GenTegra

APPLICATION NOTE

Enhanced RNA Preservation Under Extreme Conditions

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Abstract

Background. As an analyte, RNA is of significant value in research, diagnostic medicine and public health screening. However, it is among the most unstable analytes known, due to its instability to hydrolysis in water and to the ubiquitous presence of ribonucleases in the lab, clinical and field isolates. Clearly, methods are needed to enhance the stability of RNA during short-term laboratory processing and over the long term, to enable safe, unrefrigerated shipping and biobanking. Here, we describe the use of a novel RNA stabilizer, GenTegra®-RNA™ in terms of its capacity to mitigate RNA instability in the fluid phase and after the RNA and GenTegra-RNA had been air-dried together, to enable unrefrigerated RNA shipping and biobanking.

Results. Using purified RNA from cultured Hela cells, from human blood lymphocytes and from rat liver, it is demonstrated that upon air-drying, high quality RNA can be preserved after 7 months of dry state storage in GenTegra-RNA at 25°C, 37°C or 56°C, thereby exceeding a worse-case temperature swings expected for unrefrigerated biobanking or ambient temperature shipping. It is then shown that, subsequent to that 7month heat spike, purified RNA can be stored at ambient temperature for a total of 4 years with little diminishment of quality. Finally, it is shown that while still in the fluid phase, the preservatives in GenTegra-RNA greatly extend the time over which RNA may be kept in the fluid phase without refrigeration during its use in the laboratory.

Conclusions. These studies demonstrate that when stabilized by GenTegra-RNA, purified RNA can be preserved in the fluid state for at least 100hrs at 37°C, with no significant loss of quality. The data obtained via dry state storage demonstrate that RNA can be preserved in GenTegra-RNA for at yeast 4yrs in the absence of refrigeration.

Keywords

RNA preservation, Dry-state, Biobanking, Unrefrigerated Shipping

Introduction

Although RNA is of great value in research, diagnostic medicine and public health screening, it is among the most unstable analytes known, due to the intrinsic instability of the ribose-phosphate linkage with respect to hydrolysis in water and to the ubiquitous presence of ribonucleases in the lab and in clinical or field isolates (1,2). Clearly, technologies are needed to enhance the stability of RNA as an analyte during routine laboratory processing and over the long term, to enable safe, unrefrigerated shipment and biobanking.

RNA is far less stable than DNA in a biochemical sense because of the destabilizing influence of its 2' hydroxyl moiety, which faciliates general base catalysis and metal ion catalyzed addition of water across the RNA phosphate ester linkage and which, by a similar pathway, renders RNA sensitive to ribonucleases, that are ubiquitous in both the lab and field environment (1).

RNases are resistant to heat, denaturants and metal ion depletion, unlike DNases which are inactivated under those conditions (2). Both the general base and enzyme-catalyzed degradation of RNA have a high activation energy and therefore are very temperature dependent (2). Thus, low-temperature storage is a effective approach to RNA preservation. However, such cyrogenics are both expensive over time and can be an expensive and risky proposition during shipping.

RNA handling in the lab requires that it be used in the fluid phase and thus must be warmed to at least 4°C, thereby giving rise to the well-known deleterious effects of "freeze-thaw" cycling and the general observation that RNA is metastable even upon continous fluid storage at 4°C over times greater than a few hours.

When shipping RNA samples between sites, continuous low temperature preservation requires that RNA be shipped on dry ice which presents a large risk. Should the dry ice evaporate while in transit, the shipment can undergo catastropic damage (3).

The same requirement for continuous cryogenic storage of RNA during biobanking gives rise to substantial cost, due to the energy consumption associated with low temperature storage. It also presents an operations risk, due to the realistic possibility of power outage in a RNA biobank and the resulting catestropic warming that would result.

GenTegra-RNA™ was recently developed as a general solution to that RNA stability problem (4). The approach that has been taken in the design of GenTegra-RNAfor

use in the lab is based on RNA preservation in the fluid state by addition of a proprietary small molecule RNase inhibitor which, in all other aspects, is inert with respect to subsequent use of the RNA for quantitiative testing, such as qrtPCR, microarrays and sequencing (4).

Even in the complete absense of RNase activity, over the long term RNA is unstable in the presence of water, due to residual base-catalyzed addition of water to the phosphodiester linkage (1). The approach that we have taken to the design of GenTegra-RNA for long term RNA preservation, is to optimize it for ambient temperature RNA storage in the air-dried state, under conditions where nearly all water has been lost from the sample during ordinary evaporation.

Upon air-drying in GenTerga-RNA, RNA becomes embedded in a solid phase, delivered by the GenTegra-RNA containing less than 5% water by mass, so that all residual water is coordinated by the solid matrix and thus unavailable to support either enzymatic or non-enzymatic addtion of water to the phosphodester linkage, thereby obviating water-mediated strand breakage.

Having greatly reduced the rate of RNA hydrolysis via RNase inhibition and water removal, the primary residual source of RNA damage will be oxidation of the nucleic acid bases, primarily guanosine, that results from interaction with oxygen radical species induced by heat, or by metal ion activation of molecular oxygen which, subsequent to rehydration leads to depurination and beta elimination to produce a strand break (5).

GenTegra-RNA has been designed to mitigate such oxygen radical damage via inclusion of inhibitors of primary oxygen radicals and secondary peroxide radicals that are produced from them. Such free radical or peroxide radical inhibition is of special importance when monitoring RNA stability over a prolonged time-frame at elevated temperature.

Materials and Methods

RNA Purification. RNA was purified using a Qiagen RNeasy Midi Kit (Qiagen, Hilden, Germany) from freshly collected venous blood samples (Memorial Blood Centers, St. Paul, MN), from HeLa cells or from rat liver tissue. At time of purification, RNA was eluted into TE (pH 8.0), water, 1 mM EDTA, or 10 mM citrate buffer, pH 7.0. RNA concentration was measured by NanoDrop UV/VIS spectrophotometry (Thermo Scientific, Waltham, MA).

Ambient Versus 37°C Storage in the Fluid State. White blood cell (WBC) RNA (TE buffer only, no preservative) and WBC RNA with TE plus GenTegra-RNA was stored in the fluid phase at lab ambient room temperature (~25°C) or 37°C for up to 105 hours. RNA was used in all cases at 20 μg in 100 μL (0. 2 μg/μL). A "Time Zero" sample was taken at the start of the study. Samples were taken at regular intervals, thereafter, over the course of 5 days: 0, 9, 24, 33, 48, 57, 72, 81, 96, and 105 hrs. At each timepoint, 3 μL was removed and frozen until ready for analysis.

Multi-year RNA Storage in the Dry State. Purified HeLa, WBC and rat liver RNA were stored as 20 μg aliquots in a standard 96-well format, where each well contained GenTegra-RNA per the manufacturer's recommendation. Each RNA sample in GenTegra-RNA was then air-dried per the manufacturer's recommendation and the wells were sealed and then stored (unrefrigerated) for up to 4 years.

One such plate of air-dried RNA was first heated to 37°C in an oven for 7 months, then transferred to a warehouse for ambient temperature storage (~25°C) for 3.5 years. A second plate was heated to 56°C in an oven for 7 months, then transferred to a warehouse for ambient temperature storage (~25°C) for 3.5 years. A third plate was kept in a warehouse at ~25°C for the entire 4 year time period.

Subsequent to the 7 month heat treatment and later after a total 4 years of storage, all RNA samples were rehydrated with 50 μL of water. Each RNA sample was quantified by NanoDrop and then subjected to Agilent Bioanalyzer and rtPCR at the 18S locus, to yield a 313bp long amplicon fragment.

Bioanalyzer Assesment of RNA Quality. At time of analysis, frozen RNA samples were thawed, or matched dried RNA samples were rehydrated and diluted to 20 ng/μL. RNA was then denatured for 2 min at 70°C, then 1μL was run on the 2100 Bioanalyzer using the RNA 6000 Nano Total RNA Assay (Agilent Technologies, Santa Clara, CA). Such CE traces reveal a pair of prominent ribosomal (rRNA) peaks (18S, 1,9kb and 28S, 5kb) which are used as surrogate for total RNA quality in the sample (6).

Strand Breakage Density Estimation from Bioanalyzer Data. For each sample, a ribosomal RNA 18S/28S ratio was calculated by comparing measured 18S peak height to the corresponding 28S peak height. That measured ratio ($28S{:}18S_{test}$) was then normalized to the ribosomal RNA ratio measured for the matched fresh "Time Zero" sample ($28S{:}18S_{Time\ Zero}$) to generate R_n, where $R_n = 28S{:}18S_{test}/28S{:}18S_{Time\ Zero}$. Strand breaks accumulate more rapidly on the longer 28S target (5kb)

than for the shorter 18S target (1.9kb) as a function of increasing overall strand break density. Assuming Poisson statistics, the relationship between strand break density (X) and the measured 28S:18S peak height ratio can be estimated from the relationship (7)

$$X=[-\ln(R_n)]/3.1$$

rtPCR Amplification of 313b Region of 18S rRNA. To further evaluate RNA quality, a 313-nucleotide target sequence of 18S rRNA was reverse transcribed and amplified in a one-step reaction. The primers were obtained from a mammalian 18S rRNA primer set kit (Maxim Biotech Inc., Rockville, MD). The 25 µL reaction included 15 ng of RNA, 500 nM of forward primer, 500 nM of reverse primer, and AgPath-ID One-Step RT-PCR master mix (Life Technologies, South San Francisco, CA). Thermal cycling was performed on the MJ Research PTC200 with the following protocol: 45°C for 10 min; 95°C for 10min; 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; 72°C for 10 min; followed by a 4°C hold. Three microliters of the rtPCR was loaded and run, alongside a 250 bp DNA ladder (Invitrogen, Carlsbad, CA), on a 2% agarose gel containing ethidium bromide.

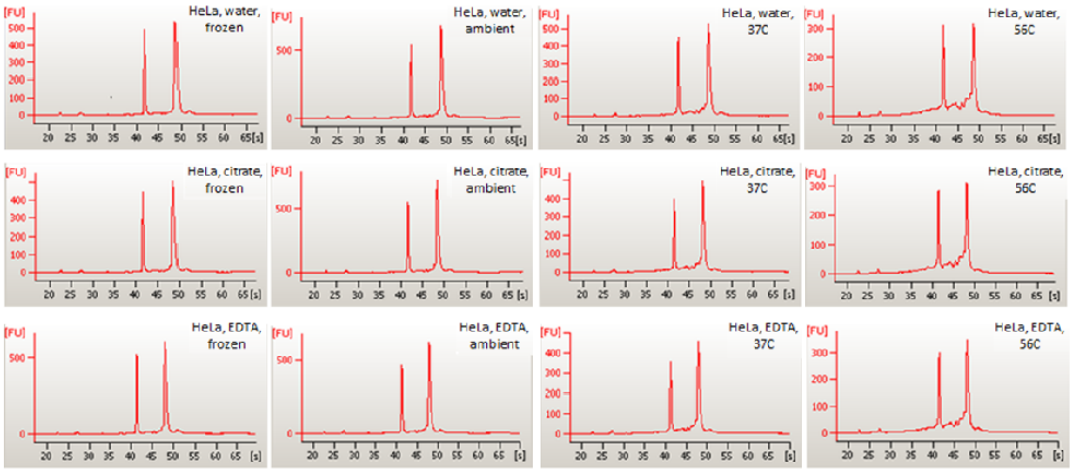
Results. RNA Quality Assessment (Bioanalyzer) Subsequent to a 7-month Heat Spike.

At the time of its original design, a dry-state preservation study was configured to test for the effects of extreme temperature on the preservation of RNA. Subsequent to air-drying, a number of Hela, WBC and Rat Liver RNA samples in GenTegra-RNA were placed, after air-drying, into ovens at 25°C or 37°C or 56C for 7 months. Upon completion of the 7month incubation, they were rehydrated and subjected to Nanodrop UV/Vis spectrometry, Bioanalyzer analysis and rtPCR at the 18S locus, to be compared to matched samples which had been kept frozen for the 7 months.

Those Bioanalyzer data are shown in Figures (1-3) . For HeLa (Figure. 1) it is seen that dry state storage for 7months at 25°C or 37°C or 56°C was indistinguishable from frozen control (i.e. RIN values @8) as assessed by Bioanalyzer analysis of 18S (1.9kb) and 28S (5kb) rRNA.

Figure 1

HeLa RNA stored dry at 25 °C, 37 °C, or 56 °C for 7 months
Frozen -20 °C control included
Bioanalyzer data



RNA	Buffer combined with GenTegra-RNA	Storage	Duration	Recovery (%)	RIN Score	28S:18S	Normalized ratio	Strand break per fragment	Break per kb
HeLa	water	-20°C	7 months	100	10.0	1.10	1.00	0.00	0.00
		Ambient		100	10.0	1.22	1.11	-0.11	-0.03
		37°C		101	9.6	1.16	1.05	-0.05	-0.02
		56°C		101	8.0	1.00	0.91	0.10	0.03
	citrate	-20°C	7 months	100	10.0	1.15	1.00	0.00	0.00
		Ambient		100	10.0	1.35	1.18	-0.16	-0.05
		37°C		101	9.3	1.22	1.06	-0.06	-0.02
		56°C		101	8.1	1.00	0.87	0.14	0.04
	EDTA	-20°C	7 months	100	10.0	1.15	1.00	0.00	0.00
		Ambient		100	10.0	1.29	1.13	-0.12	-0.04
		37°C		101	9.7	1.29	1.13	-0.12	-0.04
		56°C		101	8.5	1.11	0.96	0.04	0.01

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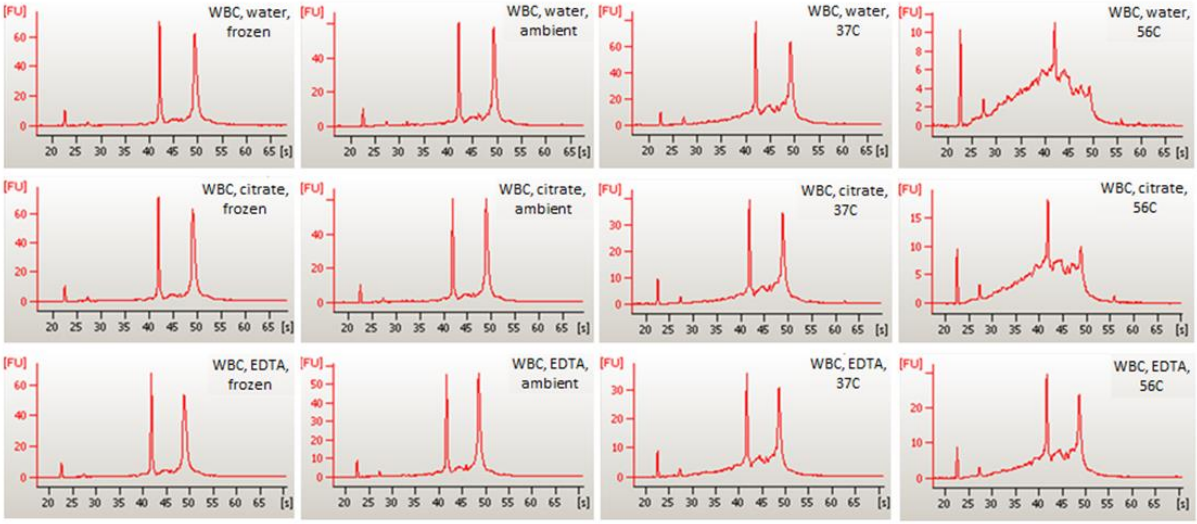
Hela RNA in water, citrate, or EDTA buffer was stored dry with GenTegra-RNA for 7 months at ambient temperature, 37°C, or 56°C. Subsequent to rehydration, the sample was analyzed by Bioanalyzer. A matched frozen control was included in the analysis.

Figure 2

WBC RNA stored dry at -20 °C and 25 °C for 7 months
Bioanalyzer Data



RNA	Buffer combined with GenTegra-RNA	Storage	Duration	Recovery (%)	RIN Score	28S:18S	Normalized ratio	Strand break per fragment	Break per kb
WBC	water	-20°C	7 months	100	9.7	0.86	1.00	0.00	0.00
		Ambient		100	9.4	0.95	1.11	-0.10	-0.03
		37°C		103	7.4	0.79	0.92	0.09	0.03
		56°C		102	3.4	0.36	0.42	0.86	0.27
	citrate	-20°C	7 months	100	9.6	0.86	1.00	0.00	0.00
		Ambient		100	9.5	0.95	1.11	-0.10	-0.03
		37°C		103	8.3	0.90	1.05	-0.05	-0.01
		56°C		102	4.3	0.43	0.50	0.70	0.22
	EDTA	-20°C	7 months	100	9.7	0.79	0.99	0.01	0.00
		Ambient		100	9.3	1.00	1.27	-0.24	-0.07
		37°C		103	8.3	0.80	1.01	-0.01	0.00
		56°C		102	7.0	0.78	0.98	0.02	0.00

Human lymphocyte (WBC) RNA in water, citrate, or EDTA buffer was stored dry with GenTegra-RNA for 7 months at ambient temperature, 37°C, or 56°C. Subsequent to rehydration, the sample was analyzed by Bioanalyzer. A matched frozen control was included in the analysis.

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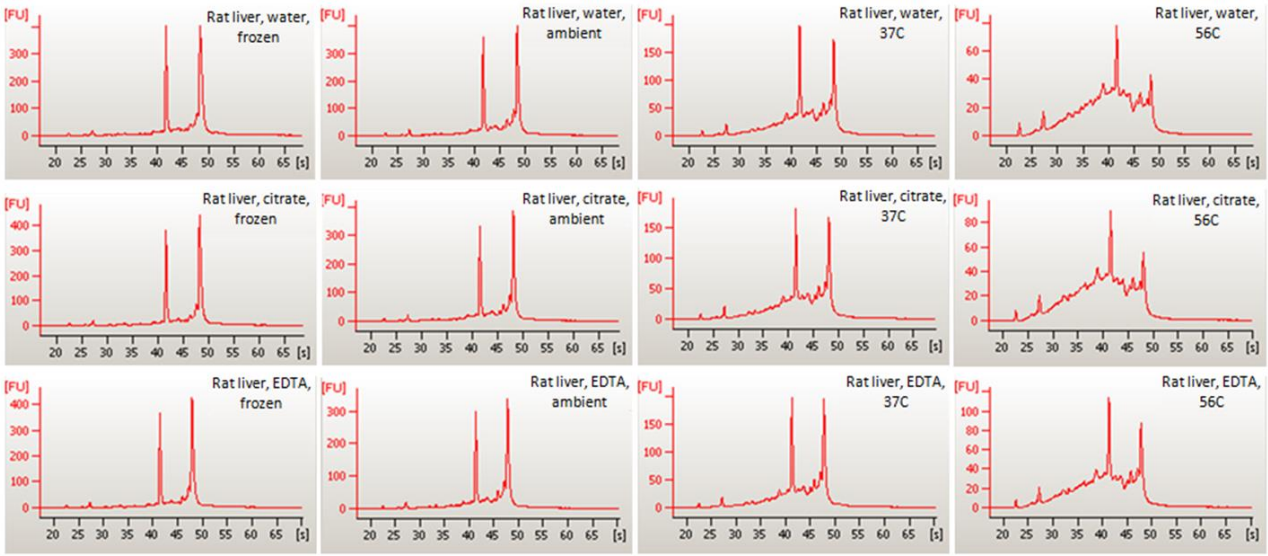
Figure 3

Rat liver RNA stored dry at 25 °C, 37 °C, or 56 °C for 7 months
Frozen -20 °C control included
Bioanalyzer data



RNA	Buffer combined with GenTegra-RNA	Storage	Duration	Recovery (%)	RIN Score	28S:18S	Normalized ratio	Strand break per fragment	Break per kb
Rat liver	water	-20°C	7 months	100	9.3	1.00	1.00	0.00	0.00
		Ambient		104	8.2	1.16	1.16	-0.15	-0.05
		37°C		105	5.7	0.81	0.81	0.21	0.07
		56°C		104	3.4	0.57	0.57	0.56	0.17
	citrate	-20°C	7 months	100	9.0	1.16	1.00	0.00	0.00
		Ambient		104	8.2	1.16	1.00	0.00	0.00
		37°C		105	5.8	0.95	0.82	0.20	0.06
		56°C		104	3.6	0.64	0.55	0.59	0.18
	EDTA	-20°C	7 months	100	9.0	1.16	1.00	0.00	0.00
		Ambient		104	8.1	1.16	1.00	0.00	0.00
		37°C		105	6.5	1.05	0.91	0.10	0.03
		56°C		104	4.5	0.81	0.70	0.36	0.11

Rat liver RNA in water, citrate, or EDTA buffer was stored dry with GenTegra-RNA for 7 months at ambient temperature, 37°C, or 56°C. Subsequent to rehydration, the sample was analyzed by Bioanalyzer. A matched frozen control was included in the analysis.

For WBC RNA (Figure. 2) it is seen that dry state storage for 7months at 25°C and 37°C was indistinguishable from the frozen control but upon heating for 7months at 56°C, RIN values dropped to @7, indicative of @ 0.3 breaks per kilobase (7). Interestingly, for those WBC RNA samples stored with additional EDTA, RNA damage at 56°C was eliminated.

For Rat Liver RNA (Figure 3) perhaps the most contaminated RNA with respect to residual cellular constituents, it is seen that dry state storage for 7months at 25°C or 37°C was still indistinguishable from the frozen control but upon heating for 7months to 56°C, RIN values dropped to @3.5, indicative of about 0.2 strand breaks per kilobase (7). Again, as for the WBC RNA, Rat Liver RNA samples stored with additional EDTA, incurred much less damage upon 7 months storage at 56°C (only about 0.1break/kb).

RNA Quality upon 4yrs of Storage at Ambient Temperature. Purified Hela, human lymphocyte (WBC) and Rat Liver RNA were stored dry in GenTegra-RNA at lab-ambient temperature for 4yrs, Subsequent to 4yrs of storage, all three sample types were rehydrated and subjected to RNA quantitation and Agilent Bioanalyzer analysis. Those data are shown in Figures 4-6).

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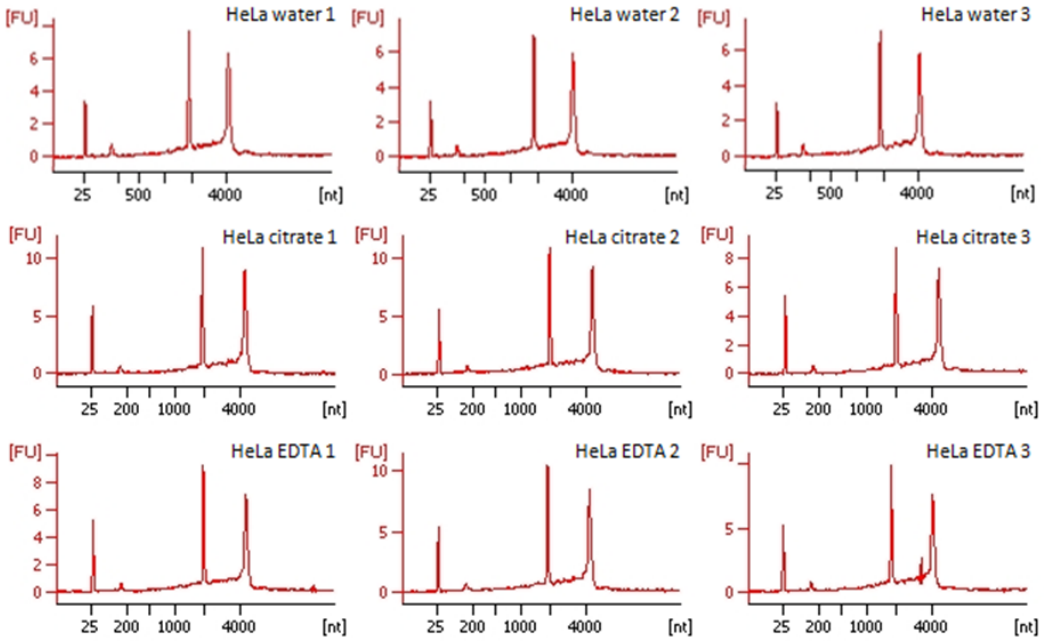
Figure 4

Hela RNA stored dry at 25 °C for 4 years
Bioanalyzer data

HeLa, ambient for 4 years



RNA	Buffer combined with GenTegra-RNA	Storage	Recovery (%)	RIN Score	Normalized ratio	Strand break per fragment	Break per kb	Average break per kb
Hela	water	Ambient, 4 years	115	8.2	0.78	0.25	0.08	0.08
			121	8.2	0.78	0.25	0.08	
			116	7.7	0.78	0.25	0.08	
	citrate	Ambient, 4 years	115	8.4	0.79	0.24	0.07	0.08
			116	7.7	0.78	0.25	0.08	
			112	8.5	0.78	0.25	0.08	
	EDTA	Ambient, 4 years	115	8.2	0.72	0.33	0.10	0.09
			118	7.9	0.78	0.25	0.08	
			115	8.3	0.72	0.33	0.10	

Hela RNA in water, citrate, or EDTA buffer was stored dry with GenTegra-RNA for 4 years at ambient temperature. The RNA samples were rehydrated and analyzed by Bioanalyzer. Top row, 3 replicates of RNA in water; middle row, 3 replicates of RNA in citrate buffer; bottom row, 3 replicates of RNA in EDTA buffer.

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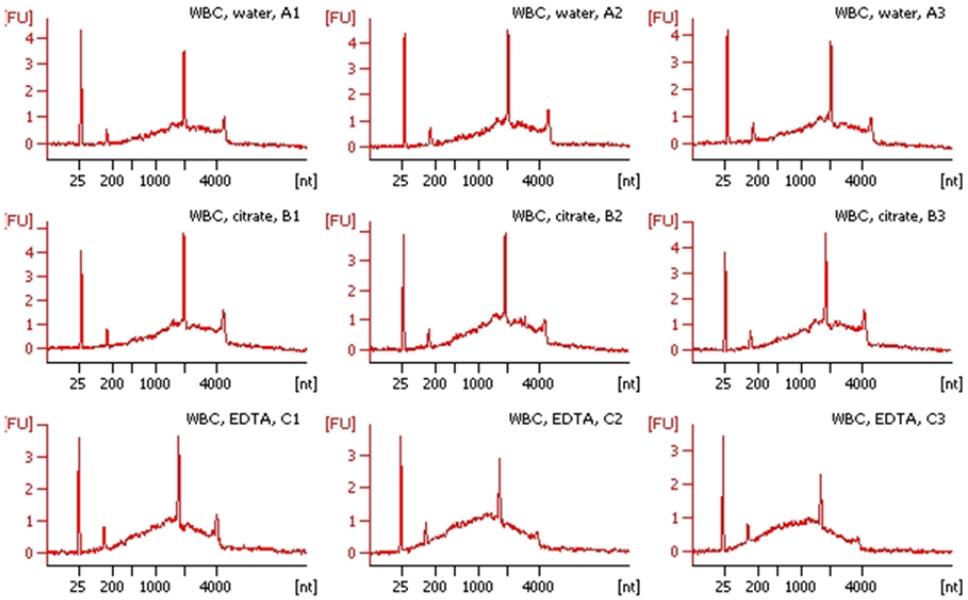
Figure 5

WBC stored dry at ambient for 4 years
Bioanalyzer Data



RNA	Buffer combined with GenTegra-RNA	Storage	Recovery (%)	RIN Score	Normalized ratio	Strand break per fragment	Break per kb	Average break per kb
WBC	water	Ambient, 4 years	116	5.3	0.30	1.20	0.37	0.37
			118	5.4	0.32	1.14	0.35	
			115	4.8	0.30	1.20	0.37	
	citrate	Ambient, 4 years	112	5.0	0.32	1.14	0.35	0.38
			111	4.1	0.26	1.35	0.42	
			111	4.8	0.32	1.14	0.35	
	EDTA	Ambient, 4 years	115	4.7	0.34	1.07	0.33	0.38
			112	3.6	0.24	1.42	0.44	
			121	3.8	0.30	1.20	0.37	

Human lymphocyte (WBC) RNA in water, citrate, or EDTA was stored dry with GenTegra-RNA for 4 years at ambient temperature. The RNA samples were rehydrated and analyzed by Bioanalyzer. Top row, 3 replicates of RNA in water; middle row, 3 replicates of RNA in citrate buffer; bottom row, 3 replicates of RNA in EDTA buffer.

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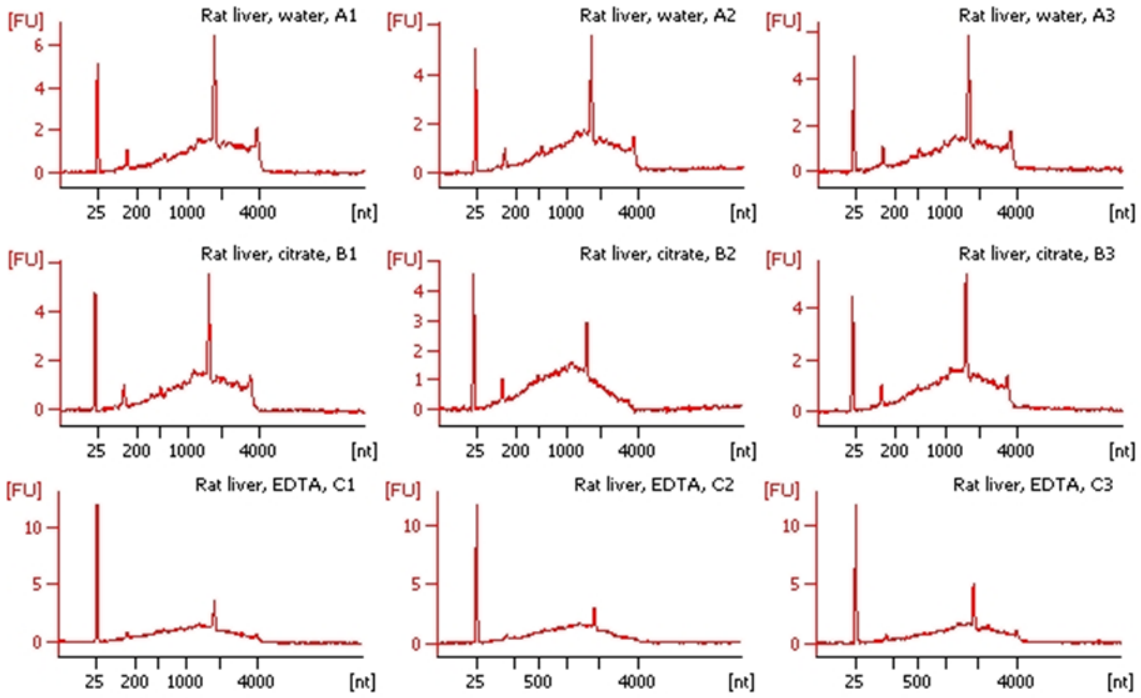
Figure 6

Rat liver stored dry at ambient for 4 years
Bioanalyzer Data



RNA	Buffer combined with GenTegra-RNA	Storage	Recovery (%)	RIN Score	Normalized ratio	Strand break per fragment	Break per kb	Average break per kb
Rat liver	water	Ambient, 4 years	110	4.9	0.24	1.42	0.44	0.50
			109	4.8	0.18	1.70	0.53	
			113	5.0	0.19	1.64	0.51	
	citrate	Ambient, 4 years	109	4.3	0.12	2.11	0.66	0.67
			110	3.8	0.13	2.04	0.64	
			111	4.3	0.10	2.25	0.70	
	EDTA	Ambient, 4 years	115	4.8	0.23	1.48	0.46	0.46
			114	4.3	0.30	1.19	0.37	
			114	4.7	0.18	1.70	0.53	

Rat liver RNA in water, citrate, or EDTA buffer was stored dry with GenTegra-RNA for 4 years at ambient temperature. The RNA samples were rehydrated and analyzed by Bioanalyzer. Top row, 3 replicates of RNA in water; middle row, 3 replicates of RNA in citrate buffer; bottom row, 3 replicates of RNA in EDTA buffer.

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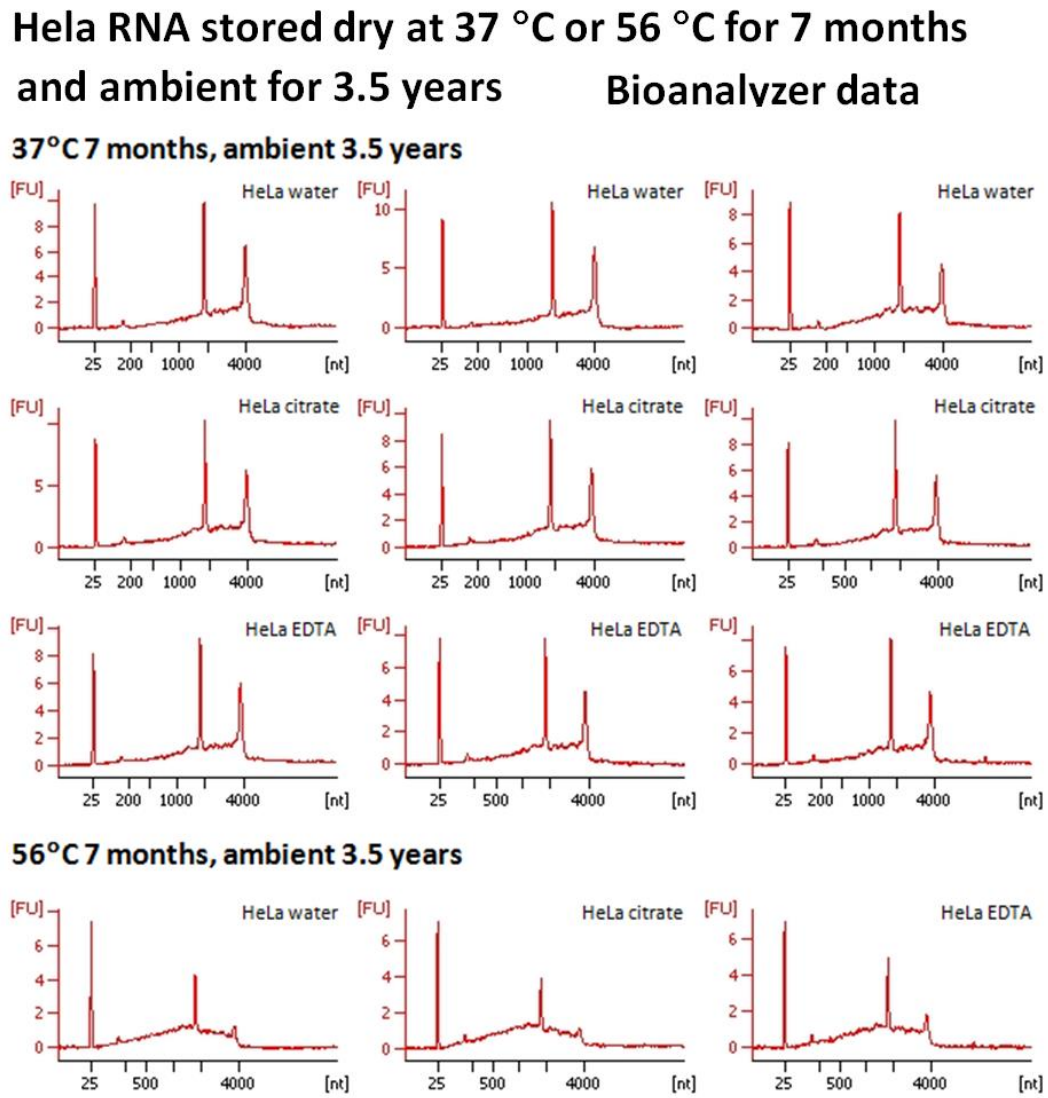
Figure 7

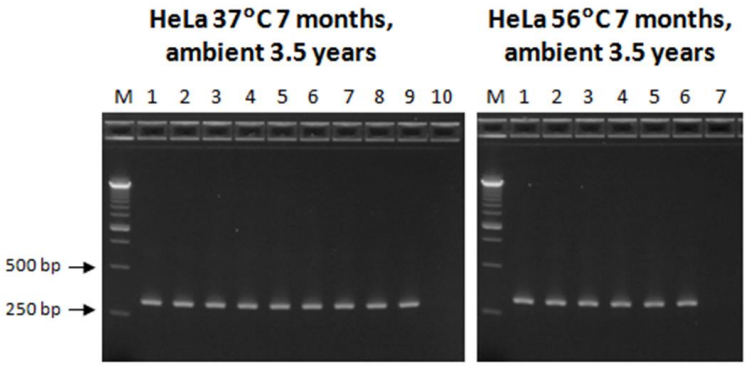


Hela RNA stored dry at 37°C (Figure 7a) or 56°C (Figure 7b) for 7 months, then at ambient temperature for 3.5 years. The RNA samples were rehydrated and analyzed by Bioanalyzer. Figure 7a shows electropherogram for samples initially stored at 37°C for 7 months, followed by ambient storage for 3.5 years. First row, 3 replicates of RNA in water; second row, 3 replicates of RNA in citrate buffer; third row, 3 replicates of RNA in EDTA buffer. Figure 7b shows electropherograms for RNA samples subjected to the initial 56°C incubation for 7 months, followed by ambient storage for 3.5 years (single replicate per buffer).

Figure 8

Hela RNA stored at 37 °C or 56 °C for 7 months followed by 3.5 years at ambient temperature

18S RT-PCR assay, 313 bp amplicon



M: 250bp ladder
Lanes 1, 2, 3: RNA in water
Lanes 4, 5, 6: RNA in citrate buffer
Lanes 7, 8, 9: RNA in EDTA buffer
Lane 10: Negative control

M: 250bp ladder
Lanes 1, 2: RNA in water
Lanes 3, 4: RNA in citrate buffer
Lanes 5, 6: RNA in EDTA buffer
Lane 7: Negative control

After rehydration of RNA, 15 ng of RNA was used as template for a one-step RT-PCR, and a portion of the reaction run on 2% agarose gel with ethidium bromide. The target amplicon is 313 bases. Figure 8a shows RT-PCR results for triplicate RNA samples stored at 37°C followed by ambient storage. Figure 8a: 250 bp ladder in lane M; RNA in water in lanes 1, 2, 3; RNA in citrate buffer in lanes 4, 5, 6; RNA in EDTA buffer in lanes 7, 8, 9; negative control in lane 10. Figure 8b shows duplicate RNA samples stored at 56°C followed by ambient storage. Figure 8b: 250 bp ladder in lane M; RNA in water in lanes 1, 2; RNA in citrate buffer in lanes 4, 5, 6; RNA in EDTA buffer in lanes 7, 8, 9; negative control in lane 10.

The trends seen after 4yrs of ambient temperature storage in GenTegra-RNA are consistent with expected differences in resulting RNA purity (8). Cultured Hela RNA

(Figure 4) showed no measurable change in rRNA quality after 4yrs (RIN Score of @8) which is as expected for the generally high quality of cell line derived RNA. Upon calculation, those Bioanalyzer data generates a strand break density of about 0.1 per kilobase after 4yrs (7).

RNA extracted from human lymphocytes (WBC) Figure 5, show evidence for the initial stages of RNA damage after 4yrs associated with RIN scores @5, which generates a calculated strand break density of about 0.4 breaks per kilobase after 4yrs (7).

Rat liver RNA (Figure 6) potentially the most contaminated sample, displays the poorest preservation after 4yrs: associated with a RIN score @4 and about 0.5 calculated strand breaks per kilobase (7).

Although the observed strand break density is measurable after 4yrs with sensitive Bioanalyzer analysis for Hela (0.1/kb) human lymphocyte (0.4/kb) and Rat Liver (0.5/kb) those strand break densities may remain too small to affect functional analysis. To confirm that expectation, we have performed a relatively “long” rtPCR reaction (313 nt) at the 18S locus to measure RNA stability functionally, post dry-state storage in Gentegra-RNA (Figure 10). In those reactions,18S primers were chosen which are common to all vertebrate 18S rRNAs and thus would perform identically on human and rat RNA.

Those data (Figure 10) show that after 4yrs of storage at ambient temperature in GenTegra-RNA, all three RNA types, in all three original buffer systems, support rtPCR at the 18S locus identically, thus confirming the general findings from RIN analysis: i.e that average template length, even for the most unstable rat liver samples is >300b and therefore suitable to support an rtPCR reaction which requires an intact 313b template.

RNA Quality Subsequent to a 7-month heat spike and 3.5 years of Storage at Ambient Temperature. At the time of its original design, this dry-state preservation study was also configured to test for the effects of extreme temperature on the preservation of RNA over a multi-year time period. Subsequent to air-drying, a number of the HeLa RNA samples in GenTegra-RNA were placed, dry, in a 37°C oven or a 56°C oven for 7 months. Upon completion of that 7-month heat treatment, they were all returned, still-dry, to lab ambient temperature, where they remained for 3.5

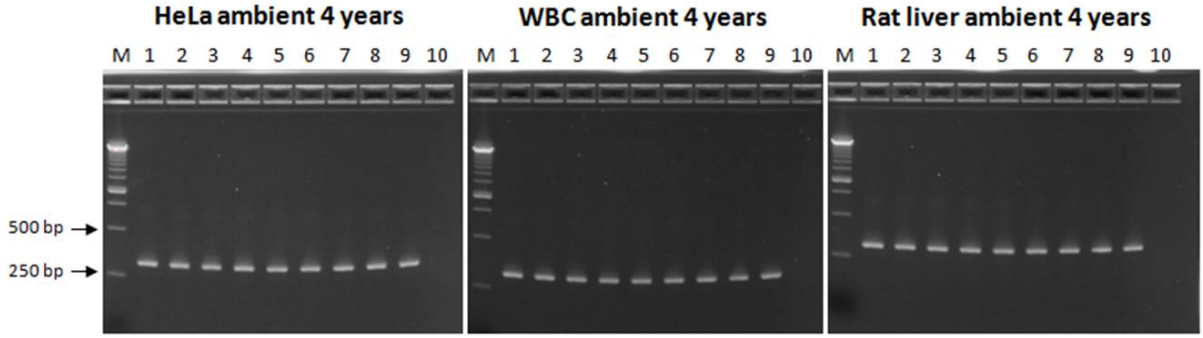
years to complete the remainder of the 4yr storage protocol. Upon completion of the 4yrs, all Hela RNA samples were rehydrated and subjected to Nanodrop UV/Vis spectrometry, RIN analysis and rtPCR at the 18S locus, to match the samples which had been kept at @25°C for the entire 4yrs.

Those Hela RNA recovery and RIN data are shown in Figures 6-7. It is seen that inclusion of a 7month treatment at 37°C followed by 3.5yrs of ambient storage produced a drop in RIN score from @8 (the value seen after 4yrs of ambient temperature storage) to @7, indicative of about 0.1 extra RNA strand breaks per kilobase that may be attributed directly to the effect of the initial 7 months of dry state storage at 37°C (7). It is further seen that inclusion of an initial 7month treatment at 56°C produced a drop in RIN score from @8 to @5, indicative of about 0.3 extra RNA strand breaks per kilobase that may also be attributed to 7 months of dry state storage at 56C in GenTegra-RNA (7).

The corresponding functional testing via rtPCR is displayed in Figure 9, where it is seen that all Hela RNA samples, independent of the extent of the initial 7month heat shock are found to be identical as assessed by rtPCR of a 313b 18S rRNA template.

Figure 9

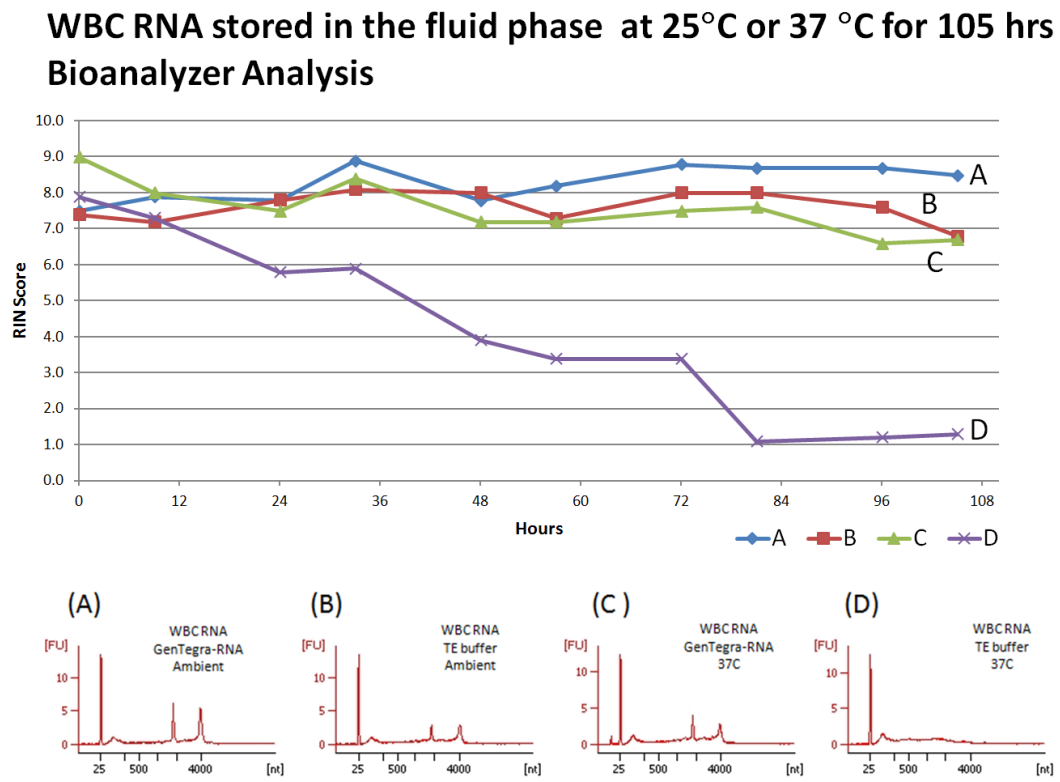
Hela, WBC, and rat liver RNA stored at ambient temperature for 4 years 18S RT-PCR assay, 313 bp amplicon



Each gel:
M: 250bp ladder
Lanes 1, 2, 3: RNA in water
Lanes 4, 5, 6: RNA in citrate buffer
Lanes 7, 8, 9: RNA in EDTA buffer
Lane 10: Negative control

18S RT-PCR analysis of Hela RNA, human lymphocyte (WBC) RNA, and rat liver RNA stored at ambient temperature for 4 years. After rehydration of RNA, 15 ng of RNA was used as template for a one-step rtPCR, and a portion run on 2% agarose gel with ethidium bromide. Triplicate RNA samples per buffer were analyzed. For each gel, 250 bp ladder in lane M; RNA in water in lanes 1, 2, 3; RNA in citrate buffer in lanes 4, 5, 6; RNA in EDTA buffer in lanes 7, 8, 9; negative control in lane 10.

Figure 10



Human lymphocyte (WBC) RNA stored in the fluid phase for up to 105 hours at 25°C or 37°C. Samples were periodically taken over the course of 5 days and analyzed by Bioanalyzer. RIN scores were plotted against time (hours). Ambient stored samples are indicated by curve A (RNA with GenTegra-RNA) and curve B (RNA in TE buffer). 37°C incubated samples are indicated by curve C (RNA with GenTegra-RNA) and curve D (RNA in TE buffer). Figure 10b shows Bioanalyzer electropherograms from samples collected after 105 hours. Curves A, B, C, and D correspond to electropherograms A, B, C, and D.

RNA Storage in the Fluid Phase. It is well-known that purified RNA is unstable over time in the fluid phase, due to residual ribonuclease activity and to general base-catalyzed hydrolysis of the phosphdiester linkage. Both reactions produce cleavage of the RNA backbone and a subsequent reduction in strand length.

In Figure 11, we display the results of an experiment in which highly-purified human lymphocyte (WBC) RNA was suspended in GenTegra-RNA or in TE buffer over a 105hr timecourse. Temperature was held at 25°C or 37°C. At each timepoint, RNA was evaluated by Agilent Bioanalyzer to ascertain RNA fragmentation based on the integrity of the 18S and 28S rRNA markers (7).

As seen in Figure 11, at storage time = 0, repeat sampling produced RIN values in the range from @8 to @9, indicative of high RNA quality. Figure 11, curve A displays the effect of ambient temperature (25°C) storage in the fluid state in samples preserved in GenTegra-RNA. As seen, there was no systematic change in RIN detected, indicating that RNA stability had remained intact in GenTegra-RNA over the full 105 hrs.

Matched WBC RNA samples stored in ordinary TE buffer were similarly stable over the same 105hr time-frame (Figure 11, curve B) indicative of the generally high quality of this RNA preparation. However, when the same RNA (in TE) was subjected to continuous 37°C heating over the 105hr time-frame (Figure 1, curve D and electrophorogram D) it can be seen that significant RNA cleavage was detected within 24hr, with nearly complete degradation at 105 hrs.

For comparision, the matched sample (Figure 1, curve C) stored in the fluid phase at 37°C in GenTegra-RNA showed no signficant change in quality over the first 100hrs of fluid phase heat treatment.

Discussion

These data have demonstrated that high-quality RNA samples are greatly stabilized by GenTegra-RNA in both the fluid phase (Figure 11) and in the solid phase phase that is generated by air-drying within GenTegra-RNA (Figures 1-8). The fluid phase data (Figure11) suggests that RNA may be routinely kept in GenTegra-RNA at room temperature or at 37C for up to 4 days.

The long term dry-state storage data (Figures 1-8) are instructive. They show that when RNA is stored in the dry-state in GenTegra at ambient temperature RNA at the highest level of purity (such as that from a HeLa cell line) does not incur any measurable RNA damage after 4yrs (Figure 4) as assessed by rRNA Bioanalyzer analysis. Human blood lymphocyte RNA, and rat liver RNA (at an intermediate level of residual purity) displays a bit more damage after 4yrs as assesed by RIN analysis, suggestive of 0.4 to 0.5 RNA breaks per kilobase after 4yrs of ambient temperature dry-state storage in GenTegra-RNA (Figures 5,6).

After 4yrs of ambient temperature storage, preliminary functional analysis (313b 18S rtPCR, Figure 10) is consistent with Bioanalyzer analysis, i.e. that RNA damage is not detected after 4yrs for an RNA template up to 300b in length.The effect of a 7 month heat-spike suggests that in the dry state, the effect of elevated temperaures and long term storage are additive: 7 months at 37°C followed by 3.5yrs at ambient temperature adding about 0.2 extra RNA strand breaks per kilobase and 7 months at 56°C adding about 0.2 extra RNA strand breaks per kilobase as measured directly by RIN.

Overall, the data presented here show that, in both the fluid phase and in the air-dried solid state, RNA stability has been enhanced by storage in GenTegra-RNA to the point where it can be used safely without refigeration: in the fluid-state at the lab bench, or upon air-drying for ambient temperature shipping and long-term biobanking, under conditions of temperature excess (i.e. 7months at 56C) which exceed any that would be encountered outside a lab environment.

Acknowledgements

Abbreviations

rRNA: Ribosomal RNA
RNAase: Ribonuclease
rtPCR: Reverse Transcriptase mediated polymerase chain reaction

Author Information

MEH is an expert in DNA dry storage methodology. He was a co-founder and Chief Scientific Officer for GenVault and GenTegra LLC.

GenTegra

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