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Effect of temperature and time on RNA detection by RT-qPCR in rodent tissue and blood samples stored in MagMAX™ Lysis/Binding Solution Concentrate: Considerations for RNA detection in specimens stored under suboptimal conditions

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Abstract

For detection of viral RNA in blood or tissue, samples are often collected into lysis buffers prior to downstream molecular analysis. Immediate sample processing and cold storage are not always possible during large-scale or field studies, or in facilities lacking a stable electrical supply. Additionally, samples may need to be transported significant distances before processing. Here, using *Peptidylprolyl Isomerase A (Ppia)*, a stably expressed gene in rodent tissues, we investigate the long-term stability and detection of RNA in guinea pig tissues stored for up to 52 weeks and in hamster blood stored for up to 12 weeks in MagMAX Lysis/Binding Solution Concentrate at -80°C, 4°C, 21°C, and 32°C.

Keywords

RNA stability; lysis buffer; storage; temperature

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Report

Under ideal conditions, it is recommended to store samples for RNA analysis either frozen (flash freezing in liquid nitrogen) or in preservation solutions such as RNAlater to maintain sample integrity and prevent degradation [1]. Although cryopreservation remains the gold standard, the use of liquid nitrogen may be impractical or unavailable in certain contexts, such as fieldwork studies or resource-limited settings. RNAlater has been proposed as an alternative, enabling room temperature storage for several days without substantial loss in RNA quality [2], which may be advantageous when collecting tissue samples under non-ideal conditions [3]. However, when handling potentially infectious material, the use of RNAlater may pose a biohazard risk, as studies have demonstrated its capacity to preserve viral infectivity [4–6].

High-consequence viral hemorrhagic fevers (VHFs) are caused by negative-sense RNA viruses that include nairoviruses, arenaviruses, filoviruses, and henipaviruses. Processing and molecular analyses of samples that are known or suspected to contain these viruses for outbreak response, ecological field studies, or research require handling under strict biocontainment and inactivation via validated methods before work can be performed at lower containment [7]. Particularly during outbreak response, these samples may be collected under resource-limited settings, inadequate cold-chain storage and face logistical difficulties in transportation to facilities for analysis. To reduce infection risk and maintain sample quality, it is ideal for samples to be immediately collected into a known inactivant that can also stabilize RNA until samples can be processed by, for example, nucleic acid extraction and subsequent reverse transcription quantitative PCR (RT-qPCR), which can confirm viral presence and determine viral loads (e.g., in pathogenesis and medical countermeasure studies) through the detection of short, specific sections of the viral genome [8–10].

MagMAX Lysis/Binding Solution Concentrate (Cat. No. Invitrogen, AM8500) has been demonstrated to be an effective inactivant for many RNA viruses [11–13]. This lysis buffer contains 55–80% guanidinium thiocyanate (GITC; CAS: 593–84-0), a chaotropic agent that protects RNA from degradation by denaturing RNases [14], and is frequently used for the inactivation of virus in tissue and blood samples for downstream RT-qPCR analysis in research, diagnostics, and ecological investigations [8,15–21]. Here, we evaluated the detection of RNA in rodent tissue stored in MagMAX Lysis/Binding Solution Concentrate at various temperatures and for different lengths of time. We aimed to mimic scenarios that may occur during field studies, outbreaks, or cold-storage failures that could result in delayed processing (i.e., RNA extraction) or suboptimal handling after sample collection, and to determine suitability for medium or long-term storage under these conditions. To perform these studies, we selected *Ppia* as the gene target due to its high conservation across mammals, its stable expression in both naïve and infected rodent tissue [22]; and because the assay targets a short amplicon (126 base pairs) aligning with best practices for RT-qPCR assays which recommend amplicon sizes of 75–150 base pairs [23].

To assess stability of tissue RNA in samples stored in MagMAX Lysis/Binding Solution Concentrate, liver, gonad, kidney, heart, lung, eye, and brain tissues were collected post-

mortem from naïve adult strain 13/N guinea pigs (n = 9; 5 males, 4 females; 879–1345 days old) from the U.S. Centers for Disease Control and Prevention (CDC) in-house colony that were euthanized for humane reasons (e.g., age, injury, or underlying conditions) by isoflurane inhalation followed by intracardiac administration of sodium pentobarbital. Tissue was maintained on ice, homogenized in MagMAX Lysis/Binding Solution Concentrate using 4 mL polycarbonate grinding vial containing pre-cleaned 3/8" 440C stainless steel grinding balls (OPS Diagnostics, Cat. No. PCVS 04–240-03) and stored at either –80°C, 4°C, 21°C, or 32°C for up to one year prior to RNA extraction and RT-qPCR analyses. To ensure sufficient material for all sampling points, whole organs were collected. Organs too large for a single grinding vial were divided and homogenized separately to ensure complete homogenization. Homogenization was performed using the 2000 Geno/Grinder (SPEX SamplePrep) using 1500 strokes/minute for 2 minutes. All tissue samples were brought to a final volume of 10 mL with MagMAX Lysis/Binding Solution Concentrate and centrifuged at 100 × g for 5 minutes to remove any particulate debris. Samples were pre-aliquoted into 300 µL volumes before storage. Post-homogenization sample processing took approximately 4 hours and was conducted at room temperature. To mimic potential storage conditions, homogenates were placed in cold storage (–80°C or 4°C), controlled room temperature (21°C [19.5–22.5°C]), or hot storage (32°C [28–32°C]) to mimic warmer equatorial conditions during field studies or outbreaks.

RNA was extracted using the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Cat. No. 4462359) in a 96-well KingFisher Apex purification system (Thermo Fisher Scientific) from 250 µL tissue homogenate after 0, 1, 2, 4, 8, 12, 36, or 52 weeks of storage. Immediately prior to extraction, 150 µL isopropanol was added to sample homogenate. Eluates were treated with Baseline-zero DNase (Lucigen, Cat. No. DB0715K), eluted in 75 µL MagMAX elution buffer, and stored at –80°C. RNA extracted from week 0 samples were assessed using the Nanodrop 1000 (Thermo Fisher Scientific) for yield and purity (A260/A280). RNA extracted from one heart sample was found to be of insufficient yield and purity at week 0 and was discarded from further analysis.

RT-qPCR set-up was performed using the OT-2 liquid handling system (Opentrons). PCRs were run on the CFX384 Real-Time PCR Detection systems (BioRad) with 10 µL reaction volume containing 2.5 µL template RNA for RT-qPCR using the SuperScript III Platinum One-Step qRT-PCR kit (ThermoFisher Scientific, Cat. No. 11732088). Each RT-qPCR reaction was performed in duplicate. Thermal cycling conditions were as follows: 50°C reverse transcription step for 15 min, 95°C denaturation for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s. RNA assessed using the reference gene, *Peptidylprolyl Isomerase A (Ppia)* [Accession #XM_003465805 (guinea pig) and XM_005086775 (hamster)], which is stably expressed in rodent models of VHFs [22]. Primers were based on the pan-species *Ppia* assay described previously, with probe sequence shifted to remove an indicated hairpin site identified in the previously described sequence. This assay spans multiple exons (based on *Mus musculus* [NM_008907], exon boundaries are not defined for guinea pig or hamster sequences) and targets the majority of splice variants (8/11) [24]. Primers were generated by Integrated DNA Technologies, and primer sequences (5'–3') were as follows: forward (400 µM; exon 1), CCC ACC GTG TTC TTC GAC; reverse (400 µM, exon 3), TCC TTT CTC TCC AGT GCT CAG; and probe (200 µM, exon 1), HEX – CCT TGG GCC –

ZEN – GCG TCT CCT TCG A – IABkFQ. Data was analyzed in Bio-Rad CFX Manager 3.1 and GraphPad Prism v10. Changes in C_t values over time were calculated for each storage condition relative to C_t values on week 0. C_t values greater than 35 were considered negative. Ordinary one-way ANOVA with Tukey multiple comparisons test was conducted using C_t values to evaluate statistical differences between samples stored at -80°C , 4°C , 21°C , or 32°C (Table S1).

We found that cold storage (-80°C and 4°C) was optimal, with minimal change (i.e., $<3.3 C_t$) to tissue RNA C_t values up to 52 weeks post-sample collection. This is supported by previous studies, which showed that storing synthetic and cellular RNA in GITC-containing buffers at -20°C , -80°C , and in liquid nitrogen for up to 18 months did not appreciably reduce RNA stability [25]. Storage at room temperature (21°C) for up to 12 weeks, and at 32°C for up to 4 weeks, demonstrated on average no significant change in C_t value (i.e., $<6.6 C_t$ or ~ 100 -fold loss) (Figure 1A, Table S2), indicating utility of these conditions up to these time points. However, extended storage at high temperatures indicated degradation of tissue RNA, with approximately a 100–1000-fold ($6.6 - 9.9 C_t$ increase) loss on average by 36 weeks at 21°C and 8 weeks at 32°C . High-temperature storage (32°C) was particularly detrimental for RNA detection, with only liver (2/9), gonad (5/9), kidney (3/9), and brain (1/9) returning quantifiable values ($<35 C_t$) at 52 weeks. Heart and lung RNA samples were particularly sensitive; little to no RNA could be detected by RT-qPCR at 36 weeks of storage at 32°C or 52 weeks at 21°C . Significance differences were observed between samples stored at cold temperatures (-80°C and 4°C) and 32°C from as early as 1 week for lung, and 2 weeks for heart (Table S1).

To assess stability of blood RNA in samples stored in MagMAX Lysis/Binding Solution Concentrate, terminal whole blood samples from naïve Syrian hamsters ($n = 10$; 5 males and 5 females; 133 days old) were collected in either lithium heparin (LiH) or potassium EDTA (KEDTA) before adding 1 volume blood to 4 volumes undiluted in MagMAX Lysis/Binding Solution Concentrate. Samples were pre-aliquoted into 300 μL volumes before storage as previously described for tissues: cold storage (-80°C or 4°C), room temperature (21°C [$19.5-22.5^{\circ}\text{C}$]), or hot storage (32°C [$28-32^{\circ}\text{C}$]). RNA was extracted as previously described for tissue from 250 μL whole blood lysate after 0, 1, 2, 3, 4, 8, or 12 weeks of storage and analyzed by RT-qPCR. The detection of *Ppia* in blood samples was more stable than in tissues; irrespective of anti-coagulant used for collection, detection levels remained consistent up to 12 weeks post-storage, even at 32°C (Figure 1B). These differences may be linked to compositional differences between matrices. Metal ions such as magnesium, zinc, iron, and lead have been shown to increase RNA hydrolysis [26,27]. Blood has lower metal ion concentrations than tissue [28], and chelators used in blood collection (e.g., KEDTA and LiH) can bind metal ions, potentially conferring further protection to RNA from hydrolysis [26,29]. Certain tissues (liver and kidney) may also contain higher levels of natural chelators such as glutathione, metallothionein and citrate providing additional protection, which could address differences observed between tissue types [30–32]. In addition, the high lipid content of tissues may interfere with the chemistry of lysis reagents by altering the pH which could accelerate RNA decay [33].

We determined that a short amplicon can be detected across all storage conditions in tissue for up to a year under cold storage, whereas room temperature or higher led to increased C_t values and decreased ability to detect RNA targets after 4 weeks of storage. RNA detection was not affected by temperature and remained stable in blood samples stored up to 12 weeks under all four conditions tested. These findings are based on detection of an amplicon size of 126 bp to mimic the conditions used for molecular detection of pathogens by RT-qPCR. Although no infectious agents were used in these studies, ratios of MagMAX Lysis/Binding Solution Concentrate to sample used herein adhered to or exceeded our current validated procedures for virus inactivation ($\geq 1:10$ for tissue and $\geq 1:4$ for blood) to ensure these data are applicable to infectious virus studies.

While these data support detection of short amplicons, future studies would be warranted to assess RNA quality, data necessary for analyses like next-generation sequencing (NGS) that require high-quality, intact RNAs to provide high-resolution transcriptomics [34], as previous studies have indicated that tissue RNAs extracted via the MagMAX kit may not be suitable for NGS/RNA sequencing studies [35]. Furthermore, our data focuses on a cellular target (not a viral target) and is derived from naïve tissue. We previously reported that *Ppia* was stably expressed in both naïve and infected rodents [22], other gene targets may not exhibit stable expression in rodents. Within living cells, various mechanisms exist to degrade both cellular and viral RNAs, such as nuclease mediated degradation. However, in our study, samples were processed and stored in a guanidinium thiocyanate-based lysis buffer, a strong protein denaturant; thus, enzyme-mediated RNA degradation is unlikely to have played a significant role. Instead, the observed RNA decay is more likely due to instability in chemical structure (e.g., hydrolysis), which is known to be accelerated at increased temperatures [36]. Here, we focus on single stranded messenger RNA; however, RNA stability in viral targets may also vary depending on RNA composition (e.g., double-stranded vs. single-stranded RNA viruses, or segmented vs. non-segmented RNA viruses). Future studies would benefit from including a broader range of targets representing diverse RNA compositions and structures. Finally, we aimed to mimic sub-optimal conditions that might be encountered in low-resource conditions; other factors not assessed herein that could negatively impact RNA stability include temperature fluctuations (e.g., cold-storage failure, day-night cycle), exposure to temperatures $>32^\circ\text{C}$, light exposure, and unregulated humidity levels.

Overall, this report provides data relevant to both laboratory and field studies, supporting the use of cold temperatures (-80°C and 4°C) for long-term storage of rodent tissue samples collected in MagMAX Lysis/Binding Solution Concentrate. Additionally, our findings indicate that under sub-optimal circumstances when cold storage is not feasible, blood and most tissue types – excluding lung and heart, which show earlier levels of degradation – can be stored in MagMAX Lysis/Binding Solution Concentrate at room temperature for up to 12 weeks, and at temperatures up to 32°C for 4 weeks, prior to RNA extraction and RT-qPCR analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability.

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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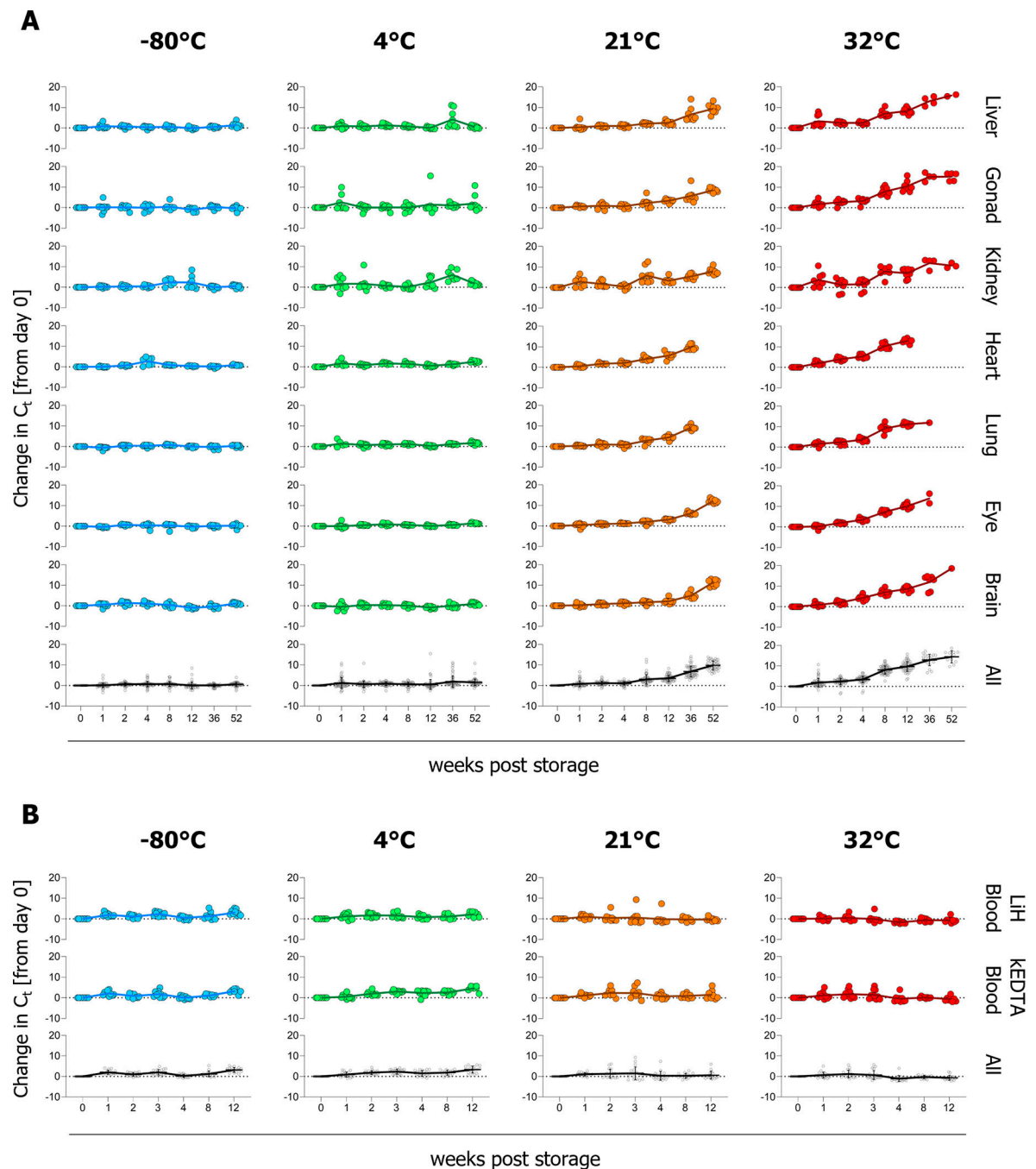


Figure 1. Tissue RNA can be detected in tissue and blood samples after long-term storage in MagMAX Lysis/Binding Solution Concentrate at -80°C , 4°C , 21°C , and 32°C . (A) Tissues (liver [n=9], gonad [testes (n=5) or ovary (n=4)], kidney [n=9], heart [n=8], lung [n=9], eye [n=9], and brain [n=9]) harvested from 9 individual naïve guinea pigs were homogenized into undiluted MagMAX Lysis/Binding Solution Concentrate and stored at -80°C , 4°C , 21°C , or 32°C for indicated periods (B) Whole blood from 10 naïve Syrian hamsters was collected into vacutainers containing lithium heparin (LiH) or potassium EDTA (KEDTA). Blood was added to MagMAX Lysis/Binding Solution Concentrate before

storing at -80°C , 4°C , 21°C , or 32°C For both tissues **(A)** and blood **(B)** RNA was extracted using the MagMAX™ Pathogen RNA/DNA Kit in a 96-well KingFisher Apex purification system at the indicated time points and quantified by RT-qPCR using primers specific for the reference gene *Peptidylprolyl isomerase A*. To determine changes in RNA levels, differences in C_t values relative to week 0 were calculated and graphed using GraphPad Prism v10. Individual animals are represented by circles, where sample-type means are indicated by the solid line. Tissue samples were assessed at all indicated timepoints, data points depicted without values indicate instances where RNA was not detectable. Due to limited terminal blood sample volume, fewer blood samples were analyzed at later time points (LiH blood: $n = 10$ at $\leq$ week 4, $n = 9$ at weeks 8 and 12; KEDTA blood: $n = 10$ at $\leq$ week 3, $n = 9$ at week 4, $n = 8$ at weeks 8 and 12). Graphs are presented by individual tissue type or blood specimen type or with all data for the corresponding time and temperature combined (All), with error bars representing the standard error of the mean.