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Highly degraded RNA can still provide molecular information: An in vitro approach

The long-term survival of RNA in *postmortem* tissues is a tricky topic. Many aged/forensic specimens show, in fact, high rates of null/inconclusive PCR-based results, while reliable outcomes were sometimes achieved from archaeological samples. On the other hand, several data show that the RNA is a molecule that survives even to several physical–chemical stresses. In the present study, a simple protocol, which was already developed for the prolonged hydrolysis of DNA, was applied to a RNA sample extracted from blood. This protocol is based on the heat-mediated (70°C) hydrolysis for up to 36 h using ultrapure water and di-ethyl-pyro-carbonate-water as hydrolysis medium. Measurable levels of depurination were not found even if microfluidic devices showed a progressive pattern of degradation. The reverse transcription/quantitative PCR analysis of two (60 bp long) housekeeping targets (glyceraldehyde-3-phosphate dehydrogenase and porphobilinogen deaminase) showed that the percentage of amplifiable target (%AT) decreased in relation to the duration of the damaging treatment ($r^2 > 0.973$). The comparison of the %AT in the degraded RNA and in the DNA samples that underwent the same damaging treatment showed that the %AT is always higher in RNA, reaching up to three orders of magnitude. Lastly, even the end-point PCR of blood-specific markers gave reliable results, which is in agreement with the body fluid origin of the sample. In conclusion, all the PCR-based results show that RNA maintains the ability to be retro-transcribed in short cDNA fragments even after 36 h of incubation at 70°C in mildly acidic buffers. It is therefore likely that the long-term survival of RNA samples depends mainly on the protection against RNAase attacks rather than on environmental factors (such as humidity and acidity) that are instead of great importance for the stability of DNA. As a final remark, our results suggest that the RNA analysis can be successfully performed even when DNA profiling failed.

Keywords:
Aged forensic samples / ddPCR / Postmortem samples / qPCR / RIN / RNA degradation

1 Introduction

In Forensics, there has been an increase in interest for RNA studies. Out of the several fields of application [1], the tissue-specific gene expression of RNA makes this molecule the ideal tool for determining the cellular origin of unknown samples [2]. In addition, most recently, tissue-specific transcriptional changes were described as a response to the death of the human organism, thus opening the frontiers to a molecular evaluation of the postmortem interval [3].

As the reliability of any PCR-based result depends mainly on the level of RNA degradation, this crucial concern needs to be considered in the evaluation of results from aged/forensic samples [4–7]. It is generally accepted that RNA is more labile than DNA and that RNA degradation occurs quite readily—in the *postmortem* period—in a tissue-specific manner [1]. Although conflicting results were obtained in studying the role of the *postmortem* period on RNA integrity, both endogenous and exogenous RNases are likely the main cause of this early process [8–16]. In addition, even *premortem* factors (such as

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Abbreviations: **DEPC**, di-ethyl-pyro-carbonate; **FS**, filtered samples; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **PBGD**, porphobilinogen deaminase; **qPCR**, quantitative PCR; **rfu**, relative fluorescent units; **RIN**, RNA integrity number; **RS**, retained samples; **RT**, reverse transcription

environmental conditions and circumstances of death) may influence the degree of RNA degradation [14–17].

The intrinsic chemical structure of the RNA molecule (in particular the –OH on the C2 of the sugar) plays a central role in the nonenzymatic degradation of this nucleic acid [18]. This hydroxyl group, in fact, implicates, at neutral pH, a 200 times weaker phosphodiesteric bond in RNA than in DNA [19]. In addition, while acidic conditions promote DNA degradation [20], RNA hydrolysis at pH 4–5 (at 90°C) is minimal [21]. Thus, in conclusion, RNA is more susceptible to the hydrolysis of the phosphodiesteric bonds, while DNA is prone to depurination about 100–1000 times more than RNA (even if this finding is not directly supported by detailed published data) [22]. Also, exogenous factors (such as metal cations, formaldehyde, UV, radiation, and ROS) are involved in RNA nonenzymatic degradation/modification [23].

All this makes the RNA analysis from aged/forensic samples a challenging topic with unpredictable rates of inconclusive or uncertain PCR-based results [24–26]. At the same time, however, since reliable results were achieved even from archaeological samples, it was speculated that the RNA molecule is "more robust than previously considered" [27], thus opening the way to further studies on RNA decay.

The simplest nonenzymatic method to induce degradation of the nucleic acids is the heat incubation in aqueous buffers. This inexpensive procedure was reported in several papers both on RNA (see Supporting Information Table 1 [6, 28–37]) and DNA samples [38]. It is worth mentioning, however, that the wide range of the hydrolytic conditions and the lack of experimental details in some cases do not allow to predict exactly the level of the two nucleic acids degradation even in the supposedly same experimental conditions. A simple protocol for the prolonged aqueous hydrolysis at 70°C of the DNA molecule was recently described [38]. In the present work, the same hydrolytic procedure was applied to a trial RNA sample, and the molecular features of the resulting degraded RNAs were compared with those of the corresponding DNA samples.

2 Materials and methods

The flowchart of the experiment performed in the present study is described in Supporting Information Fig. 1. The details of each step are reported as follows.

2.1 Hydrolysis medium

The degradation rate of RNA can be influenced by the chemical composition of the hydrolysis medium [20]. Therefore, two aqueous media (named medium A and D, respectively) were employed in the present experiment. Medium A was a nuclease-free water purchased from Ambion (USA, cat. no. AM9938). Medium D was 0.1% di-ethyl-pyro-carbonate (DEPC)-water. Medium D was prepared following standard protocols [39] using Millipore water (18.2 MΩ/cm) plus DEPC (Aldrich; cat. no. 159 220). The protocol involved the overnight dissolution of the DEPC and then the solution being autoclaved at 120°C for 20 min. Both media were stored at room temperature until use.

2.2 Trial sample

The trial sample employed in this study was prepared by pooling the RNA samples extracted from the peripheral blood of 42 healthy subjects, who provided informed consent in agreement with the Ethical Committee of the A.U-O of Trieste (Italy). Before RNA extraction, blood samples were stored at room temperature for 1–2 h. RNA was extracted by Trizol method following standard procedure (including DNAaseI treatments). RNA samples were stored at −80°C in 0.1% DEPC-water for about 3 years, until the present study. Samples have been submitted to two cycles of thawing/freezing before the present analyses because two different RNA aliquots were collected for other studies. The resulting RNA sample, named "pooled sample", was of about 1.3 mL and characterized using physical and molecular methods, whose results are described later. Since both the quality and the amount of this sample were considered suitable for the aims of the present study (see below), the sample was split in two aliquots of 600 μL each and precipitated by adding Na–acetate pH 7.4 (at final concentration of 0.3 M) and 2.5 volumes of ethanol at –20°C. After two washes with 70% ethanol, the two samples were redissolved in 3.3 mL of mediums A and D, respectively. The resulting samples were named "sample A" and "sample D". These two samples were then quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific) apparatus and the final concentration was adjusted, in each sample, at 60 ng/μL by adding the corresponding medium. Finally, both "sample A" and "sample D" were tested by the Agilent 2100 Bioanalyzer system. All the recommended procedures to avoid the risk of contamination (use of masks, gloves, overcoats, sterile disposable plastics, etc.) were followed for the preparation of the trial samples, as well as in the course of the subsequent procedures performed on the samples.

2.3 Hydrolysis of the samples

The two RNA samples underwent the same hydrolytic treatments simultaneously. Aliquots of 12 μg in a volume of 200 μL were made from each sample (A and D). These were incubated at 70°C in a thermo-block for increasing intervals of time (from 6-36 h), each one in duplicate experiments. According to the length of incubation (in hours), these samples were named A-6, A-12, A-18, A-24, A-36 and D-6, D-12, D-18, D-24, D-36, respectively. To avoid evaporation of the samples during the hydrolytic treatments, 1.7 mL Eppendorf tubes were sealed with Parafilm (Whatman). The *time 0* controls (A-0 and D-0, respectively) were performed as well.

After incubation, each sample was immediately centrifuged in Ultracel 3 K Amicon Ultra columns (Millipore, MA, USA) for 30 min at 12 000 rpm. These devices allow the

separation of molecules with a weight <3000 Da. The filtered samples (FS) were then recovered and stored at −20°C until micellar electrokinetics chromatography (MEKC) analysis. The retained samples (RS), containing the resulting RNAs, were washed with nuclease-free water (Ambion) and then recovered following the manufacturer's recommendations. Since the employment of these columns can interfere with the accuracy of the NanoDrop assessment [38], all the RS were precipitated by the addition of Na–acetate pH 7.4 (at final concentration of 0.3 M) and 2.5 volumes of ethanol at −70°C, washed twice with 70% ethanol and re-dissolved in 40 μL of nuclease-free water (Ambion). The samples were then split into three tubes and stored at −80°C until further use.

2.4 Analysis of the FS by MEKC

To assess the rate of depurination, the filtrated samples were analyzed by MEKC. This analysis was performed only on samples treated for 24–36 h by using a MDQ (Beckman, USA) apparatus. The analytical conditions of the MEKC system have been described elsewhere [40]. The FS obtained from the separation through Ultracel 3 K Amicon Ultra columns were dried using a Concentrator 5301 (Eppendorf International, Germany) at 60°C, redissolved by adding 12.5 μL of 1% HCl and analyzed in replicate runs. Twelve micrograms of DNA sample "FM" [38] was treated at 70°C for 6 h as positive control of hydrolysis. For calculations, it was assumed that the hydrolysis of 1 μg of RNA provides, in a final volume of 12.5 μL, about 60 μM [40] of each RNA base.

2.5 Spectrophotometric analysis of the RS

One microliter aliquots from each sample were quantified using the NanoDrop spectrophotometer. Absorbances at 260 nm and 280 nm were determined for each of the samples by triplicate measurements.

2.6 Assessment of the RNA integrity number

To assess the RNA Integrity Number (RIN) [41] of the samples, the Agilent 2100 Bioanalyzer was used. A Total RNA Assay was performed following the manufacturer's recommendations on 260–370 ng of each treated sample. For samples A and D, 60 ng were used. Also 250 ng of a control RNA sample (purchased from Ambion) was also included in the analysis.

2.7 cDNA synthesis

Reverse transcription (RT) was carried out in a final volume of 20 μL by using the RETROscript kit (Ambion) following the manufacturer's recommendations. RT was performed, in triplicate, from 270 ng of the "pooled sample." Single RTs were performed on 280 ng of each degraded sample as well as on the *time 0* controls. In addition, to produce a calibration curve, fourfold dilutions (from 360 to 1.4 ng) of the "pooled sample" were retrotranscripted in parallel (these samples were named S1, S2, S3, S4, and S5, respectively). Negative controls (both "no enzyme" and "no template") were carried out.

2.8 Quantitative PCR

This assay was performed on all 24 samples (20 degraded RNA samples and four *time 0* controls) and five calibration samples. Two 60 bp targets located within the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were amplified in a duplex format in this study. PCR primers and TaqMan probes were designed by using http://www.ncbi.nlm.nih.gov/ and the Primer Express software (Vers. 2.0 Applied Biosystems). The sequences of the six probes are reported in Supporting Information Table 2. A CFX96 Real-Time System (Biorad) thermocycler was employed through 40 cycles of PCR at the following conditions: denaturation at 95°C for 20 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. A volume of 1.5 μL of each cDNA was assessed (in triplicate) by quantitative PCR (qPCR) using the Jump Start 2x Ready Mix Taq (Sigma-Aldrich®). Primers concentration is reported in Supporting Information Table 2. All the tests were performed in a final volume of 15 μL in a 96-well plate. Negative controls performed alongside the preparation of the samples were included, as well as negative qPCR controls. The raw data were analyzed by the provided software (CFX Manager ver. 2.0). The percentage of amplifiable targets (%AT) was calculated as reported in [38].

2.9 ddPCR

This assay was performed by probing the 60 bp GAPDH target on a selection of samples. Specifically, each of the three cDNAs from 270 ng of the "pooled sample" was assessed by triplicate tests. Triplicate assays were also performed on the cDNA retrotranscribed from samples S1, S2, S3, S4, and S5 (e.g., the calibrators in the qPCR assay), as well as from samples A-36 and D-36.

PCR reaction mixtures were prepared in a total volume of 20 μL containing 10 μL 2× Supermix for Probes without dUTP (Bio-Rad), forward and reverse primers 900 nmoles/μL each, GAPDH MWG probe 250 nmoles/μL, 1.0 μL of cDNA (or its dilutions), and nuclease-free water. Twenty microliters of PCR reaction mixture were transferred to a sample well in a disposable droplet generator cassette (Bio-Rad) and 70 μL of droplet generation oil (Bio-Rad) was then loaded into the oil well for each channel. The cassettes including eight reaction mixtures were loaded into a QX200 Droplet Generator (Bio-Rad) to create emulsions. Emulsified PCR reactions were transferred to a 96-well PCR plate and PCR was run on a 2720 Thermal Cycler (Applied Biosystem) submitting the following thermal profile: denaturation/hot start at 95°C for 10 min and then 40 cycles of 94°C for 30 s and 59°C for 60 s,

followed by 10 min enzyme deactivation at 98°C. Plates were read on a Bio-Rad QX-200 droplet reader using QuantaSoft v1.7.4.0917 software from Bio-Rad. At least one negative control well without cDNA was included in every run. Each well was then read using the QX200 Droplet Reader (Bio-Rad) and droplets analyzed for emission in the FAM wavelengths. Two analytical thresholds were applied to the raw data to assess the number of positive droplets. The first analytical threshold was fixed at 4000 droplets for each sample while the second one was calculated sample to sample by the software.

2.10 End-point PCR

Three human blood-specific targets (ALAS2, CD93 and HBB), usually employed in forensic genetics for body fluid and tissue identification [2], were used on a selection of cDNAs. Primers sequences and PCR conditions are those reported in [42]. Thirty-three cycles of PCR were performed in a final volume of 12.5 μL by using the Multiplex PCR kit (Qiagen). Negative controls were included. The amplicons were separated by capillary electrophoresis using an ABI 310 sequencer (Applied Biosystems). An analytical threshold of 50 rfu (relative fluorescent units) was used for amplicon call. The following arbitrary scores were used to describe the amplicon height expressed as rfu: –: <50 rfu; +: 51–500 rfu; ++: 501–3000 rfu; +++: >3000 rfu.

2.11 Data analysis

Microsoft Excel 2007 was used for calculations and graphs. For statistical analyses (one-way ANOVA test), significance was assumed with p-values < 0.05.

3 Results and discussion

Since high amounts (at least 500 μg) of RNA were needed to perform the designed hydrolytic procedure, a unique trial sample was prepared by pooling the RNA samples extracted from the peripheral blood of 42 donors. The strategy of pooling different samples together prevents possible issues stemming from both interindividual and sample-to-sample variabilities [4–6, 43]. The resulting "pooled sample" was then split in two and redissolved in two different media, leading to "sample A" and "sample D."

3.1 Assessment of the depurination

The release of the bases was assessed in the RNA samples treated for 24 and 36 h. None of the two purinic moieties was detected by MEKC (LOD: 1.5–2.0 μM; limit of quantification: 6.5–120 μM). Measurable levels of adenine and guanine were observed in the control DNA sample in agreement with our expectation [38, 40]. No peak was found in the negative controls.

All these data together confirm that the release of the purinic moieties from RNA molecules is much lower than in DNA [22]. In particular, the data of the MEKC analysis allowed us to calculate that the release of the two nucleobases was at least 700–900 times lower in RNA than in DNA samples treated under the same experimental conditions [38]. Therefore, depurination, which represents one of the main mechanisms of DNA degradation [44], was negligible in the heat-mediated hydrolysis of the RNA performed in this study.

3.2 Spectrophotometric assessments

The results of this analysis are reported in Table 1. No difference was found in the OD_{260}/OD_{280} ratios of the hydrolyzed samples when compared to the *time 0* controls. Differently, reduced OD_{260}/OD_{280} ratios, related to the degree of depurination, were found in DNA samples treated under the same hydrolytic conditions [38].

3.3 RIN assessment

RIN is a number between 0 and 10 that is calculated by using the area values of the 18S and 28S fractions. The score 10 reflects fully intact RNA, while the score 0 reflects fully degraded RNA [41]. The RIN value has been considered for about a decade a good index of RNA integrity [31] even if recent reports [33, 34] questioned its reliability.

The RIN value of the "pooled sample" was about 5.6, while that of the deriving samples "A" and "D" were 4.7 and 4.6, respectively. It is worth mentioning, however, that all these values are in agreement with the source of the samples [43, 45], as well as their manipulations over time [4–7, 43, 45], and do not interfere with the aim of the present study.

As reported in Table 1, the average RIN values of the hydrolyzed samples ranged from 2.1 to 2.4. No relation with the length of incubation was found for both incubation media ($r^2 \leq 0.2$). Beyond the RIN values found here, however, the visual evaluation of the profiles produced by the Agilent 2100 Bioanalyzer (see Fig. 1) allows the identification of progressive and comparable patterns of degradation for both hydrolysis media.

3.4 qPCR

In this study, two 60 bp short housekeeping targets were chosen for the analysis. Figure 2 shows the averaged Cq of the six measurements of each set of samples. Increments of the Cq, related to the length of incubation ($r^2 > 0.973$), were observed in both targets. In agreement with lower levels of gene expression, the Cq of the PBGD target was always higher than the GAPDH. Interestingly, Cq was always scored even in the samples incubated at 70°C for 36 h. No difference was found between the samples treated in the two different media. Therefore, RNA appears to be resistant to the slight acidic conditions [21] of medium D [46], as opposed to the

Table 1. Sample analyzed in the present study

	Medium A				Medium D			
L.o.H.	OD_{260}/OD_{280}	RIN	GAPDH	PBGD	OD_{260}/OD_{280}	RIN	GAPDH	PBGD
0	1.93 ± 0.02	3.2	265.5 ± 51.5	229.2 ± 15.0	1.94 ± 0.02	3.3	291.6 ± 26.8	257.9 ± 44.8
6	1.94 ± 0.03	2.2	101.6 ± 20.7	83.9 ± 19.5	1.95 ± 0.04	2.1	128.3 ± 22.4	113.6 ± 13.3
12	1.93 ± 0.02	2.1	38.4 ± 6.4	43.6 ± 13.6	1.94 ± 0.02	2.3	46.7 ± 6.8	46.1 ± 13.7
18	1.94 ± 0.02	2.4	15.2 ± 2.4	16.1 ± 4.8	1.94 ± 0.03	2.4	15.0 ± 3.1	12.6 ± 3.4
24	1.95 ± 0.02	2.3	11.1 ± 3.1	13.1 ± 4.7	1.96 ± 0.02	2.3	14.3 ± 2.2	13.1 ± 2.8
36	1.93 ± 0.03	2.3	2.3 ± 0.4	1.4 ± 0.6	1.95 ± 0.04	2.4	2.8 ± 0.3	2.0 ± 1.0

L.o.H.: length of hydrolysis at 70°C; OD_{260}/OD_{280}: average ratio of the absorbance as assessed by NanoDrop. For the "pooled sample," the ratio was 1.93 ± 0.02; RIN: average RIN value from duplicate assessments of each sample by Agilent Analyzer; GAPDH and PBGD: average quantity of sample found in 280 ng of RT template, as assessed by UV absorbance.

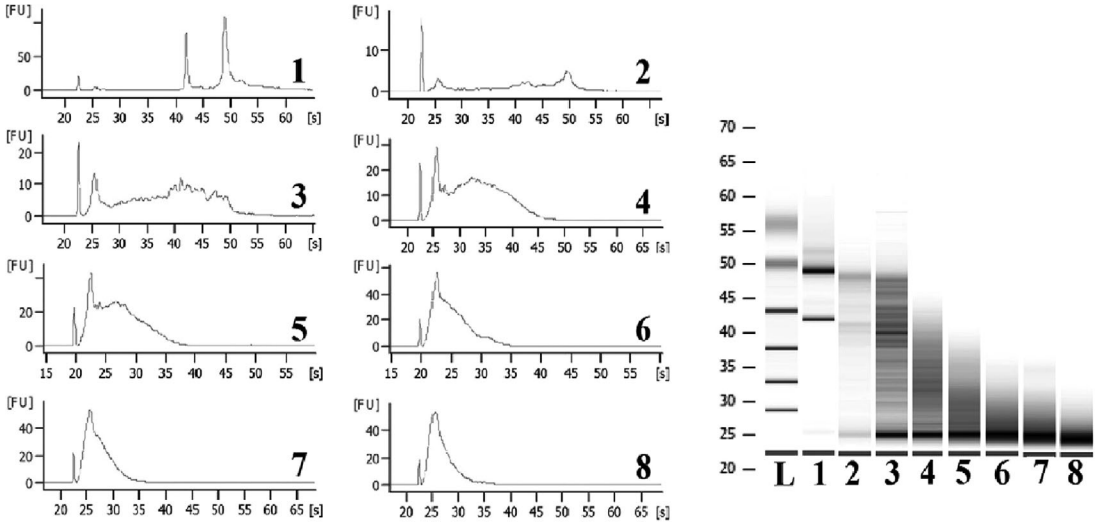

Figure 1. Results of the Agilent 2100 Bioanalyzer assay. 1: control sample from Ambion (RIN = 10); 2: sample A (RIN = 4.7); 3: sample A-0 (RIN = 3.2); samples 4–8: samples treated for 6, 12, 18, 24, and 36 h at 70°C. L: ladder.

DNA molecule [20, 21, 38, 44]. No Cq was found in the negative controls.

Quantification of the two targets was performed by comparison with the calibration data ($y = -3.3x + 28.6$ ($r^2 = 0.982$) for GAPDH; $y = -3.3x + 36.9$ ($r^2 = 0.987$) for PBGD. In particular, Table 1 shows the amount (±standard deviation) of each target found in 280 ng of sample, while Supporting Information Fig. 2 shows the %AT. Interestingly, about 0.5–1% of each of the two targets was still detected in the samples treated for 36 h. This finding marks the main difference with the DNA molecule, as no 62 bp long fragment within the hTERT (human telomerase reverse transcriptase) target was probed in 300 ng of DNA incubated at the same hydrolytic conditions for 36 h [38] (see Section 3.7). Lastly, no relation was found between the RIN of the samples and their %AT ($r^2 = 0.261$).

3.5 ddPCR

Two working sessions were performed by ddPCR [47] to probe the 60 bp long GAPDH target. The employment of this method allowed us to calculate that 1 ng of the "pooled sample" contained approximatively 5822 copies of the GAPDH target (see Supporting Information Table 3). This value falls within the range found in 72 tissues (from 700 copies/ng RNA of breast tissue to 10 000 copies/ng RNA of muscle tissue) [48], but it is lower than those extrapolated from other papers [49, 50]. However, even if the absolute number (of copies) found here could be inaccurate, this is of scarce practical consequence for the aim of the present study (see below).

The analysis of the five calibration samples S1–S5 showed a correlation ($r^2 = 0.989$) between the amount of RNA used for the retro-transcriptions and the number of copies found by the ddPCR. The replicate assay of the most degraded samples showed 49 ± 17 and 46 ± 2 targets/μL in samples 36-A and 36-D, respectively. These values, corresponding to 0.96% and 0.89% of the loaded targets, respectively, are in excellent agreement with the data of the qPCR assay (0.82% and 1.00%, respectively). Less than 0.6 copies/μL were detected, on average, in the negative controls.

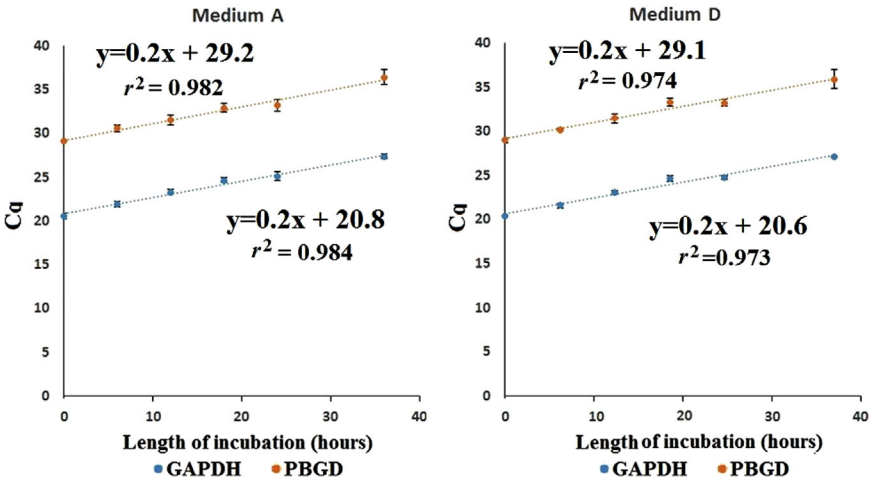

Figure 2. RT/qPCR analysis of the degraded samples. The average Cq value ± standard deviation from $n = 6$ test is plotted for each set of samples. X-axis: length of incubation at 70°C (in hours). *Y*-axis: Cq.

Table 2. Results of the end-point PCR analysis

Sample	Volume	HBB (61 bp)	ALAS2 (103 bp)	CD93 (151 bp)
A-12	0.5 and 2.0	+++	+++	+++
A-18	0.5 and 2.0	+++	+++	+++
A-24	0.5 and 2.0	+++	+++	+++
A-36	0.5	+++	–	–
A-36	2.0	+++	++	–
D-36	0.5	+++	–/+	–
D-36	2.0	+++	++	–
S5	1.0	+++	++/+++	+++
S5	0.25	+++	+/++	+++
S5	0.06	+++	–	++

Sample: list of the samples tested by this method; S5: control sample S5 (RT from 1.4 ng of the "pooled samples" used as the lowest point of the calibration scale); Volume: volume (μL) of the RT reaction used as template for the present test. The molecular weight of each of the targets is provided in the bracket.

3.6 End-point PCR

Table 2 summarizes the results obtained from the selected cDNA samples. The 151 bp long CD93 marker was never found in the most degraded samples A-36 and D-36. These samples, in fact, provided only the amplicons of the HBB and ALAS2 markers (whose molecular weight is 61 and 103 bp, respectively). ALAS2, however, dropped out when less cDNA (0.5 μL) was used as PCR template (see Supporting Information Fig. 3). In agreement with lower levels of degradation, the remaining samples provided all the three blood-specific markers, either by using 7 or 28 ng of RNA as template (e.g., 0.5 and 2.0 μL of cDNA). The positive control provided all three amplicons down to 17.5 pg of RNA (e.g., 0.25 μL of cDNA), while ALAS2 dropped out when using 4.4 pg of RNA (e.g., 0.06 μL of cDNA). No amplicon was scored in the negative controls.

These results show that, when enough template is suitable, blood-specific markers with low molecular weight are still recoverable even from RNA samples incubated at 70°C for 36 h.

3.7 Comparison with DNA samples

The main goal of this study was to investigate the ability of RNA to provide PCR-based results in experimental conditions that were proved not to supply any data for DNA. This is possible by evaluating the percentages of the targets that are still amplifiable in DNA and RNA samples, which underwent the same damaging treatment. In our case, the absolute quantification data provided by the ddPCR assay enables us to calculate that 1 ng of the RNA "pooled sample" contains approximatively 5822 copies of the GAPDH target. On the other hand, by assuming that each human DNA molecule weighs approximatively 6–7 pg [51, 52], it seems correct to calculate that about 286 copies of a haploid genome (each containing a single-copy gene) are required, on average, to give 1 ng of DNA. Thus, as reported in Table 3, the estimated number of the original RNA and DNA targets that underwent qPCR assessments was of the same order of magnitude. Table 3 also shows the %AT found in RNA and DNA samples after the hydrolytic treatments. The %ATs were always higher in RNA samples, reaching three orders of magnitude after 24 h of incubation. About 0.8% of the targets were still amplifiable in RNA samples after 36 h of incubation, while no Cq was found in DNA samples after the same length of treatment. Thus, these results show that RNA has the intrinsic ability to provide PCR-based results much better than DNA after the same prolonged incubation in water at 70°C. However, no general rule can be argued, as the degradation rate also depends on the nucleotide sequence both in RNA [33, 34] and DNA [54].

4 Concluding remarks

The understanding that RNA could be more chemically stable than DNA in specific conditions without RNases effect [21, 55] is not fully established. At the same time, no data exist on the level of PCR-based results that can be recovered from the two nucleic acids submitted to the same damaging procedure. In the present study, a RNA sample from blood

Table 3. Percentage of amplifiable targets in heat-damaged samples

Target	N.A.	6 h	12 h	18 h	24 h	36 h
GAPDH (60 bp)	RNA (122 200)	36 ± 7	14 ± 2	5 ± 1	4 ± 1	0.8 ± 0.1
hTERT (62 bp)	DNA (85 800)	5 ± 2	0.3 ± 0.1	0.04 ± 0.02	≈0.001(*)	–

Target: target probed. In bracket, the length of the qPCR amplicon. N.A.: nucleic acid. In bracket, the number of targets used for each qPCR assay. The data for hTERT (human telomerase reverse transcriptase) are from [38]. -: no Cq. See also Footnote.
For RNA, the number of GADPH targets put into each qPCR assay was calculated as follows: 1.5/20 of the RT (280 ng of RNA) = 0.075 × 280 × 5822 (e.g., number of targets/ng RNA) = 122 262. For DNA, the number of hTERT targets put into each qPCR assay was calculated as follows: 300x286 (e.g., nanograms of DNA as assessed by UV absorbance x number of single gene copies/ng of DNA) = 85 800. (*) Percentage assayed at levels lower than the limit of quantitation but still clearly evidenced.

underwent the same prolonged heat-mediated hydrolytic protocol recently performed on a trial DNA sample [38]. The results of both qPCR and of ddPCR assays showed that RNA maintains the ability to be retro-transcribed in short cDNA fragments after 36 h of incubation at 70°C. Moreover, even the end-point PCR of two blood-specific targets (HBB and ALAS2) provided reliable results, in agreement with the cellular origin of the RNA sample.

Our data are then in agreement with the hypothesis that the long-term survival of the RNA samples depends mainly on the protection against RNAase attacks [2, 4–17, 24–26, 33, 34, 56, 57] rather than on environmental factors (such as humidity, acidity, etc.), which are instead of great importance for DNA stability [44].

In real case-work analysis of aged/forensic samples, it is impossible to have detailed and accurate data on the storage conditions of the sample; in addition, no relation exists between the aging of the sample itself and the level of degradation of the nucleic acids, which can be still recovered from it [1, 27, 58]. However, since our results indicate that the RNA analysis could be successfully performed even in those cases where DNA testing failed, the recovery of both nucleic acids could be of practical usefulness in some circumstances. Thus, for example, a reliable molecular identification of body fluid by the analysis of cell-specific mRNA could be obtained from a specimen that gave no (or very partial) DNA profiles [42]. Moreover, in sexual assault cases it could be possible to identify a vaginal-specific microbiome targeting 16S rRNA [59] when DNA profiling was unsuccessful. It is clear, however, that further studies are needed to confirm the real impact of this promising finding.

In the present work, even the 151 bp long sequences of the CD93 target were achieved from samples treated at 70°C for 24 h by end-point PCR. It is therefore of interest to establish if severely degraded RNA samples, such as those tested here, could be used as a reliable template for a NGS-based approach [60, 61], focusing on forensically relevant transcripts as well. As a future perspective, the desirable development of commercial kits for the determination of body-fluid markers should include RNA samples with known (controlled) degree of degradation. These "degraded control samples" could be then used to standardize the inter- and intralaboratory workflow in proficiency testing and in ISO/IEC validation procedures.

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