

## Short-term stability study of RNA at room temperature

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Running Title: Room temperature RNA storage

### Abstract

The quality of RNA preserved in different stabilization matrices was investigated after 2 weeks of storage at room temperature. RNA samples in RNastable (Biomatrica), GenTegra (IntegenX) and RNashell (Imagene) were compared to RNA stored at -80 °C (the current gold standard for RNA preservation) and with liquid or dried RNA stored at room temperature without additives in this multi-center study. One center prepared all of the RNA samples and five participating laboratories applied the samples to the matrices and stored them for 2 weeks at room temperature. Samples were shipped to three testing laboratories, where the 336 RNA samples were rehydrated and then analyzed for RNA recovery, purity, and integrity. Parallel RNA quality analysis and real-time PCR analysis were performed at each of the three testing laboratories. Each of the RNA matrices tested was shown to be fit-for-purpose for short-term room temperature storage in terms of total RNA recovery and rRNA integrity. All but one of the matrices were judged to be fit-for-purpose for mRNA integrity assessed by real-time PCR analysis. In a follow-up study, RNase-contaminated samples were shown to provide accurate real-time PCR results when stored for up to 3.5 months in either RNashell or RNastable.

## Introduction

Novel storage technologies that allow preservation of purified nucleic acids at room temperature (RT°) are emerging. Manufacturers guarantee high sample quality while users enjoy the advantages of simplified sample handling and shipping, improved logistics, and lower energy costs (1) since dehydrated samples are stored or shipped at RT°. DNA preservation at RT° is now well-documented (2), but the field of RT° RNA preservation is still emerging, as RNA is commonly known to be a fragile macromolecule susceptible to degradation by ubiquitous ribonucleases (RNases) (3). Room temperature storage solutions for biospecimens (e.g. dried blood spots) or nucleic acids (FTA or Guthrie cards on filter paper) are becoming increasingly popular. However, few studies have focused on the storage of RNA at ambient temperature or at RT°. Hernandez et al. (2009) investigated the recovery and integrity of commercial RNA isolated from liver tissue. In their study, aliquots were stored for 4 weeks in RNASTable or at -80 °C. Equivalent recovery, purity and integrity was shown between RNASTable storage and storage at -80 °C. However, only a limited number of RNA samples were tested and other RT° storage matrices such as RNAshell and GenTegra were not considered (4). Wan et al. (2) conducted a comparison study of RT° storage matrices from Biomatrix and IntegenX (formerly GenVault) for DNA, whereas only RNASTable (Biomatrix) technology was assessed for RNA. RNA samples stored for 11 days at RT° in RNASTable had a slightly higher RIN and a complete RNA recovery compared to storage at -80 °C. In these publications, the RNA storage products were not compared between suppliers and the experimental condition, RNA RT° storage without matrix, was not assessed, as samples were only compared to the -80 °C control. Thus, we conducted this multicenter study to compare various storage products and conditions (e.g. -80 °C storage, RT° storage of dried or liquid RNA).

Three RNA RT° storage matrices are commercially available: GenTegra (IntegenX), RNASTable (Biomatrix) and RNAshell (Imagene). Biomatrix and IntegenX have developed RNA storage systems that utilize simple microcentrifuge tubes or 96-well plate formats, whereby RNA in solution is mixed with the stabilization matrices, then samples are dehydrated and stored at RT°, thus protecting the RNA from hydrolysis and nuclease degradation (5, 6). The Imagene product uses an alternative RNA preservation system whereby RNA is dried and encapsulated under anhydrous and anoxic conditions in metal RNAshells thereby protecting RNA from oxidation (7). These three RT° storage technologies are based on the principle of anhydrobiosis, the capacity of some multicellular organisms to survive for years or decades in the complete absence of water but to revive after rehydration.

In order to obtain reliable and accurate RNA and gene quantification, it is essential to control preanalytical variables related to sample storage conditions (e.g. storage duration, additives, vials, temperature, etc.) (8), not only those related to biospecimen collection, handling and processing. In this multi-center study, the efficiency and fitness-for-purpose of RT° short-term storage and shipment systems to preserve RNA integrity, recovery and gene expression was assessed. A period of two weeks at RT° was chosen because it is sufficient for common world-wide shipments.

One center prepared RNA from 7 human blood samples and shipped the RNA to five international centers (Figure 1A). At these five centers, the RNA was aliquoted into the RT° preservation matrices: RNASTable, GenTegra and the control condition “dried RNA no matrix” (Figure 1B). RNA in RNAshells was prepared by the manufacturer (Figure 1B). Dried RNA samples were stored at RT° for 2 weeks, and then shipped to 3 testing laboratories at ambient temperature where the samples were rehydrated and assessed for RNA recovery, RNA Integrity Number (RIN) and gene

quantification by real-time PCR (Figure 1A). The RT° storage systems GenTegra, RNAstable and RNAsheIl were compared to three controls: (a) RNA stored at -80 °C (the current best practice); (b) RNA dried without matrix (“dried RNA no matrix”) stored at RT° to investigate whether dehydration is sufficient for high quality RNA preservation; and (c) liquid RNA stored at RT° (“liquid RNA no matrix”) to assess the kinetics of the natural degradation process of RNA at RT°.

## Materials and Methods

### Materials

Biomatrix (San Diego, CA, USA) supplied RNastable 25-Tube kits (ref. 93221-001; lot nb. 41D162) and GenVault (IntegenX, Pleasanton, CA, USA) supplied GenTegra RNA 25-Tube kits (ref. GTR5025-S; lot nb. 1009160830) and Imagene (Evry Cedex, France) supplied RNashells®.

### RNA extraction

Informed consent from donors was obtained prior to collection, CNER Ethics Protocol ID [201107/02](#). 25 ml of blood from each of 7 healthy human volunteers was collected in PAXgene Blood RNA tubes (BD Biosciences, Erembodegem) at IBBL (Integrated BioBank of Luxembourg). PAXgene tubes were stored at RT° for 2 hr before automated RNA extraction on QiaCube (Qiagen, Venlo) with the PAXgene Blood RNA kit (PreAnalytiX, Venlo; ref. 762174) at IBBL. For each donor, extracted RNA concentration was adjusted to 50 ng/μl. Samples were stored at -80 °C prior to shipment on dry ice to the five preparation centers.

### RNA sample processing for RT° storage

The 5 preparation centers performed parallel RNA processing following the manufacturers' instructions. 20 μl of RNA/tube (1 ug RNA) was aliquoted into RNastable, GenTegra and empty RNase-free tubes ("dried RNA no matrix" and "liquid RNA no matrix"). Samples were incubated for 5 min at RT°, mixed and dried in a speedvac without heat for 2 hr for GenTegra and "dried RNA no matrix" and for 1 hr for RNastable. Immediately after drying, all RNA tubes were recapped. RNastable tubes were packed in heat-sealed pouches containing desiccant. Dried RNA samples were stored for 2 weeks at RT° (18 – 28 °C) weeks. RNashells were prepared with 20 μl of RNA/shell by the manufacturer. The dried samples were shipped at ambient temperature to the testing centers; the testing centers rehydrated the RNA samples with RNase-free water.

### RNase contamination of RNA samples

RNA from the same donor, donor 3, was extracted and half of the volume contaminated with RNases by contact with human skin for 3 seconds. The quantity of added RNases was not determined. The RNA samples were immediately analyzed for RNA Integrity Number (RIN). Aliquots of contaminated and pure RNA were prepared for -80 °C and RNastable, and stored for 1, 2, 3.5 and 12 months, while RNashell and "liquid RNA no matrix" were stored for 1, 2 and 3.5 months. Samples were stored at RT° except for the -80 °C condition. At the specified time points, samples were rehydrated and analyzed for RIN, and by real-time PCR for ACTB and ORM1 expression (Lab 3, see below).

### RIN analysis

RIN analyses were performed with the RNA 6000 Nano kit on 2100 Bioanalyzer (Agilent).

### RNA quantification

RNA samples were quantified by spectrophotometry on a Take3 plate in a Synergy Mx instrument (BioTek) before and after dehydration. For GenTegra and RNastable samples, the

spectrophotometer was blanked with RNase-free water containing their respective matrices in solution. RNA purity was measured by OD260/OD280 and OD260/OD230 ratios.

### Reverse transcription and real-time PCR

All RNA samples were reverse-transcribed and analyzed by real-time PCR for high abundant [ACTB (beta-actin) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)] and low abundant [HMBS [hydroxymethyl-bilane synthase, alias PBGD] house-keeping genes, and low abundant genes PLAUR (plasminogen activator, urokinase receptor), IL1B (interleukin-1 beta) and ORM1 (orosomucoid 1). All 3 labs followed their standard real-time PCR protocols, described below. All Cq values were compared to the -80 °C controls ( $\Delta Cq$ ).

Lab 1: 4  $\mu$ l of total RNA (equivalent to 0.2  $\mu$ g total RNA) from each sample in the different storage matrices was reverse transcribed into cDNA using the SuperScript® III First-Strand Synthesis SuperMix (Life Technologies) on a thermal cycler (Bio-Rad). Reference RNA (Clontech) was run in parallel to control for the reverse transcription efficiency. 10x-diluted cDNA was amplified using the Fast SYBR Green Master Mix (Life Technologies Europe BV, Gent, Belgium) and 300 nM primers [(Table 4); Sigma-Aldrich, Bornem, Belgium] in 20  $\mu$ l reactions in a 7500 Fast Real-Time PCR System (Life Technologies) (40 cycles: 15 sec at 95 °C and 1 min at 60 °C). The No Template Control (NTC) assay was run in parallel and technical triplicates were performed. The specificity of the PCR amplicons was controlled by melting curve analysis.

Lab 2: RNA was reverse transcribed using oligo(dT) from the iScript Select cDNA Synthesis Kit (BioRad, Hercules, CA). The reverse transcription was performed in a 20  $\mu$ l volume at 42 °C for 60 min. Real-time PCR reactions were performed in duplicate in 96-well plates using the iCycler Thermal Cycler (BioRad). The 20  $\mu$ l reactions consisted of 10  $\mu$ l of 1X iQ™Supermix, 500 nM of each primer (Table 4), 4  $\mu$ l of cDNA template, and water.

Lab 3: Reverse transcription was conducted on RNA samples using TaqMan High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with random hexamers, according to the manufacturer's protocol. RNA was used at 4  $\mu$ l except for the recleaned samples for which 12  $\mu$ l was used per 20  $\mu$ l reaction. All real-time PCR reactions were processed on an ABI 7500 real-time PCR machine in triplicate. cDNA was used at 1.6  $\mu$ l volume per 20  $\mu$ l reaction with cycling conditions (1 cycle of 50 °C for 2 min followed by 95 °C for 10 min, 50 cycles of 95 °C for 15 sec followed by 60 °C for 1 min). Controls consisting of sterile molecular grade water were negative in all runs. ACTB (Hs03023880\_g1) and ORM1 (Hs01590791\_m1) probes were both purchased from Applied Biosystems, Inc.

### RNA re-cleaning

RNA samples stored at -80 °C or with the RNastable and GenTegra matrices were cleaned with the PicoPure RNA Isolation kit (Arcturus Engineering, Inc. Mountain View, CA) to investigate the amplifiability of RNA stored in GenTegra. Experiments were performed in Lab 3. Briefly, 18  $\mu$ l of each sample was diluted with 32  $\mu$ l water in a 0.5 ml microcentrifuge tube and then the manufacturer's

protocol was followed for RNA isolation. Roughly 18 µl of re-cleaned RNA was produced from each sample and 12 µl of purified RNA was used for reverse transcription and real-time PCR.

### Statistical analysis

Statistical analysis was performed with SigmaPlot using the Shapiro-Wilk test for normality testing. When the normality test failed, an ANOVA1 test on ranks (Kruskal-Wallis) was executed followed by Dunn's test for multiple comparisons versus the -80 °C control condition. When the normality test passed, ANOVA1 was followed by a Holm-Sidak test where conditions were compared to the -80 °C control group.

For comparisons between donors, the all pairwise multiple comparisons method (Tukey test) was applied.

The coefficient of variation between donors, for each laboratory and each gene, was calculated to give the inter-donor variability for real-time PCR.

Combined uncertainty was determined according the Guide to the expression of Uncertainty in Measurement (GUM) (9). Combined uncertainty between the 3 testing laboratories was calculated

following the formula: combined uncertainty =  $\sqrt{(SD_{lab\ 1}^2 + SD_{lab\ 2}^2 + SD_{lab\ 3}^2)}$ .

## Results

### Total RNA recovery and purity

To evaluate the RNA recovery, quantification was performed on samples prepared with each of the matrices before drying and after rehydration (Figure 2A). Inter-individual recovery for the same storage condition did not significantly change from donor to donor (data not shown). RNA recovery from RNASTable, GenTegra and RNAShell ranged from 96 to 106% and was not significantly different from the -80 °C control condition. Dried RNA samples stored with no matrix for 2 weeks at RT° showed the lowest, and the only significantly different, RNA recovery percentage (81%).

RNA purity showed average 260/280 ratio values between 1.9 and 2.1 for five of the conditions (RNASTable, RNAShell, -80 °C, “dried RNA no matrix”, and “liquid RNA no matrix”). However, the 260/280 ratio was only 1.2 for the RNA samples stored in the GenTegra matrix. The 260/230 ratio values were highly variable between conditions, and also among the samples in each condition. These 260/230 ratio values were 0.5 +/- 0.1 (Standard deviation) for RNASTable, -0.3 +/- 4.7 for GenTegra, 1.0 +/- 0.2 for RNAShell, 1.2 +/- 0.2 for “liquid RNA no matrix”, and 3.4 +/- 3 for “dried RNA no matrix”.

### rRNA integrity assessment by RIN

Ribosomal RNA integrity was assessed by determining the RNA Integrity Number (RIN; Figure 2B) (10) using an Agilent Bioanalyzer. All donor RNAs in different storage conditions gave RIN values > 7 and sharp 28S and 18S rRNA peaks. When all RT° storage conditions were compared to the -80 °C control, no significant differences were detected, indicating that the rRNA integrity was not affected when total RNA was stored for 2 weeks in the preservation matrices. The RIN value was >8 for RNAShell, RNA stable and GenTegra; while the RIN value were 7-8 for “liquid RNA no matrix” and “dried RNA no matrix”. The RT° storage without matrix (whether liquid or dry RNA) showed higher analytical variability in RIN measurements. Data obtained in the “dried RNA no matrix” condition suggested that RNA drying in the absence of any stabilizing compound did not impact rRNA integrity when analyzed by a Bioanalyzer.

### mRNA integrity assessment by real-time PCR quantification

Since the RNA was prepared from each donor separately and then aliquoted in different storage conditions, any difference in real-time PCR cycles (delta cycle quantification ( $\Delta Cq$ )) reflects differences in the storage conditions rather than the original gene expression. Each laboratory followed its best practice real-time PCR protocol. Data were analyzed for each storage condition (Table 1) and graphed separately for each of the 3 testing laboratories (Figures 3A-C).

Overall, the -80 °C condition gave the best real-time PCR quantification results in testing laboratory 1 (Table 1 and Figure 3A). RNASTable and RNAShell showed real-time PCR Cq values similar to those of RNA samples stored at -80 °C ( $\Delta Cq$  of +0.11 and +0.18 respectively (all genes  $\Delta Cq$  values are highlighted in Table 1)), for “liquid RNA no matrix” and “dried RNA no matrix” samples, the average Cq values increased by 0.54 and 0.64 respectively compared to the -80 °C control condition. For RNA stored in the GenTegra matrix, high Cq values were detected for all genes; an increase of +6.45  $\Delta Cq$  was detected compared to the -80 °C conditions.

Real-time PCR data from the second testing laboratory showed the same trend (Table 1 and Figure 3B). RNAsheIl and RNAsTable performed equivalently to -80 °C control. For the “dried RNA no matrix” condition the Cq values were 0.2 Cq lower compared to Cq values of the “liquid RNA no matrix” condition, and both conditions were within 1 Cq ( $\Delta$ Cq of +0.74 and +0.95 respectively) when compared to the -80 °C condition. In contrast, the  $\Delta$ Cq was +12.97 for the RNA samples stored in GenTegra..

The real-time PCR data from the third testing laboratory (Table 1 and Figure 3C) showed that the storage system RNAsTable gave the best gene quantification results ( $\Delta$ Cq of -0.07), followed by -80 °C, RNAsheIl ( $\Delta$ Cq of +0.33) and “dried RNA no matrix” ( $\Delta$ Cq of +0.39). Compared to -80 °C, “liquid RNA no matrix” showed a value 1.55 Cq higher than the -80 °C control and the Cq of GenTegra was significantly higher by +11.23 Cq.

Statistical analysis of real-time PCR data between all storage conditions showed that only the comparison of GenTegra to -80 °C resulted in a significant difference; this was observed for all six of the analyzed genes in each of the three testing laboratories. In addition, the difference “liquid RNA no matrix” versus -80 °C control condition was statistically significant only for gene ACTB tested in laboratory 3 (Figure 3A-C).

#### *Inter-laboratory Variability*

To get an inter-laboratory overview of real-time PCR quantification results for the different RT° storage conditions, average Cq values and combined uncertainty for ACTB were calculated, as this gene was analysed in all 3 laboratories (Table 2). In general, results between laboratories were concordant even if each testing laboratory followed its own real-time PCR procedure. After 2 weeks of storage in RNAsTable or RNAsheIl, very similar real-time PCR results were obtained when compared to RNA that was stored at -80 °C ( $\Delta$ Cq of +0.03 and of +0.05, respectively). The Cq for the negative control “dried RNA no matrix” only increased 0.56 Cq; this showed that RNA dried without stabilization matrix was also amplifiable by real-time PCR. RNA stored in liquid state without matrix at RT° presented a 1.32 Cq higher value when compared to the -80 °C control. In this study, the GenTegra samples had Cq values that were consistently higher (+11.37 Cq) than the negative control “liquid RNA no matrix”.

Real-time PCR analytical variability for ACTB from the three testing laboratories for the different storage conditions was the lowest for “dried RNA no matrix” (combined uncertainty: 1.04), followed by RNAsTable (combined uncertainty: 1.33), “-80 °C” (combined uncertainty: 1.40), RNAsheIl (combined uncertainty: 1.90) and “liquid RNA no matrix” (combined uncertainty: 2.44). Analytical variability was highest for RNA stored at RT° in the GenTegra matrix (combined uncertainty: 7.92) (Table 2).

#### **RNA RT° stability study of pure and RNase-contaminated samples**

As liquid RNA storage for 2 weeks at RT° was not contraindicated for real-time PCR, a second phase of this study investigated the RNA quality after storage for up to 3.5 months at RT°, by RIN measurement and real-time PCR gene quantification. Moreover RNA samples contaminated with skin RNases were analyzed to assess the efficiency of the RT° storage systems.



For pure RNA, all the RIN values for RNAsheIl, RNAsable and -80 °C remained >7 for all time points (GenTegra was not assessed) (Figure 4A). Only the condition “liquid RNA no matrix” showed degradation of pure RNA, where after 1 month the RIN decreased from 7.5 (intact RNA) to 3.2. After 2 months the RIN was 4.1, and after 3.5 months the RIN could not be determined (N/A). These results confirm the 2-week stability study and additionally show that RNA integrity is preserved for at least 3.5 months when pure RNA is stored in RNAsheIl, RNAsable or at -80 °C. Furthermore, RNA integrity is preserved for as long as 12 months with the RNAsable matrix (the only RT° condition assessed for 12 months, besides -80 °C storage).

RNase-contamination of RNA immediately resulted in a decrease in RIN from 7.5 (intact RNA) to 4.1 just prior to application on any storage condition (Figure 4A). Contaminated RNA preserved for 1 month in RNAsheIl gave the highest RIN (5.6) of all RNase-contaminated samples, but RIN at the 3.5-month time point could not be determined. RIN values for RNAsable, -80 °C and “liquid RNA no matrix” were low (between 4.6 and 1.2). The analytical variability of RIN measurements is known to be high (F. Betsou, personal communication). A clear time-course tendency could only been seen for “liquid RNA no matrix” stored at RT° where rRNA integrity continuously decreased. Contaminated RNA preserved for 12 months in RNAsable resulted in a RIN value of 2.2, highly similar to the -80 °C condition with a RIN value of 2.8.

Real-time PCR data for ACTB and ORM1 showed similar expression profiles as a function of the storage conditions (Figure 4B and 4D, respectively). The stability study of ACTB gene quantification in pure RNA samples showed minimal quantification differences between 1-month-old samples and 3.5-month-old samples or even 12-month-old RNAsable and “-80 °C” samples. Surprisingly, for pure RNA stored in RNAsheIl a 0.4 lower Cq value was detected after 3.5 months storage and for “liquid RNA no matrix” Cq levels increased by 0.72 Cq (from Cq 17.71 to Cq 18.43) over 2.5 months. For contaminated samples, there was a sharp increase in Cq levels over time for the condition “liquid RNA no matrix” [ $\Delta$ Cq ACTB of +5.35 (from Cq 28.14 to Cq 33.49)]. This increase in Cq was much higher than for pure samples stored in “liquid RNA no matrix” (+0.72 Cq). Thus, the difference in amplification cycles between pure and contaminated RNA for each of the three time points was used to estimate the degradation rate of liquid RNA at RT°. At the 1-month time point, the difference was 10.4 Cq; at 2 months the difference increased to 12.82 Cq, and at 3.5 months a 15.06 cycle difference was attained; suggesting that RNA degradation in these conditions increases exponentially and can be expressed by the equation  $541.09 e^{1.27x}$  ( $R^2=0.982$ ) (Figure 4C). After 3.5 months storage, real-time PCR quantification showed that contaminated RNA in RNAsheIl performed equally as well in real-time PCR as -80 °C storage (+0.06  $\Delta$ Cq ACTB; +0.13  $\Delta$ Cq ORM1) whereas RNAsable showed a visible Cq increase (+1.72  $\Delta$ Cq ACTB; +1.32  $\Delta$ Cq ORM1) when compared to the -80 °C control (Figure 4B, D). Assessment of RNA in RNAsable preserved for up to 12 months showed that real-time PCR data for a given condition do not change considerably over time.

### GenTegra

The cause of the low real-time PCR performance observed for all GenTegra samples (cf. Figure 3) was further investigated for genes ACTB and ORM1 (Table 3). To assess whether a constituent of the GenTegra matrix was interfering with the real-time PCR reaction, the quantity of RNA used for cDNA preparation was increased 3-fold for all samples and showed, as expected, ACTB Cq values which were 1.2 Cq and 1.1 Cq lower, respectively, for the conditions “-80 °C” and RNAsable; (ORM1 Cq

values were 1.4 and 1.6 Cq lower) compared to the initial cDNA input. However, for the GenTegra samples the ACBT Cq values increased by 17.7 cycles (Cq=46) (ORM1 Cq values were not detectable); suggesting an inhibitory effect of the GenTegra matrix on some step of the analytical process. By performing RNA sample clean-up, the Cq values for the GenTegra samples diminished by 21.7 cycles and eventually were measurable for ORM1. For RNastable and -80 °C samples, the purification resulted in slightly increased Cq values; most likely because sample purification caused detectable RNA quantity loss.

## Discussion

In this study, our objective was to assess the fitness-for-purpose of RT° RNA storage matrices for use in the context of the ISBER proficiency testing schemes which require intercontinental shipments of RNA (11). We assessed the 2-week stability of RNA at ambient temperature which corresponds to roughly 3 times the longest shipment duration in the proficiency testing schemes. These findings can also be extended to any biobanking application where shipment of RNA is needed.

RIN measurement is now broadly implemented in the scientific community as the common RNA quality control tool which provides an unambiguous and comprehensive index of the overall RNA quality of the starting material (10, 12). In general, samples with RIN values ranging from 10.0 to 7.0 are considered good quality (13). However, even RIN values of 4 give good gene microarray results (14). RIN essentially evaluates the global ribosomal RNA integrity and is not the most sensitive RNA quality control tool because there is no direct correlation between ribosomal and degradation of a specific messenger RNA. Bioanalyzer RIN values also do not provide information on RNA purity or potentially inhibitory substances. It is important to stress that there is no standardized RNA quality metric that can be used as a strict threshold for RNA applications as variables such as downstream protocol, analysis platform and specificity/sensitivity of the kits, have to be considered. Therefore, the goal of the present study was to compare various storage matrices using different RNA quality metrics, and not to set absolute recommendations.

Nucleic acids differ in their resistance to degradation. Compared to DNA, RNA is more easily cleaved in mild alkali solutions (15) and at high temperatures (16). Surprisingly, in terms of oxidation, RNA has been shown to be more resistant to oxidative stress than DNA (17); this might explain why liquid RNA and dry RNA without matrices at RT° both performed surprisingly well in our experiments. Besides oxidation and hydrolysis, the most important factor for RNA degradation is the presence of ribonucleases (RNases). These are either endogenous RNases, released during the harvesting of the cells, or exogenous RNases, added involuntarily during sample handling. In our study, endogenous RNases were inactivated by the RNA-stabilizing solution of the PAXgene Blood RNA tube, thus resulting in highly pure RNA samples with high rRNA integrity (RIN >7) and high mRNA quality even after 2 weeks of RT° storage in liquid conditions. These data are contradictory to the common opinion that RNA degrades rapidly at RT° which probably originates from a period when the RNA extraction kits were not yet optimized. Increased RNA recovery percentages were detected for the GenTegra condition and for the liquid RNA stored without matrix, actually RNA recovery percentages above 100% might result from spectrophotometry analytical variability but RNA recovery percentages above 100% for RNA stored in RT° storage matrices have already been reported (2, 18). Our study showed that pure extracted RNA is stable in solution for up to 2 months at RT° and that successful real-time PCR experiments with liquid RNA stored for 3.5 months at RT° could even be performed. These results are in agreement with a recent RNA degradation study by Opitz et al. (19).

In all three of the RNA RT° storage matrices that we tested, RNA was stored in the dehydrated state so as to prevent enzymatic reactions. Our results showed that simply by dehydrating RNA without any matrix, in clean RNase-free tubes, it was possible to preserve rRNA and mRNA integrity for high quality down-stream applications.

After two weeks storage at RT°, RNA stored in RNastable and RNashell, and RNA stored dry or liquid without matrix were shown to preserve rRNA integrity and mRNA quality for downstream real-time

PCR. Only the GenTegra technology gave unsatisfactory real-time PCR results, which were worse than liquid RNA stored at RT°, our negative control. Further investigations strongly suggested a real-time PCR and/or RNA to cDNA conversion inhibitory effect of the GenTegra matrix. RNA stored in GenTegra tubes was not degraded as shown by high RIN numbers (Figure 2B) and gel electrophoresis (data not shown) but RNA was impure, as assessed by RNA purity ratio measurements. Both 260/280 and 260/230 ratio values for RNA stored in GenTegra matrices were lower and less reproducible than in other conditions studied. At the end of our experiments, we communicated our results to the suppliers. The GenTegra supplier came to the conclusion that there had been a batch error in the production of the GenTegra tubes. A small test of new GenTegra tubes gave much better real-time PCR results. The GenTegra tubes have previously been successfully used in microarray and real-time PCR applications (20, 21). Dried RNA in GenTegra tubes and dried RNA stored at 25 °C showed similar actin real-time PCR Ct values when assessed after 2 weeks of storage, suggesting that RNA samples stored in GenTegra matrices are in general fit for real-time PCR amplification (18).

Preliminary results from a deliberate addition of skin RNases and longer RT° storage showed that storage of low quality RNA require an absolute RNase-free environment for liquid RNA storage at RT° as RNA degradation rates, indirectly assessed by RIN and real-time PCR quantification, increase rapidly in RNase-contaminated samples. Interestingly, for initially pure RNA stored in liquid state without additives at RT°, intermediate RNA integrity (RIN 4) could be determined after 2 months storage at RT° and real-time PCR data with less than a 1 Cq increase could be obtained even after 3.5 months RT° storage. In our hands, RNAsheII and RNAsTable both performed equally well for pure RNA samples. However, better RIN and real-time PCR results were obtained in RNAsheII when compared to RNAsTable, suggesting that partial protection against degradation of non-pure RNA samples might be conferred by the completely inert environment of RNAsheII. When RT° matrix matrices were compared to “liquid RNA no matrix”, both RNAsTable and RNAsheII provided degradation protection, actually RNAsheII performed as well as the gold standard -80 °C control. These real-time PCR results were in agreement with RIN results. In general, we observed that the RT° RNA stabilization matrices preserve the original quality state of the sample.

In terms of user-friendliness, the RNAsheII encapsulation requires an RNAsheII encapsulation station either installed on site or at the manufacturer’s site. Thus the RNAsheII technology may not avoid initial RNA shipment on dry ice before encapsulation, but offers unambiguously good storage conditions. RNAsTable and GenTegra are both very user-friendly. The GenTegra technology offers the easiest sample handling, as Biomatrix’s RNAsTable technology requires a semi-permeable membrane to be applied on each tube before the sample drying process. In our study, 4 of the 5 preparation centers commented that the membranes were difficult to work with. However, results were consistent, independent of whether the semi-permeable membrane was applied or not. From an environmental point of view, RT° storage solutions are more ecological compared to frozen storage as also underscored by Jensen et al. (1) and samples are not subjected to risk of power failure.

In conclusion, in this study the preservation of high quality RNA was demonstrated for 2-week storage (including shipment) with RT° storage matrices RNAsTable and RNAsheII, and also for liquid and dried RNA without matrix at RT° when the initial RNA samples were highly pure. However,

variability in RIN analytical results was consistently higher when RNA was stored without stabilization matrix. These data suggest that short-term storage in RNAsable and RNAsell is equivalent to RT<sup>o</sup> liquid or dry storage without additive, whereas GenTegra performance was poorer than RNA storage without additives at RT<sup>o</sup>. These observations are important for researchers or biorepository managers who are concerned about the impact that shipment conditions will have on their RNA samples. To further assess the characteristics and cost-effectiveness of RT<sup>o</sup> storage matrices in the long term, it will be necessary to conduct long-term stability studies over several years or even decades. The conclusion from this study is that shipment and short-term storage for up to 2 weeks in certain RT<sup>o</sup> storage matrices, but also storage without stabilization additives, are warranted as fit-for-purpose for RNA recovery, purity, integrity and real-time PCR applications.

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## References

- 1 Jensen G. Room Temperature Biological Sample Storage. 2009.
- 2 Wan E, Akana M, Pons J, et al. Green technologies for room temperature nucleic acid storage. *Current issues in molecular biology*. 2010;**12**(3):135-42.
- 3 Clinical and Laboratory Standards Institute C. Quantitative Molecular Methods for Infectious Diseases; Approved Guideline- 2nd edition- MM06-A2. 2010;**30**(22).
- 4 Hernandez GE, Mondala TS, Head SR. Assessing a novel room-temperature RNA storage medium for compatibility in microarray gene expression analysis. *BioTechniques*. 2009 Aug;**47**(2):667, 70.
- 5 Liu Z, Cohen, L., Martinez ,H., Iverson, B. and Nuñez, R. GenTegra RNA Preserves the Quality and Integrity of Purified RNA During Ambient Temperature Transport.
- 6 Ohgi S, Coulon, L., Muller, R., Muller-Cohn, J., Clement, O. Stabilizing RNA at room temperature in RNAsable. *Biotechniques*. 2010;**48**(6).
- 7 Colotte M, Coudy, D., Tuffet, S., Bonnet, J. Adverse effect of air exposure on the stability of DNA stored at room temperature. *Biopreservation and biobanking*. 2011;**9**(1):47-50.
- 8 Betsou F, Lehmann S, Ashton G, et al. Standard preanalytical coding for biospecimens: defining the sample PREanalytical code. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2010 Apr;**19**(4):1004-11.
- 9 Joint Committee for Guides in Metrology J. Evaluation of measurement data - Guide to the expression of uncertainty in measurement. 2008. p. 1-134.
- 10 Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC molecular biology*. 2006;**7**:3.
- 11 Poloni F, Ashton G, Coppola D, et al. Biorepository Proficiency Testing for the Quality Control of biospecimens for the global biobanking community. *Biopreservation and biobanking*. 2011;**9**(4):415-17.
- 12 Raman T, O'Connor TP, Hackett NR, et al. Quality control in microarray assessment of gene expression in human airway epithelium. *BMC genomics*. 2009;**10**:493.

- 13 Player A, Wang, Y., Rao, M., Kawasaki, E.,. Gene expression analysis of RNA purified from embryonic stem cells and embryoid body-derived cells using a high-throughput microarray platform. Current protocols in stem cell biology (2007) John Wiley and Sons; 2007. p. Unit 1B.2.
- 14 Roberts L, Bowers J, Sensinger K, Lisowski A, Getts R, Anderson MG. Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling. Genomics. 2009 Nov;**94**(5):341-8.
- 15 Dallas A, Vlassov, A.V., Kazakov, S.A. Transesterification and Hydrolytic Cleavage of Nucleic Acids Catalyzed by Metal Ions. In: Zenkova MA, editor. Artificial Nucleases: Springer-Verlag; 2004. p. 61-89.
- 16 Pasloske BL. Ribonuclease Inhibitors. In: Schein CH, editor. Nuclease Methods and Protocols: Humana Press Inc.; 2001. p. 105-12.
- 17 Thorp HH. The importance of being r: greater oxidative stability of RNA compared with DNA. Chemistry & biology. 2000 Feb;**7**(2):R33-6.
- 18 Martinez H, Beaudry, G., Veer, J., Robitaille, M., Houde, M., Wong, D., IversonB. and Nuñez, R. Ambient Temperature Storage of RNA in GenTegra™ for use in a RT-qPCR test for the detection of GCC mRNA. BioTechniques. 2010;**48**(4):328-9.
- 19 Opitz L, Salinas-Riester G, Grade M, et al. Impact of RNA degradation on gene expression profiling. BMC medical genomics. 2010;**3**:36.
- 20 Barnes MG, Tsoras, M., Thompson, S. D., Martinez, H., Iverson, B. and Nuñez, R. Ambient temperature stabilization of purified RNA in GenTegra™ for use in Affymetrix Human Exon 1.0 ST arrays. BioTechniques. 2010;**48**(6):468-9.
- 21 Litterst C, Martinez, H., Iverson, B, Nunez, R. Ambient Temperature Stabilization of RNA derived from Jurkat, HeLa and HUVEC Cell Lines for Use in RT-qPCR Assays.



## Figure legends

Figure 1: Study design. A. Sample flow. Rehydration and testing of 336 samples: total RNA was extracted from blood of 7 human donors and shipped on dry ice to 5 preparation centers: NIH (National Institutes of Health), CCHMC (Cincinnati Children's Hospital Medical Center), IBBL (Integrated BioBank of Luxembourg), UMN (University of Minnesota), NBK (National Biobank of Korea). Dehydration was executed the same day for all preparation centers. RNA was shipped to Imagene where RNAsheils were prepared also the same day. Dried RNA samples were stored and shipped at RT° to 3 testing laboratories: IBBL (lab 1), CCHMC (lab 2) and NIH (lab 3). After exactly 14 days, RNA was rehydrated. Quantity recovery and rRNA integrity were assessed in lab 1, while mRNA quality was analyzed by real-time PCR for genes routinely tested in each testing laboratory. A total of 378 samples were analyzed and data were compiled. B. Experimental design illustrating the comparison of RNA stored in the 6 different storage conditions analysed: GenTegra, RNAsheils, dried RNA no matrix, liquid RNA no matrix and -80 °C.

Figure 2: Total RNA recovery and rRNA integrity after 14-day storage under 6 different RT° storage conditions. A. RNA quantification data expressed relative to initial input RNA. RNA was quantified by spectrophotometry after rehydration and data are shown as mean recovery percentages +/- SD from 5 RNA samples / donor from 7 donors for RNAsheils, GenTegra and "dried RNA no matrix" (n=35). For -80 °C, RNAsheils and "liquid RNA no matrix", data are shown as means +/- SD from 7 donors (n=7). \*: p < 0.05 (Dunn's test compared to -80 °C control condition). B. rRNA integrity as measured by RIN. Data are shown as means +/- SD from 5 RNA samples/donor from 7 donors for conditions -80 °C, RNAsheils, GenTegra, and "dried RNA no matrix". For RNAsheils and "liquid RNA no matrix", data are shown as means +/- SD from 7 donors (n=7). No statistically significant differences in RIN values were detected when compared to the -80 °C control.

Figure 3: Messenger RNA quality assessment by real-time PCR in 6 different storage conditions. A. Laboratory 1: mean Cq values for each storage condition are shown for ACTB (□), GAPDH (□), HMBS (■) and PLAUR (■). B. Laboratory 2: mean Cq values for each storage condition are shown for ACTB (□), GAPDH (■) and IL8 (■). C. Laboratory 3: mean Cq values for each storage condition are shown for ACTB (□) and ORM1 (■). For each laboratory, mean values for the conditions RNAsheils, GenTegra, and "dried RNA no matrix" were calculated from 5 RNA samples/ donor from 7 donors (n=35). For the conditions -80 °C, RNAsheils, and "liquid RNA no matrix" means were obtained from 7 donors (n=7). \*: p < 0.05 (Dunn's test compared to -80 °C).

Figure 4: Time-course study of RNA quality under different RT° storage conditions following contamination with RNase. Pure and RNase-contaminated RNA samples from the same donor were stored under 4 conditions: frozen at -80 °C, RNastable, RNAsheII, or “liquid RNA no matrix”. A. RIN measured immediately after RNase-contamination (T0) (□) or after 1 (□), 2 (■), 3.5 (■) or 12 (■) months storage. N.d.: not done (experiment was not performed), N/A: not applicable, RIN could not be calculated from the electropherogram by the Agilent 2100 Expert software. B. ACTB gene quantification was measured by real-time PCR after 1 (□), 2 (■), 3.5 (■) or 12 (■) months storage. N.d.: not done. C. Graphical representation of RNA degradation process over 1, 2 or 3.5 months as assessed by ACTB real-time PCR quantification. D. ORM1 gene quantification was measured by real-time PCR after 1 (□), 2 (■), 3.5 (■) or 12 (■) months storage. N.d.: not done



Figure 1B

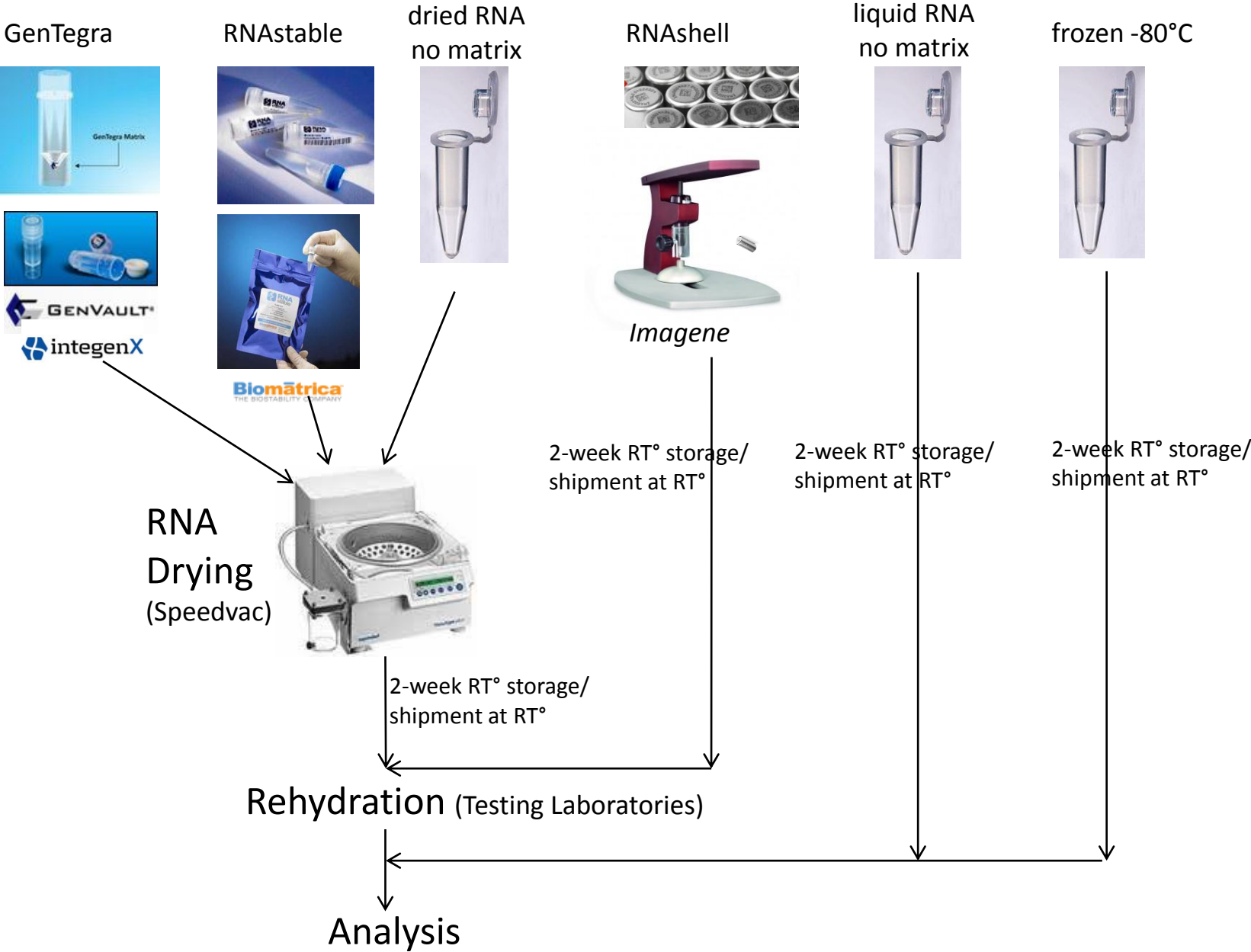
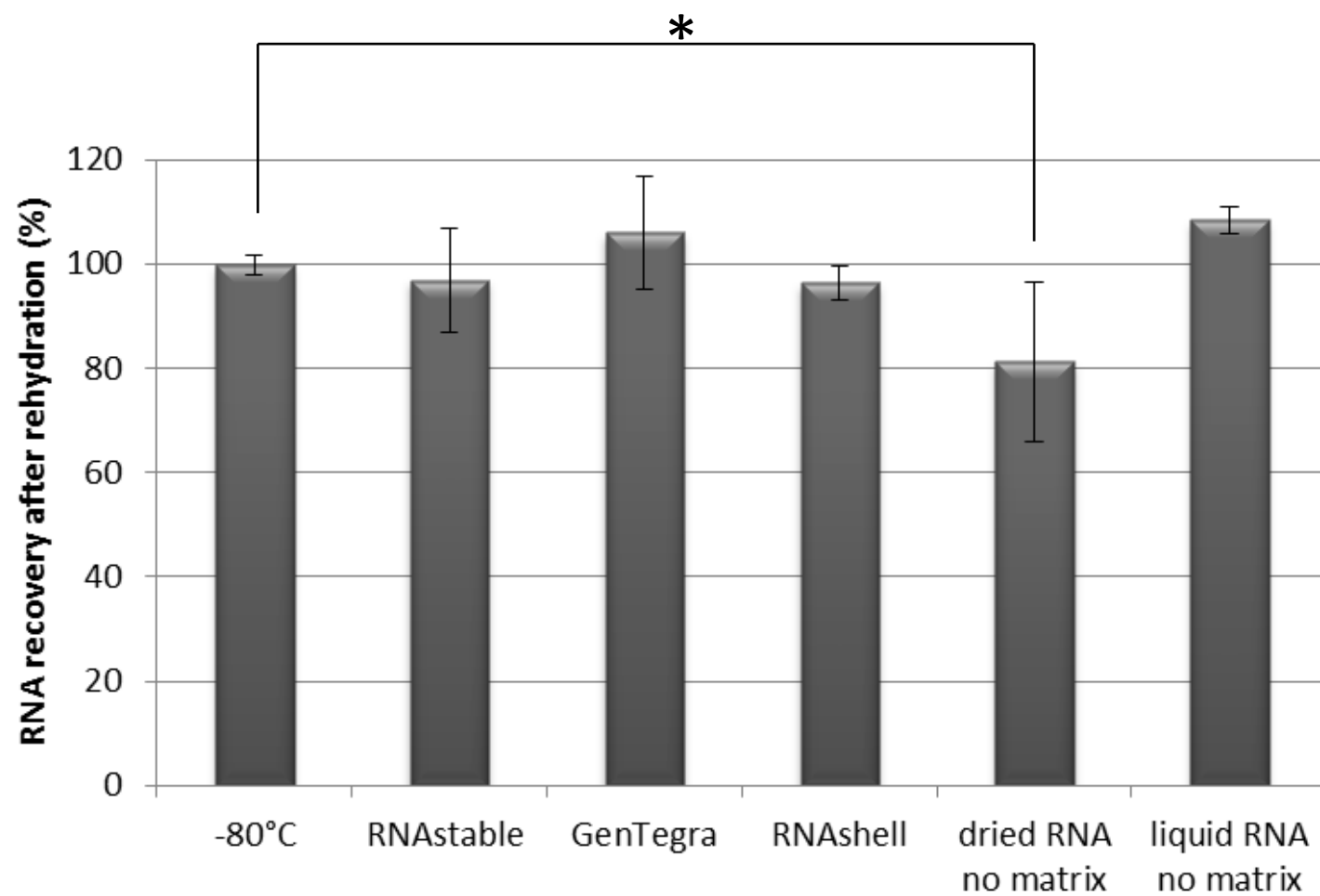


Figure 2A



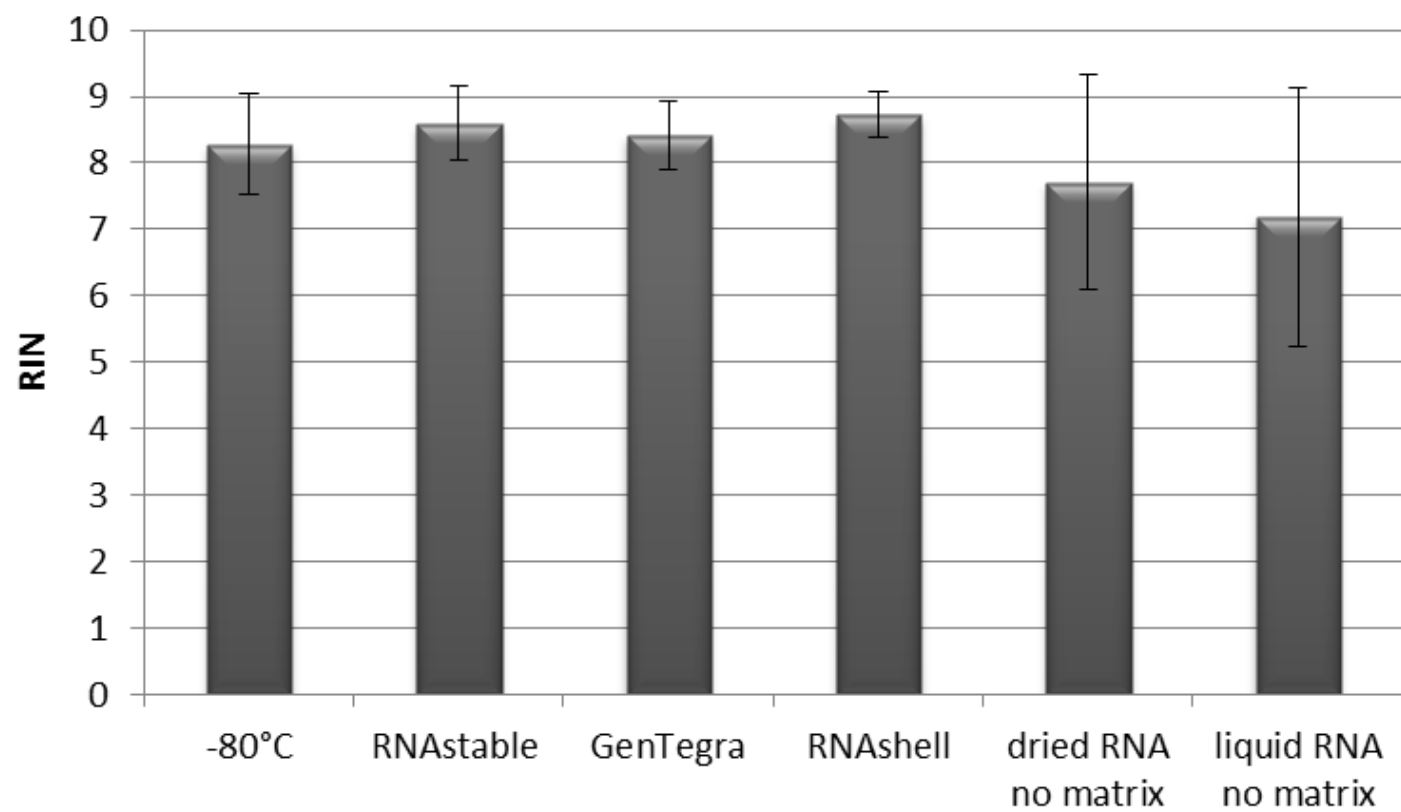


Table 1

Table 1: Summary of RNA quality assessment by real-time PCR in three testing laboratories.

Lab 1	-80°C		RNAstable			GenTegra			RNAshell			dry RNA no matrix			liquid RNA no matrix		
gene	mean Cq	SD	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq
ACTB	18.71	0.77	18.76	0.94	18.76	26.41	3.23	26.41	18.99	0.64	18.99	19.31	0.71	19.31	19.72	1.96	19.72
GAPDH	22.50	0.68	22.61	2.16	22.61	27.93	1.77	27.93	22.63	0.75	22.63	22.77	0.92	22.77	22.70	1.12	22.70
HMBS	31.39	1.48	31.35	1.35	31.35	37.44	2.01	37.44	31.59	1.39	31.59	32.35	1.21	32.35	31.87	1.73	31.87
PLAUR	25.42	0.77	25.72	1.12	25.72	32.03	3.25	32.03	25.54	0.66	25.54	26.16	0.77	26.16	25.89	1.28	25.89
all genes					24.61			30.95			24.69			25.15			25.05
Lab 2	-80°C		RNAstable			GenTegra			RNAshell			dry RNA no matrix			liquid RNA no matrix		
gene	mean Cq	SD	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq
ACTB	15.90	1.14	15.97	0.86	15.97	31.74	7.08	31.74	15.51	1.76	15.51	16.78	0.70	16.78	17.40	1.31	17.40
GAPDH	20.75	0.75	20.32	0.82	20.32	35.08	3.14	35.08	20.71	1.32	20.71	21.48	1.67	21.48	20.69	1.11	20.69
IL-1	23.27	0.67	23.20	0.72	23.20	32.00	3.97	32.00	22.90	0.79	22.90	23.89	0.70	23.89	24.68	1.24	24.68
all genes					19.83			32.94			19.71			20.72			20.92
Lab 3	-80°C		RNAstable			GenTegra			RNAshell			dry RNA no matrix			liquid RNA no matrix		
gene	mean Cq	SD	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq	mean Cq	SD	ΔCq
ACTB	17.46	0.27	17.42	0.36	17.42	28.03	1.45	28.03	17.72	0.27	17.72	17.67	0.32	17.67	18.93	0.62	18.93
ORM1	28.26	0.88	28.16	0.93	28.16	40.15	1.76	40.15	28.67	0.93	28.67	28.83	0.87	28.83	29.90	0.97	29.90
all genes					22.79			34.09			23.20			23.25			24.42

Lab 1 and Lab 2 used SybrGreen technology, Lab 3 used TaqMan assays. For the conditions RNAstable, GenTegra and “dried RNA no matrix” standard deviation (SD) was calculated for each gene on 35 data points (5 samples for 7 donors). For the conditions -80 °C, RNAshell and “liquid RNA no matrix” standard deviation (SD) was calculated for each gene on 7 data points (7 donors, each data point investigated in technical replicates). ΔCq is the difference in Cq between the test condition and the -80 °C condition. ΔCq values from different genes showed low variability within the same storage condition, suggesting that all 6 investigated genes had similar PCR performance and intra-laboratory average quantification for all analyzed genes could be calculated (“all genes ΔCq”).

Figure 3A

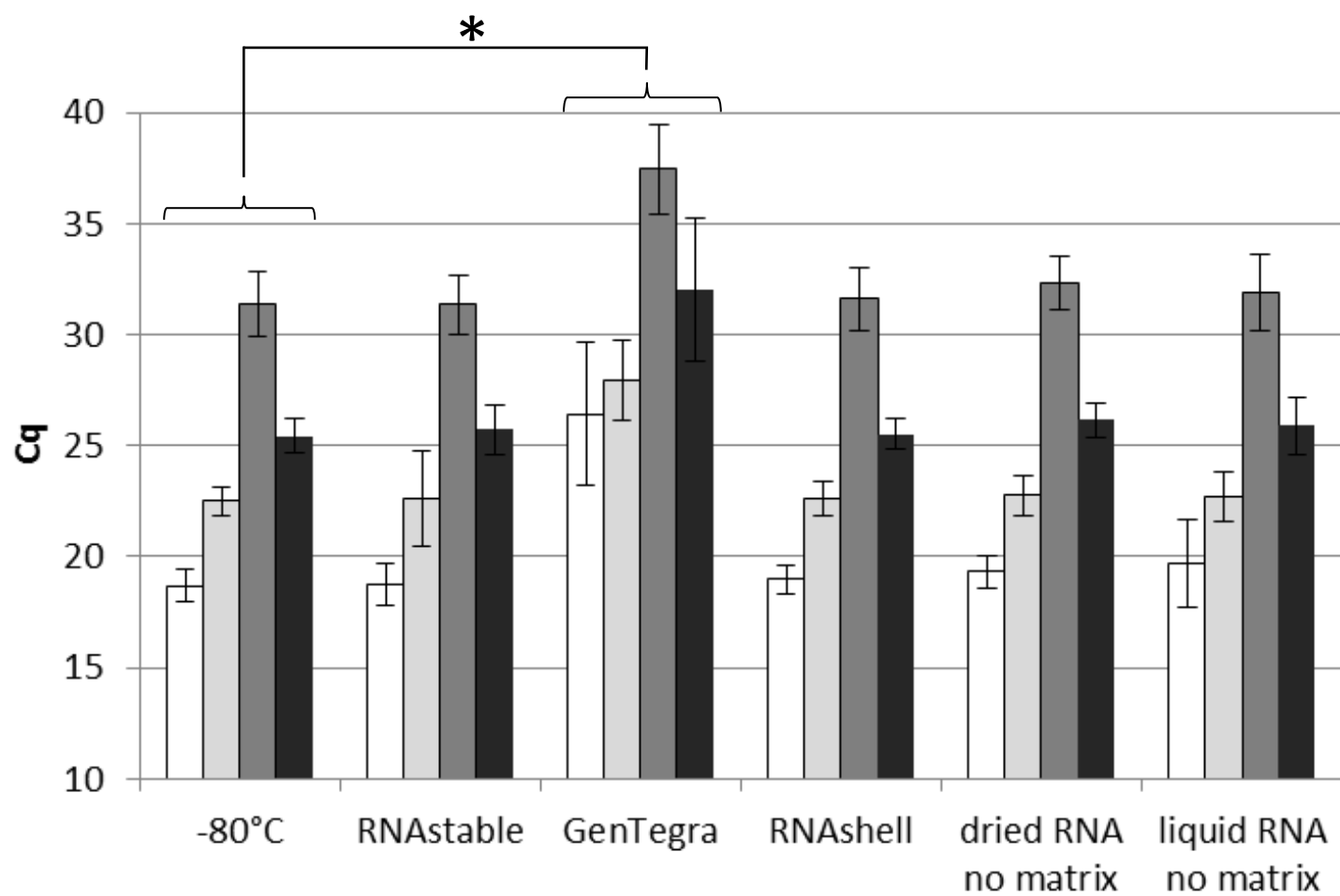




Figure 3B

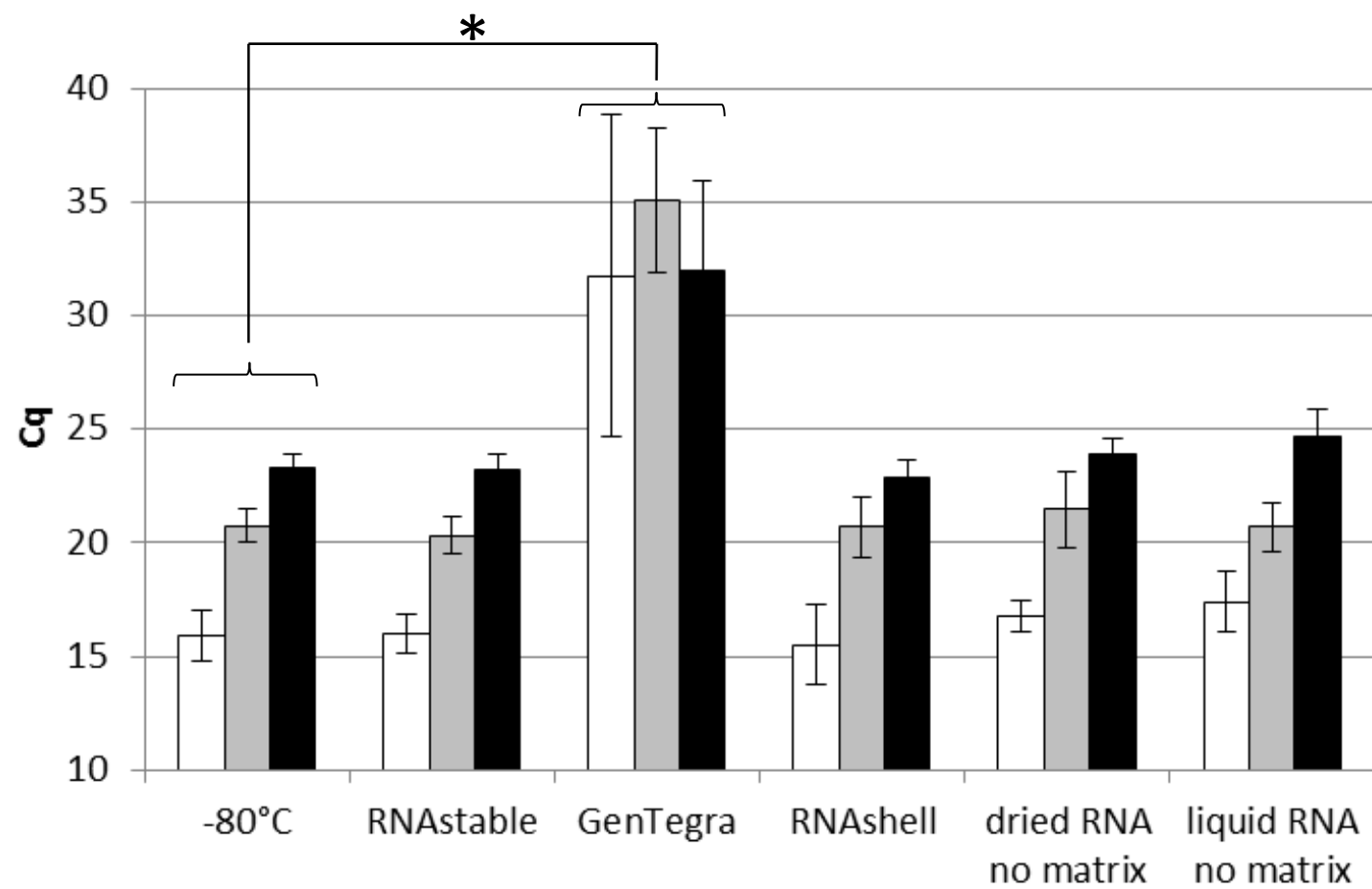


Figure 3C

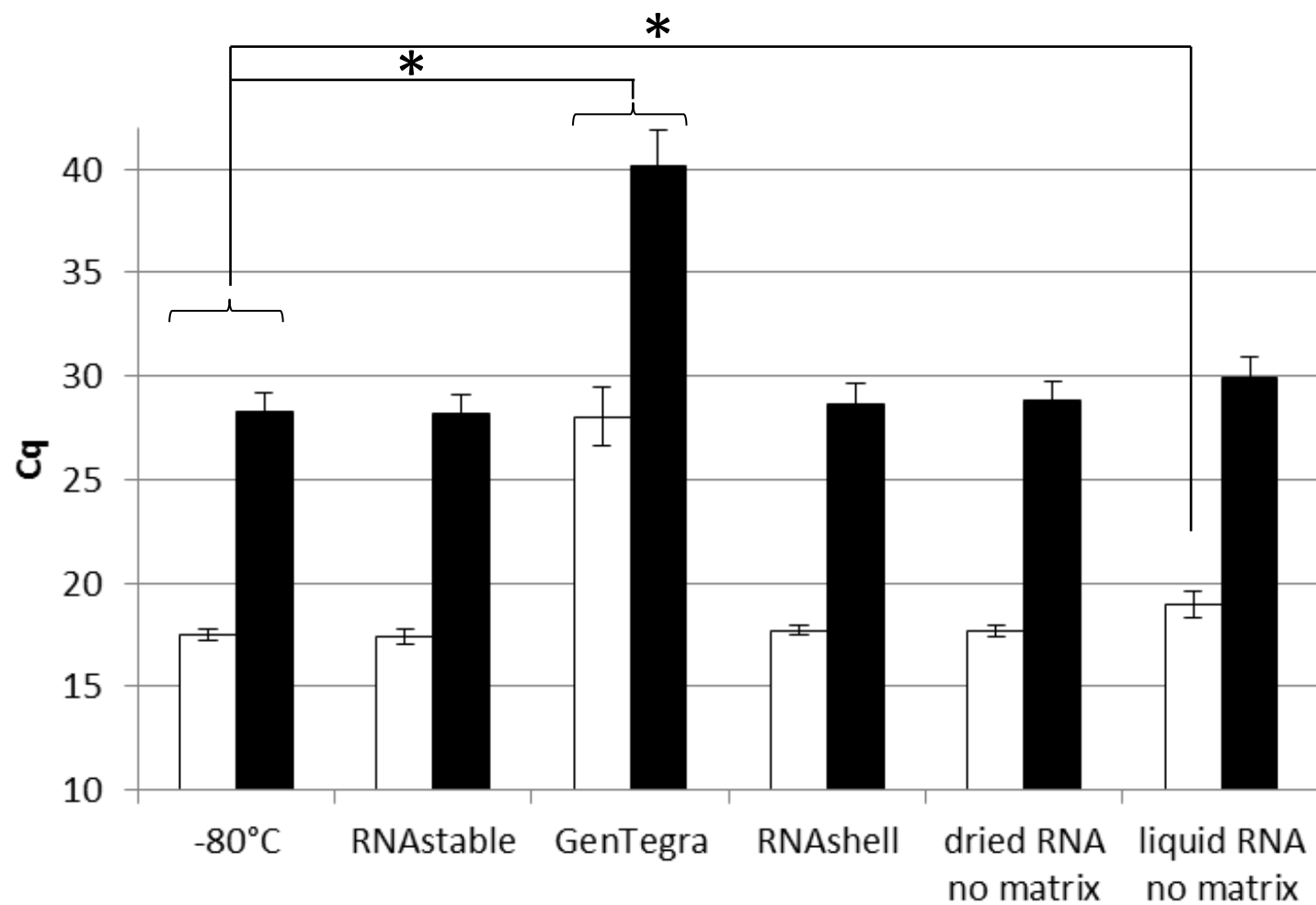


Table 2

Table 2: mRNA quality and inter-laboratory variability assessment by ACTB real-time PCR for RNA stored for 2 weeks in different storage conditions.

	ACTB		
Inter-laboratory	mean Cq	Combined uncertainty (%)	$\Delta$ Cq
<b>-80°C</b>	17.36	1.40	
<b>RNAstable</b>	17.38	1.33	0.03
<b>GenTegra</b>	28.73	7.92	11.37
<b>RNAshell</b>	17.41	1.90	0.05
<b>dried RNA no matrix</b>	17.92	1.04	0.56
<b>liquid RNA no matrix</b>	18.68	2.44	1.32

For the conditions RNAstable, GenTegra and “dried RNA no matrix”, the mean Cq values were calculated from 3x 35 samples (3 laboratories, each laboratory tested 5 samples from 7 donors). For the conditions -80 °C, RNAshell and “liquid RNA no matrix”, the mean Cq values were calculated from 3x 7 samples (3 laboratories tested one sample from 7 donors).  $\Delta$ Cq is the difference in inter-laboratory mean Cq between the condition and the -80 °C condition.

Figure 4A

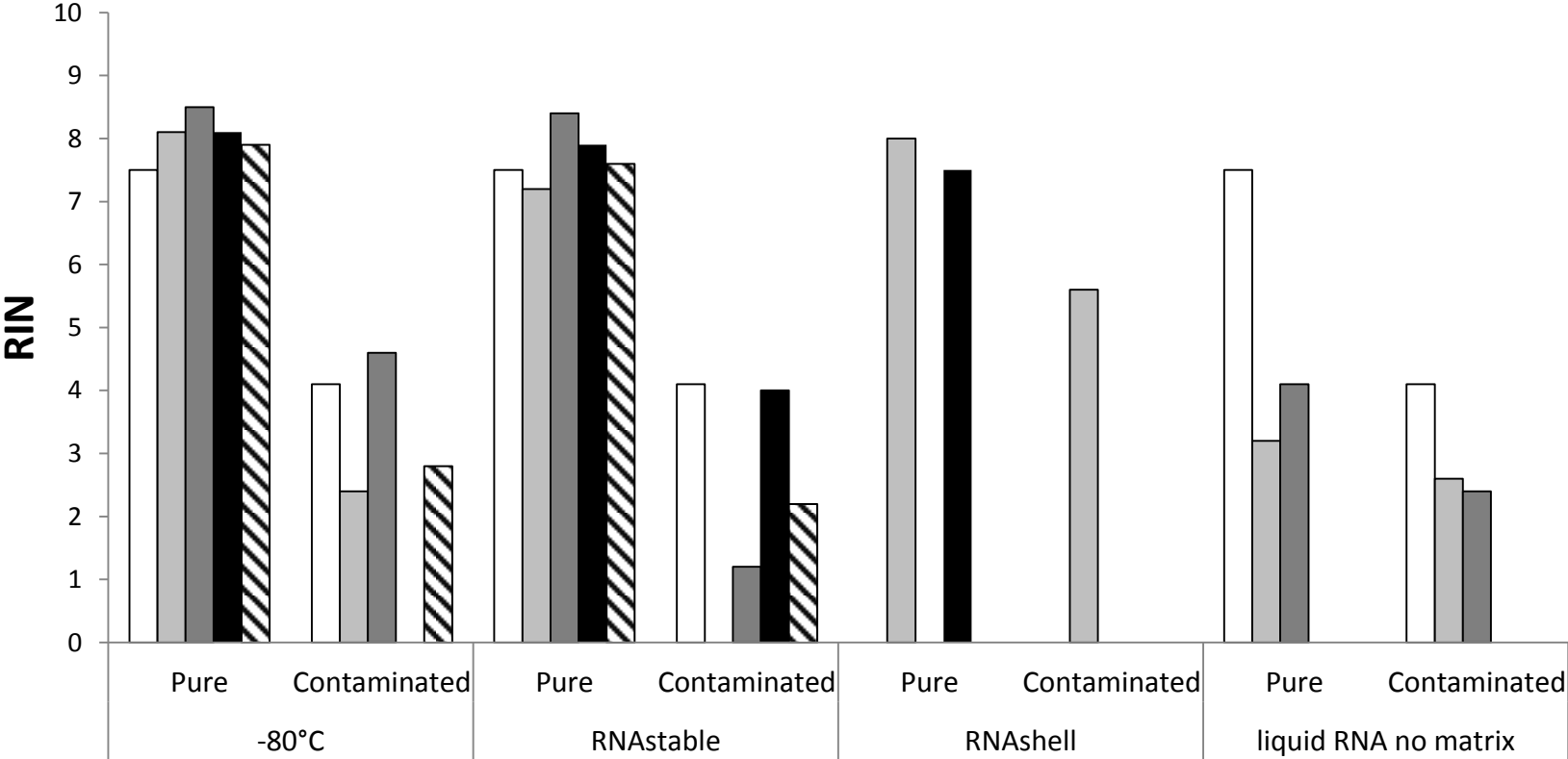


Figure 4B

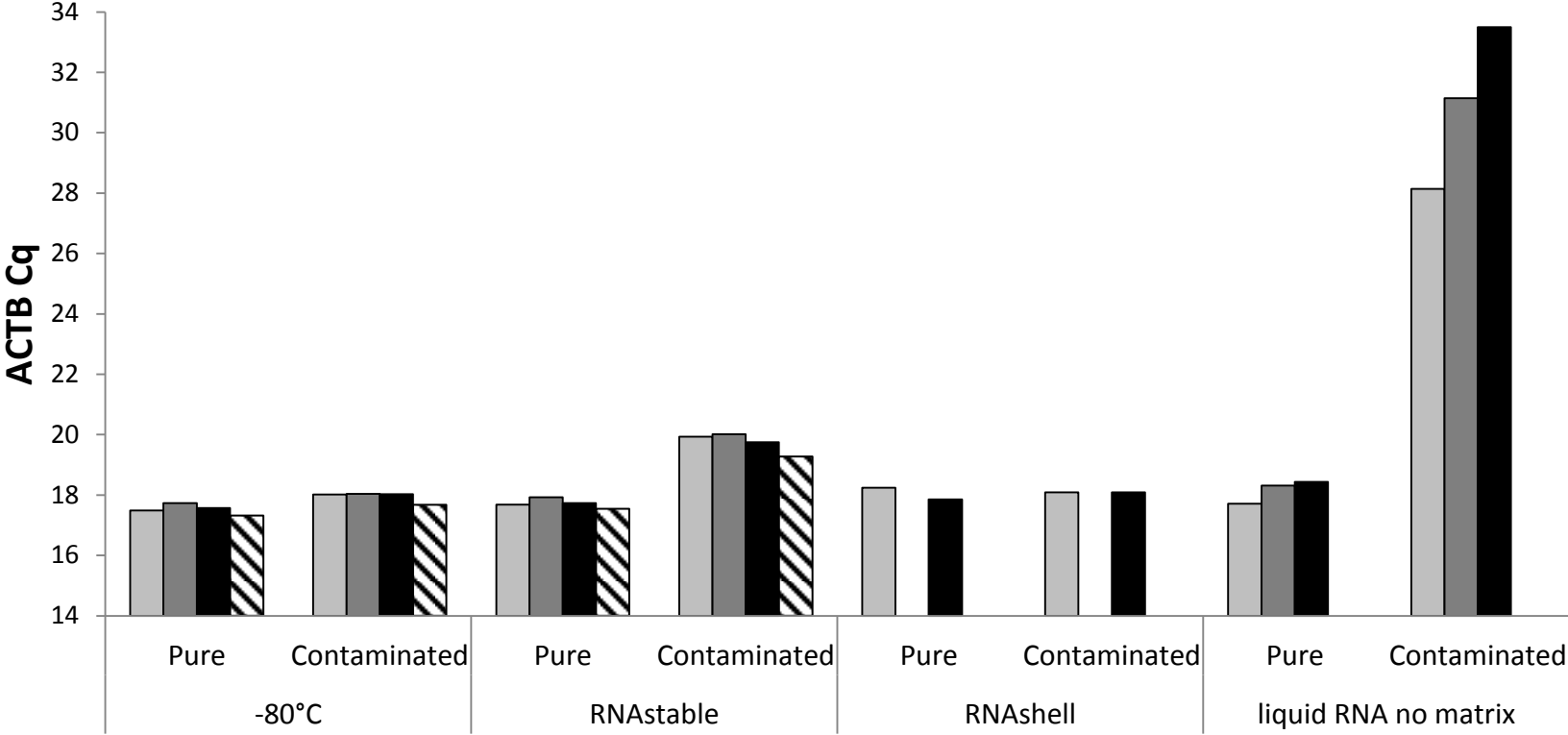


Figure 4C

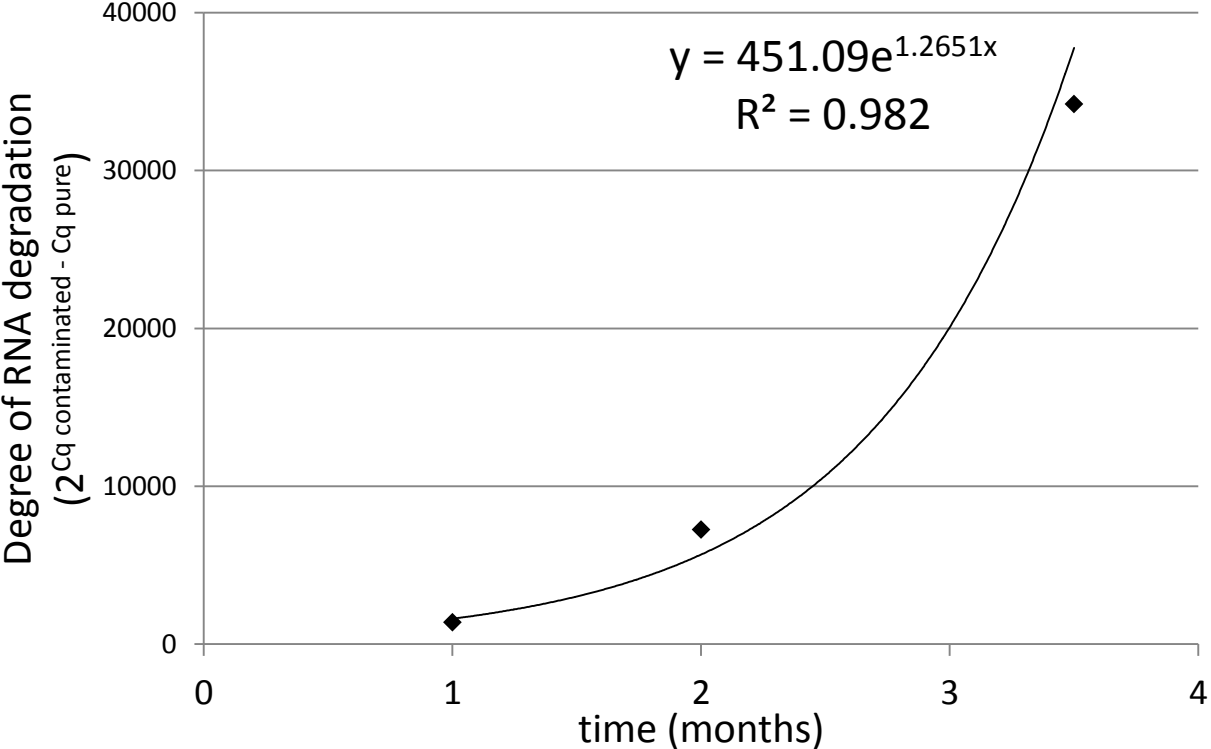


Figure 4D

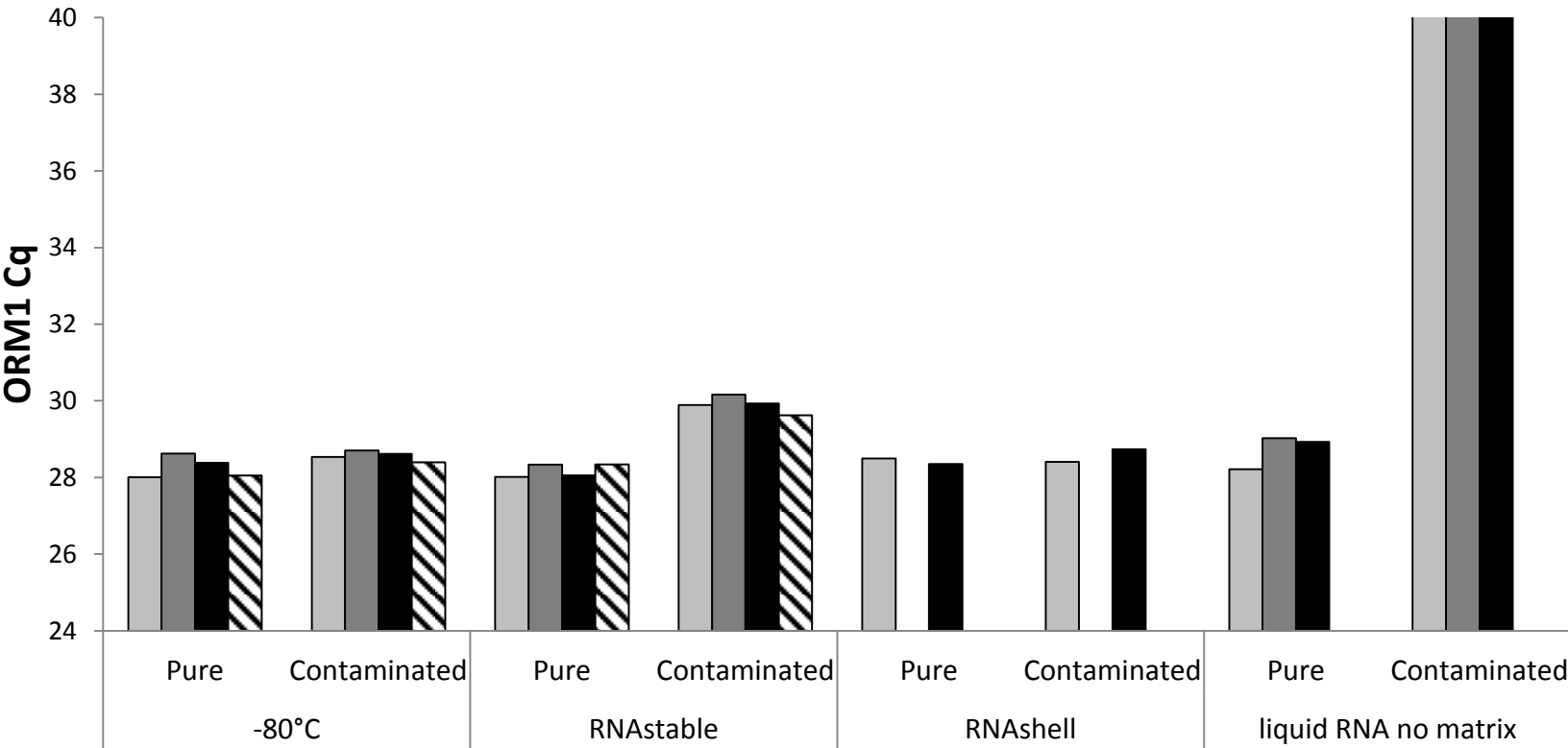


Table 3

Table 3: Impact of RNA quantity input and RNA purification on ACTB and ORM1 real-time PCR results when RNA from one donor was stored at -80 °C, in GenTegra or RNASTable matrices. Und.: undertermined

	<b>Cq ACTB</b>			<b>Cq ORM1</b>		
	-80°C	GenTegra	RNASTable	-80°C	GenTegra	RNASTable
4 ul RNA	17.28	28.90	17.39	27.48	und.	27.75
12 ul RNA	16.07	46.61	16.26	26.04	und.	26.19
12 ul purified RNA	23.63	24.95	24.08	33.89	35.07	34.23



Table 4

Table 4: Real-time PCR Primer sequences		
	<b>Forward (5' -&gt; 3')</b>	<b>Reverse (5' -&gt; 3')</b>
ACTB (Lab1+ Lab2)	ACT GGA ACG GTG AAG GTG AC	AGA GAA GTG GGG TGG CTT TT
ACTB* (Lab3)	Hs030238802_g1	
GAPDH (Lab1+Lab2)	CCA CCA GCC CCA GCA AGA GC	CAA GGT GCG GCT CCC TAG GC
PLAUR	GAC CTC TGC AGG ACC ACG AT	CGA TAG CTC AGG GTC CTG TTG
HMBS	GGC AAT GCG GCT GCA A	GGG TAC CCA CGC GAA TCA C
IL1B	TGT CAT TCG CTC CCA CAT TCT G	TGC TAC TTC TTG CCC CCT TTG
ORM1*	Hs01590791_m1	
*commercial Taqman probes from Applied Biosystems		