Application Note

GenTegra

Ambient Temperature Stabilization of Feline and Canine Tumor Cell RNA for Use in Gene Expression Assays

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Abstract

GenTegra®-RNA is a revolutionary technology for the stabilization, transport and storage of purified RNA. GenTegra-RNA , developed by GenTegra LLC is an inert chemical matrix that inactivates trace RNase in the liquid phase, providing an added level of RNA stability during sample handling, with or without ice. In the dry-state, GenTegra-RNA preserves RNA integrity by protecting samples from hydrolysis and oxidation, imparts thermal stability at temperatures ranging from -80°C to 76°C/169°F and ensures quantitative recovery of RNA upon rehydration. In this study, we report that GenTegra-RNA preserves the integrity of purified RNA derived from feline and canine tumor cell lines at ambient temperature during storage in the liquid and dry states, with no effect on performance in gene expression analysis.

Introduction

RNA samples derived from tumor cells are frequently used for gene expression analysis in mammalian cancer research. Once purified from cells or tissue, RNA stability must be maintained to avoid the loss of valuable samples. Preventing degradation of RNA, which is primarily induced by RNases that co-purify with nucleic acids or are introduced from the environment, is a major challenge, as is protecting purified RNA during long-term storage and transport. The Modiano laboratory at the University of Minnesota faces these challenges when working with RNA purified from canine osteosarcoma, canine hemangiosarcoma and feline mammary tumor cells. The use of purified RNA is an important part of the Modiano lab's ongoing research to understand the biology and pathogenesis of cancer. RT-qPCR-based gene expression analysis of transcripts such as IGFBP2 and AURKA is used as part of the lab's work to understand the basis, risk, origin and progression of cancer. To address the difficulties faced by scientists working with purified RNA specimens, GenTegra LLC has developed GenTegra-RNA a groundbreaking technology that permits long-term storage and transport of purified RNA at ambient temperature in the dry state, while providing an added level of stability for RNA in the liquid state, empowering short-term analysis. GenTegra-RNA is composed of an inert, water soluble, chemical matrix, which provides protection from oxidation and hydrolysis in the dry state, conveys thermal stability, and allows quantitative recovery of RNA samples. GenTegra-RNA simplifies sample handling in the liquid state, by inactivating trace nucleases, eliminating the need to handle RNA on ice. Here, we demonstrate that storage of purified RNA in GenTegra-RNA has no effect on RNA integrity or performance in RT-qPCR, making GenTegra-RNA a safe, convenient method of stabilization, transport and storage of purified RNA.

Materials and Methods

RNA Purification, Application, Storage and Recovery

Total RNA was purified from canine osteosarcoma, canine hemangiosarcoma and feline mammary tumor cells using RNeasy spin column technology (Qiagen, Valencia, CA), according to the manufacturer's instructions. One specimen was purified from each cell type, and the RNA samples purified from osteosarcoma, hemangiosarcoma and mammary tumor cells were labeled OSCA 32, Emma HSA, and K12, respectively.

RNA specimens were either applied to GenTegra-RNA tubes immediately after isolation and quantitation (for dry phase storage at 25°C and 60°C and liquid phase storage at 25°C and 37°C, or stored overnight at-80°C prior to application to GenTegra-RNA tubes (for dry phase storage at 37° C. An aliquot of each RNA sample was stored at -

80°C as a frozen control.

Triplicates of 1, 5 or 10µg aliquots of each RNA sample were applied to GenTegra-RNA tubes. Following application, one set of RNA samples was stored at 25° C or 37°C for 24 hours in the liquid state. A second set of RNA samples was dried overnight (16hr) in a FastDryer (GenTegra, Pleasanton, CA) according to the manufacturer's instructions. Samples were then stored in the dry state for three weeks at 25°C, 37°C or 60°C. Following the dry storage period, the samples were rehydrated in 20µL of molecular-grade water according to the manufacturer's instructions.

RNA Quantitation and Gel Electrophoresis

RNA was quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Information about RNA integrity was gathered from an electrophoretic trace and a RNA Integrity Number (RIN) was obtained using the RNA 6000 Nano Chip on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) according to the manufacturer's protocol. RIN values are given on a scale of 1 to 10, with 1 being the most degraded profile and 10 being the most intact.[1]

Denaturing agarose gel electrophoresis (Ambion, Austin, TX) of 11µg of RNA was carried out according to the manufacturer's protocol to access the quality of RNA before application to GenTegra-RNA tubes and then again after storage in GenTegra-RNA tubes.

Statistical Analysis

Statistical analysis was performed using Student's t-test to determine the difference in values between samples stored in GenTegra™ DNA tubes and samples stored at -20°C. Statistical significance was determined for p-values <0.05. All analyses were performed using JMP 5.0.1 (SAS).

cDNA Synthesis and Quantitative Real-Time PCR (qPCR) Analysis

Synthesis of cDNA from 11µg of RNA was carried out using QuantiTect Reverse Transcription Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). qPCR was performed on an Eppendorf Mastercycler ep realplex with FastStart SYBR Green Master Mix (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. GAPDH was used as an endogenous control.

Results

Storage of tumor cell RNA samples with GenTegra-RNA.

RNA aliquots were incubated in the liquid state at 25°C or 37°C for 24 hours in the presence and absence of GenTegra-RNA and compared to control samples stored at -80°C (Figure 1). There was no difference in RNA integrity for any of the samples stored at 25°C or 37°C in the presence of GenTegra™ and the frozen controls. On the other hand, the Emma HSA samples exhibited early signs of degradation following storage at 37°C in the absence of GenTegra-RNA.

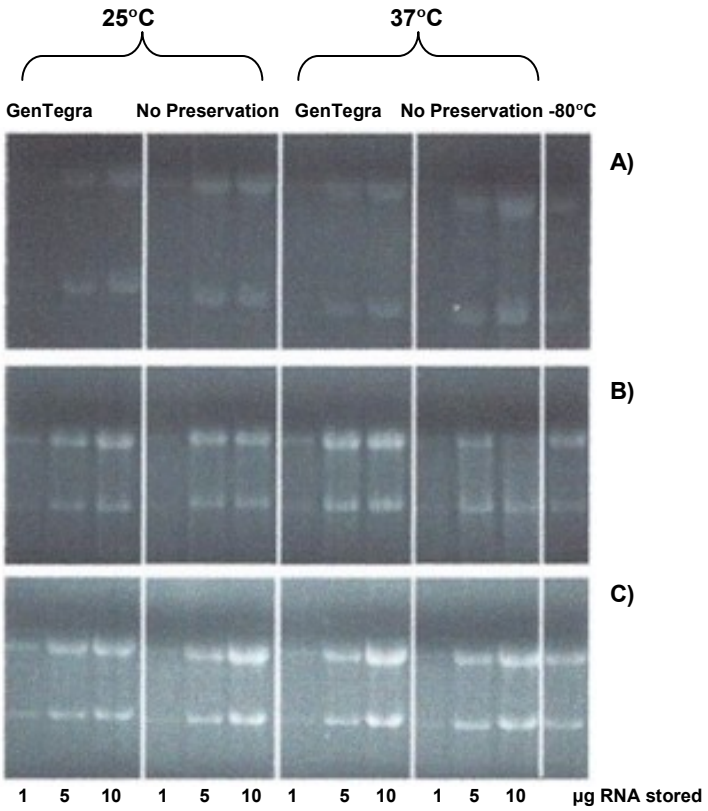

***Figure* 1.** *Gel analysis* of *OSCA* 32 *(A), Erma HSA* (B) *and K12 (C) RNA samples stored in the presence or absence of GenTegra-RNA in the liquid state for 24 hours at 25°C or 37°C, and controls stored at –80°C.* 1, 5, or *10µg* of *each RNA sample was stored under each condition.*

An additional set of RNA aliquots were dried overnight in the presence or absence of GenTegra™ and stored at 25°C, 37°C or 60°C for comparison with a frozen control stored at -80°C. Storage at 37°C and 60°C were used to assess the ability of RNA samples

stabilized in the GenTegra-RNA to withstand extreme temperatures that often occur during transport. According to FedEx shipping guidelines, packages can reach temperatures of approximately 60°C (140°F) for up to one day during transport[2]. At the end of the three week storage period, RNA samples were rehydrated in 20µL of molecular-grade water and quantitated via NanoDrop 1000. Quantitative recovery was achieved, regardless of storage condition, and there was no significant difference in percent recovery between samples stored in the presence or absence of GenTegra-RNA and frozen controls (data not shown).

Samples and controls were then subjected to Bioanalyzer analysis to determine a RIN for each storage condition. The RIN of samples K12, Emma HSA and OSCA 32 for each storage condition are summarized in Table 1. Hashed lines indicate that calculation of a RIN was not possible due to extensive degradation. Samples labeled *N/A* exhibited electropherograms consistent with the other samples stored under the same condition, but a RIN was not generated due to an error in the Bioanalyzer analysis. Complete electropherograms (Figure 2) and gel images generated by the Bioanalyzer (Figure 3) are shown for one representative sample, Emma HAS.

Sam-ples	-80°C	25°C		37°C		60°C	
	Frozen Control	Gen-Tegra	No Gen-Tegra	Gen-Tegra	No Gen-Tegra	Gen- Tegra	No Gen-Tegra
K12	10.0	10.0	7.6	N/A	- -	7.9	- -
EMMA HAS	10.0	10.0	7.6	9.6	- -	8.2	- -
OSCA 32	10.0	10.0	N/A	10.0	- -	7.8	- -

- - **No RIN generated due to extensive degradation**

N/A **No RIN due to error in Bioanalyzer analysis**

Table 1. RIN of samples stored in dry state for three weeks at various temperatures.

The RINs of samples stored in the presence of GenTegra-RNA for three weeks at 25°C or 37°C were nearly identical to the RINs of control RNA stored at -80°C (compare Figure 2Aand B with G). Remarkably, even storage at temperatures as high as 60°C for three weeks did not appreciably alter the RIN when GenTegra™ was present, with a difference of only 1.8 between samples stored at 60°C in the presence of GenTegra-RNA and frozen controls (compare Figure 2C and G). On the other hand, obvious degradation was observed in RNA stored at 25°C, 37°C or 60°C in the absence of GenTegra-RNA (compare Figure 2D, E and F with G). At 25°C, the three week storage period resulted in a decrease of 2.4 between the samples stored with no GenTegra-RNA and frozen controls. At 37°C and 60°C, it was not possible for the Bioanalyzer to calculate a RIN, due to the extensive degradation exhibited by the no matrix samples. Figure 3 demonstrates that elevated temperatures resulted in

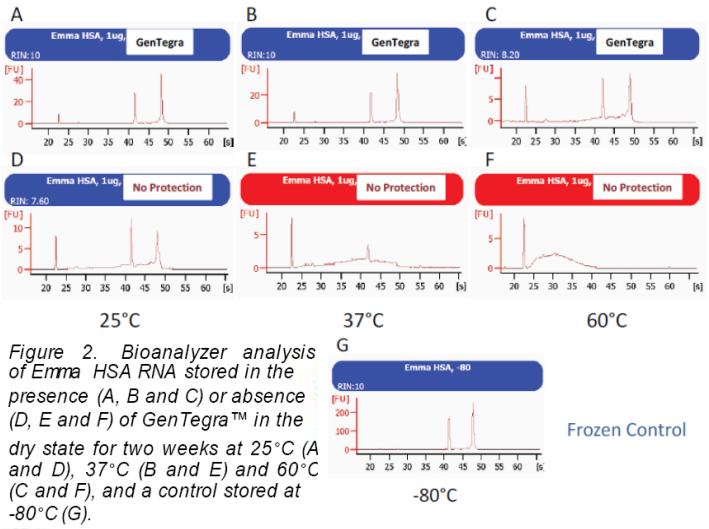

Figure 2. Bioanalyzer analysis of Emma HSA RNA stored in the presence (A, B and C) or absence (D, E and F) of GenTegra™ in the dry state for two weeks at 25°C (A and D), 37°C (B and E) and 60°C (C and F), and a control stored at -80°C (G).

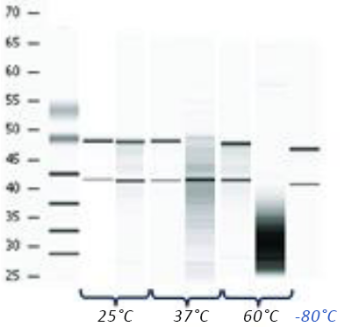

Figure 3. Gel image generated by Bioanalzyer analysis of Emma HSA RNA stored in the presence or absence of GenTegra™ in the dry state for two weeks at 25°C, 37°C and 60°C, and a control stored at -80°C.

extensive degradation in the samples stored without GenTegra-RNA but had little to no effect on the samples stored in the presence of GenTegra-RNA. Following Bioanalyzer analysis, 11µg of each the three RNA samples stored in the presence of GenTegra-RNA for 24 hours at 25°C in liquid form, and for three weeks at 60°C in the dry state, was used for cDNA synthesis and compared with controls stored at -80°C. The concentration of cDNA samples stored at -80°C (Table 2).

Sample	Storage Condition		260/280	ng/µL cDNA (from 1µg RNA)
K12	Liquid, 25°C	GenTegra-RNA	1.77	990.4
K12	Dry, 60°C	GenTegra-RNA	1.76	964.3
K12	-80°C	Frozen	1.87	1430.3
EMMA HAS	Liquid, 25°C	GenTegra-RNA	1.76	1031.3
EMMA HAS	Dry, 60°C	GenTegra-RNA	1.78	1059.8
EMMA HAS	-80°C	Frozen	1.77	1299.2
OSCA 32	Liquid, 25°C	GenTegra-RNA	1.79	1252.2
OSCA 32	Dry, 60°C	GenTegra-RNA	1.78	1175.1
OSCA 32	-80°C	Frozen	1.79	1162.6

Table 2. Concentration of cDNA synthesized from RNA samples stored under various conditions.

cDNA generated from the RNA samples described in Table 2 was then subjected to qPCR analysis of the GAPDH transcript. There was no significant difference between the GAPDH Ct values for samples stored in the presence of GenTegra-RNA in the liquid state at 25°C, or in the dry state at 60°C and controls stored at -80°C (Figure 4).

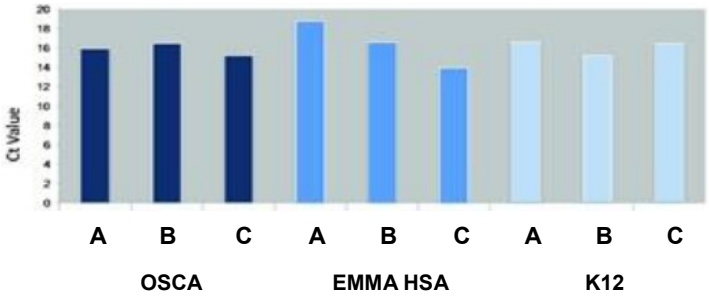

Figure 4. RNA samples stored in presence of GenTegra-RNA for 24 hours in liquid state (B) at 25°C, or in dry state for three

Finally, a dilution series was performed in order to examine PCR efficiency for analysis of the IGFBP2 and ARUKA transcripts using cDNA generated from the OSCA 32 sample following storage at 25°C for three weeks in the presence of GenTegra-RNA. The cDNA was either used undiluted, or diluted 1:10, 1:25 or 1:50 prior to qPCR analysis. Figure 5 demonstrates that the Ct values correlated with the cDNA dilutions. R2 values of 0.9576 and 0.9976 were demonstrated or IGFPB2 and ARUKA respectively. Thus, even in the undiluted samples, there was no inhibitory effect of the GenTegra-RNA on PCR efficiency.

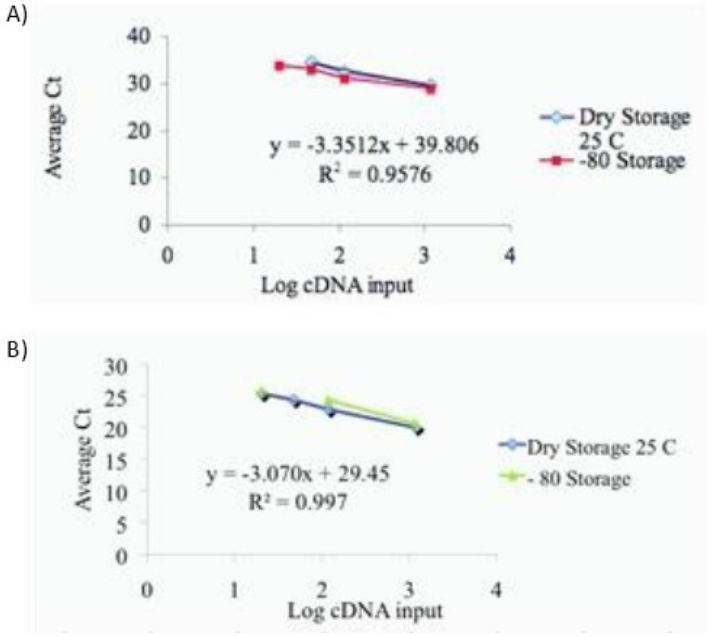

Figure 5. Dilution series using cDNA generated from the OSCA 32 sample stored at 25°C for three weeks in the presence of GenTegra-RNA for qPCR. IGFBP2 (A) and ARUKA (B) transcripts were examined.

Discussion

In the present study, we examined the use of GenTegra-RNA for storage of total RNA purified from canine osteosarcoma, canine hemangiosarcoma and feline mammary tumor cells. GenTegra-RNA is an inert chemical matrix that has the ability to inactivate trace RNase, stabilizing the RNA in the liquid phase for short-term sample handling. Very high quality RNA (RIN of 10) was used for this study. However, even with this exceptional starting material, early signs of RNA degradation were observed in the Emma HSA samples following a 24 hour storage period at 37°C in the absence of GenTegra-RNA. These signs of degradation were not observed when the RNA was stabilized with GenTegra-RNA.

GenTegra-RNA ensures integrity, stability, and quantitative recovery of purified RNA samples, enabling workflow flexibility. In an ideal scenario, immediately after RNA isolation from cells or tissues,

an aliquot destined for prompt use (i.e. for quantitation, Agilent Bioanalyzer analysis or any downstream application) is stabilized in GenTegra-RNA and kept in the liquid phase for short-term analysis, while aliquots tagged for long-term storage or transport are dried following stabilization in GenTegra-RNA. In the dry-state, GenTegra-RNA further preserves RNA integrity, protecting RNA samples from hydrolysis, oxidation and exposure to temperatures ranging from –80°C to 76°C.

Here, we have shown that the integrity of RNA stored in the dry-state, at 25ºC, 37ºC and 60ºC is preserved in the presence of GenTegra-RNA. Conversely, samples stored under the same conditions without GenTegra-RNA exhibited substantial degradation. In the most remarkable case, a RIN could not be generated for a sample stored at 60°C for three weeks in the absence of GenTegra-RNA, due to extensive degradation, while the same sample stored in the presence of GenTegra-RNA had a RIN of 8.2, which was nearly the same as the control stored at –80°C. A decrease in RIN of 2.4 was observed in samples stored in the absence of GenTegra-RNA at 25ºC, compared to frozen controls, while the same samples stored with GenTegra-RNA had no decrease in RIN. Furthermore, the substantial degradation observed in samples stored without GenTegra-RNA at 37ºC and 60°C demonstrates the ability of GenTegra-RNA to protect against heat-catalyzed hydrolysis and oxidation of the RNA Storage at 37ºC and 60°C was used primarily to assess the ability of RNA samples stabilized in the GenTegra™ matrix to withstand the extreme temperatures that often occur during transport. According to FedEx shipping guidelines, packages can reach temperatures of approximately 60°C (140°F) for up to one day during the shipping process". Thus, GenTegra-RNA eliminates concerns about sample loss due to high temperature or shipping delays.

Finally, we used RNA stored in the presence of GenTegra-RNA for RT-qPCR assays. RNA purified from all tumor cell types stored in the presence of GenTegra-RNA in liquid form at 25ºC, and in the dry state at 60°C, provided yields of cDNA similar to the frozen controls, and all samples exhibited equivalent performance to the frozen controls in qPCR quantification of GAPDH transcripts. Serial dilutions were performed on cDNA generated from the OSCA 32 sample following storage in GenTegra-RNA prior to use in qPCR analysis of the IGFBP2 and ARUKA transcripts. There was no difference in PCR efficiency using the cDNA generated from the GenTegra-RNA stored sample compared with the typical PCR efficiency observed for IGFBP2 and ARUKA demonstrating that the inert chemical matrix of GenTegra-RNA does not inhibit cDNA synthesis or performance in qPCR analyses.

Conclusion

In conclusion, GenTegra-RNA provide high levels of protection for storage and transport of purified RNA at ambient temperature. It also provides an added level of stability for RNA in the liquid state, enabling safe short-term sample handling at ambient temperature. GenTegra-RNA is compatible with cDNA synthesis and qPCR-based expression analysis. Thus, GenTegra-RNA assures preservation of irreplaceable RNA samples without interfering with downstream analyses.

References

[1]RNA Integrity Number (RIN) - Standardization of RNA Quality Control. Publication Number: 5988-8322EN, 2006.

[2]Fedex Packaging Pointers: Perishable Shipments. 2006. http://www.fedex.comlus.services.pdf/PKG_Pointers_Perishable.pdf