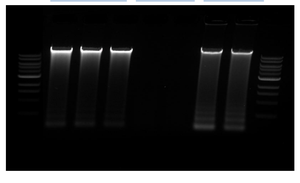



**Figure 2. Protection of DNA integrity in whole blood at ambient temperature. (A)** Blood from four separate donors was collected in VACUETTE DNAgard Blood tubes and stored at ambient temperature. Blood was also collected in $K_2$-EDTA tubes and stored frozen at -20°C. After 5.5 months of storage, DNA was purified and genotyped on the Human CytoSNP-12 SNP Genotyping Array (Illumina). **(B)** The gDNA from blood stored in VACUETTE DNAgard Blood tubes for 9 months at ambient temperature was compared by agarose gel electrophoresis to the gDNA isolated from blood stored in $K_2$-EDTA tubes under the same conditions or frozen at -20°C (samples were analyzed in triplicate).

## Results II – DNA stabilization in saliva

A.




B.

| | DGS | -80 °C |
|---|---|---|
| DNA yield (µg/mL) | 32.21 | 30.10 |
| $A_{260/280}$ | 1.88 | 1.91 |

**Figure 3: Preservation of DNA in saliva for over a year at 50°C versus saliva stored in -80°C freezer.** Fresh saliva (200 µL) was mixed with DNAgard Saliva (DGS) (150 µL) and stored at 50°C for 14 months. 200 µL aliquots of the saliva from the same individual was stored at 50°C and -80°C as a negative control (NP) and positive control respectively. The purified DNA from all samples was analyzed by gel electropheresis (**A**). DNA quantification using PicoGreen assay showed that saliva in DGS provided slightly higher DNA recovery than a frozen control (**B**).

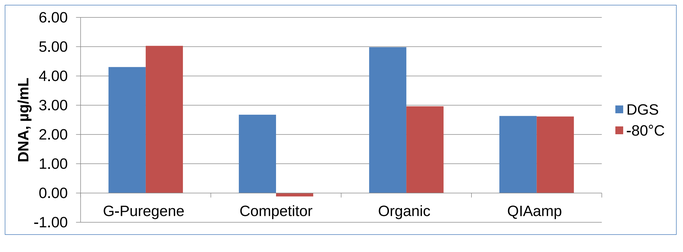
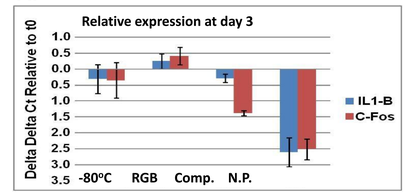


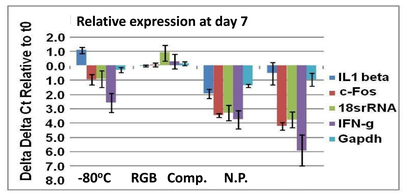

**Figure 4. DNAgard saliva isolation method compatibility.**
Fresh saliva (200 µL) was mixed with DNAgard Saliva (150 µL) and stored at ambient temperature for two weeks. A control saliva sample was stored at -80°C for the same period of time. DNA from all samples were isolated according to the manufacturer's protocol and quantified using PicoGreen florescence assay.

## Results III – RNA stabilization in blood

A.

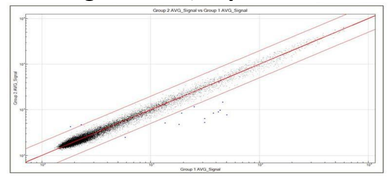



B.



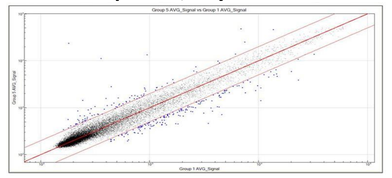

C.

RNAgard Blood, day 3



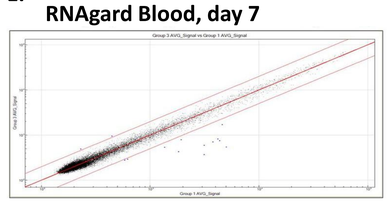
D.

Competitor, day 3



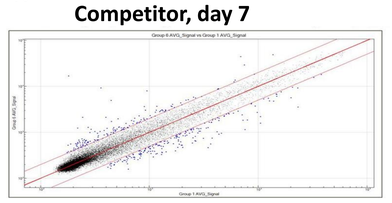
E.

RNAgard Blood, day 7



F.

Competitor, day 7



G.

Number of genes > 2-fold expression

| | RNAgard Blood | Competitor's Blood Tube |
|---|---|---|
| Day 3 | 14 | 240 |
| Day 7 | 14 | 338 |

**Figure 5. Transcript analysis in human blood stored in RNAgard Blood.** Human whole blood from a single donor was collected and stored as described in materials and methods. **RT-qPCR analysis:** After 3 days **(A)** and 7 days **(B),** RNA was purified and the expression levels of multiple transcripts quantified relative to "time 0". **Microarray analysis:** Expression profile of 34,000 genes was analyzed in RNAgard Blood and competitor samples after 3 days **(C,D)**, and 7 days **(E,F)** of storage at room temperature. Expression was quantified relative to levels at the time of blood collection. **(G)** Number of genes with > 2-fold change in expression levels relative to "time 0".

## Summary

**DNAgard Blood protects DNA integrity in whole blood stored at ambient temperature:**

- **DNAgard Blood protects DNA in blood samples subjected to harsh shipping conditions that damage unprotected specimens (figure 1).**
- **Blood storage in DNAgard Blood at room temperature is as effective as freezer storage for genotyping (figure 2).**

**DNAgard Saliva protects DNA integrity in saliva samples stored at ambient temperature:**

- **DNAgard Saliva preserves genomic DNA integrity in saliva samples for over one year at ambient temperature (more than six years based on accelerated aging (figure 3).**
- **DNAgard Saliva generally provided higher DNA recovery and better quality compared to freezer control samples. Genomic DNA from DNAgard Saliva can be isolated with almost all common purification methods (figure 4).**

**RNAgard Blood protects RNA in blood stored at room temperature:**

- **Gene expression remains unaltered in blood samples stored for 3 or 7 days at room temperature in RNAgard Blood, while storage in a leading competitor stabilizer and even freezer storage do not prevent changes in gene expression levels (figure 5A and 5B).**
- **The total gene expression profile in blood stored for 3 or 7 days at room temperature in Biomatrica RNAgard Blood is highly similar to fresh blood, outperforming a leading competitor and freezer storage (figure 5C – 5F).**