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The effect of long-term -80°C storage of thyroid biospecimens on RNA quality and ensuring fitness for purpose

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Abstract

Aims—To establish whether RNA degrades in long-term storage at -80°C and whether RNA integrity numbers (RINs) determine ‘fitness for purpose’ in severely degraded RNA.

Methods—RNA was extracted from 549 thyroid biospecimens stored at -80°C for 0.1–10.9 years then their RINs correlated with storage time. RT-PCR for 65, 265, 534 and 942 base pair amplicons of *hydroxymethylbilane synthase* was used to measure amplicon length in RNA from cryopreserved and FFPE biospecimens that were equally degraded according to RIN.

Results—Storage time did not correlate with RIN. Longer amplicons were obtained from cryopreserved samples than FFPE samples with equal RINs.

Conclusions—RNA does not degrade in thyroid biospecimens stored for long periods of time at -80°C . Although RINs are known to predict amenability to analytical platforms in good quality samples, this prediction is unreliable in severely degraded samples.

INTRODUCTION

Preserving biospecimen quality during long-term storage is of key importance for biobanks. The gold standard for preserving nucleic acid is cryopreservation, with tissue samples being snap frozen as soon after collection as possible and then stored, usually in -80°C freezers or

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Contributors

WM carried out some of the RNA extractions and RIN analysis, did all the size RT-PCR work, compiled all of the data, did the statistical analyses and wrote the manuscript. The remaining authors, with the exception of GAT and FB did the remaining RNA extractions and RIN analyses. FB provided financial assistance for the writing of the manuscript and significant intellectual input into the manuscript. GAT has overall control of the Chernobyl Tissue Bank, had the idea for the paper and provided significant intellectual input into the manuscript.

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using a liquid nitrogen (-196°C) system. A liquid nitrogen facility is considered to be optimal because biospecimens are stored below the 'glass transition point' (T_g) of water (calculated to be -135°C), at which measureable enzymatic activity probably ceases.¹ However, liquid nitrogen storage facilities are impractical in many instances and consequently cryopreserved biospecimens are commonly stored at -80°C , and are therefore potentially slowly degrading because of low-level but long-term enzymatic activity.

The Chernobyl Tissue Bank (CTB) was established in 1998 in response to the increase in thyroid cancers in people who were children at the time of the Chernobyl nuclear accident in 1986 and, due to their geographical proximity to the reactor, were exposed to radioiodine.² The CTB has been active in Ukraine and Russia, amassing a collection of thyroid cancer biospecimens that are a unique resource for researchers interested in radiation-induced cancer. The CTB collection includes formalin fixed paraffin embedded (FFPE) and cryopreserved tissue biospecimens, with the latter stored in -80°C freezers. DNA and RNA are extracted from the cryopreserved biospecimens, then quality assessed before being issued to research projects. For RNA (which is the most easily degraded nucleic acid) the quality assurance procedure in every extraction includes Bioanalyzer nano-electrophoresis analysis, which returns RNA integrity numbers (RINs) that correlate to the extent of RNA degradation in the sample.³ RINs vary from 1 (RNA completely degraded) to 10 (RNA completely intact) and are calculated using an algorithm that takes into account RNA-degradation fragments as well as the relative signal intensities of the 18S and 28S ribosomal subunits.⁴ Correlation between a sample's RIN and its amenability to downstream analytical platforms has been shown.⁵⁻⁸

We have analysed the RIN data from 549 CTB RNA samples to establish whether the RNA has degraded in the tissue samples while they have been stored at -80°C for 11 years. In addition, a further analysis was carried out on a small cohort of samples that had undergone a freeze-thaw cycle due to a freezer failure and were found to be degraded, with very low RINs that were similar to those obtained from FFPE samples. We compared the lengths of the cDNA amplicons that could be obtained from these cryopreserved and FFPE samples with similar low RINs to establish whether they were equally amenable to reverse-transcription polymerase chain reaction (RT-PCR).

METHODS

Tissue samples

Frozen samples of papillary thyroid carcinoma were obtained from the CTB (<http://www.chernobyltissuebank.com>). The CTB is approved to collect, store and issue human biospecimens by Institutional Review Boards of the Institute of Endocrinology and Metabolism, Kiev, Ukraine, the A. Tsyb Medical Radiological Research Centre, Obninsk, Russia and the Imperial College Research Ethics Committee.

Effect of storage time on RIN

Thyroid biospecimens ($n=549$, of which 458 were tumour-normal pairs from the same thyroid) were collected following thyroidectomy in Ukraine and Russia from 1998 to 2009,

snap frozen (freezing rapidly using submersion in dry ice cooled isopentane) in 2 mL polypropylene cryovials then stored in -80°C freezers in their country of collection. Warm ischaemic time (from cessation of blood flow to excision of the tissue) was not recorded but cold ischaemic time (from excision of the tissue to freezing) was <30 min in all instances, during which time the biospecimens were at room temperature. The vast majority of the biospecimens were 5 mm^3 , but any exceeding this threshold were cut to size while remaining frozen (using a scalpel that had been prechilled on dry ice) on a stainless steel tray placed on bed of dry ice. RNA was extracted from the biospecimens in London between November 2007 and August 2009 using the RNEasy Mini kit after they had been homogenised in a TissueLyser (both Qiagen, Hilden, Germany). The optional DNase digest was included in the extraction protocol to prevent DNA from coeluting with the RNA and driving an overestimation of RNA yield.⁹ The purified RNA was quantified by spectrophotometry using a NanoDrop (Thermo Scientific, Wilmington, USA) then its integrity assessed using Nano Chips run on a Bioanalyzer (Agilent Technologies, Santa Clara, USA). The RNA extractions were carried out in batches, each batch consisting of 11 thyroid biospecimens (including tumour–normal pairs where possible) plus one control for the extraction process itself (a cell pellet returning an RIN ≥ 9.5). Storage time was calculated as the number of days between the date of thyroidectomy and the date of RNA extraction. Statistical analyses were carried out using SigmaPlot V.13.

Relationship between RIN and transcript length in degraded samples

An additional cohort of frozen biospecimens ($n=40$) had undergone a freeze–thaw–refreeze cycle and yielded only degraded RNA (mean RIN 2.2 and with one exception, RIN <3). These were excluded from the calculations on the effect of storage time on RIN. However, as such poor RINs are also typical of those obtained from FFPE samples, the relationship between RIN and transcript length in frozen and FFPE samples was assessed. RNA was randomly selected from frozen biospecimens with RINs >8 ($n=10$), RINs 5–7 ($n=16$) and RINs <3 ($n=10$). RNA was also extracted from 12 FFPE samples (also randomly selected) using the RNEasy FFPE kit (Qiagen), all of which subsequently returned RINs <3 . There was no statistically significant difference in RIN between the cryopreserved and FFPE RIN <3 cohorts. RNA ($10\text{ }\mu\text{L}$ but $<2\text{ }\mu\text{g}$) was cDNA converted in a total volume of $20\text{ }\mu\text{L}$ using random hexamer primers and the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Paisley, UK). Then, $2\text{ }\mu\text{L}$ of the cDNA was subjected to 35 cycles of PCR using a combination of primers that together generated amplicons of 65, 265, 534 or 942 base pairs (bp) of the *hydroxymethylbilane synthase* (HMBS) transcript (RefSeq NM_000190.3). The PCR product ($10\text{ }\mu\text{L}$) was then separated by agarose gel electrophoresis, stained with SybrSafe DNA stain (Thermo Fisher) and imaged on a Dyversity system (Syngene, Cambridge, UK).

RESULTS

Effect of storage time on RIN

Storage times varied between 0.1 and 10.9 years. For the 549 samples that had not undergone the freeze–thaw cycle there was no correlation between storage time and RIN (Spearman's rank correlation coefficient= -0.0004), with median RINs for each full year in

storage varying between 7.3 and 8.2 (figure 1). For the biospecimens that had undergone the freeze–thaw cycle (n=40), there was also no effect of storage time on RIN: they were all equally degraded with a mean RIN of 2.2 (SD 0.5) and, with one exception (an RIN of 4.8), all had RIN $\leq$ 2.8. So, all the freeze–thaw samples but only 1% of the unfrozen samples had RINs $\leq$ 5.

RINs from tumour tissue were 0.2 units lower than from normal tissue taken from the same thyroid: median RINs were 7.6 (tumour) compared with 7.8 from normal (n=229 tumour–normal pairs, p=0.004, Wilcoxon signed rank test).

Relationship between RIN and transcript length in degraded samples

Endpoint PCR for four amplicons of 65, 265, 534 and 942 bp of HMBS was performed on cDNA from cryopreserved biospecimens with RIN $\geq$ 5 and 2 cohorts of biospecimens with RINs $\leq$ 3: cryopreserved samples that had undergone the freeze–thaw event and FFPE samples. In cryopreserved samples, amplicons of 942 bp were obtainable whenever the RIN $\geq$ 8.0 and in 87% of the samples with RINs 5–7; amplicons of 534 bp were obtained in all the remaining cryopreserved samples, including the freeze–thaw cases with RIN $\leq$ 3.0. In contrast, no FFPE samples had amplicons as long as 534 bp: maximum amplicon lengths were 265 bp (58% of the samples) and 65 bp in the remaining 42% of samples (figure 2).

DISCUSSION

Using a large sample size (549 biospecimens), we found that thyroid biospecimens did not degrade after 11 years storage at -80°C . This information verifies results from previous studies that used smaller sample sizes and other tissue types to find no deterioration in RNA quality in 51 colon biospecimens after 40 months, 82 pancreatic tumours after 7 years or in 153 endocrine tumours after 27 years (all at -80°C).^{10–12} Thus, we conclude that despite being warmer than the Tg, the RNA content of cellular biospecimens in a -80°C freezer is stable. Only two studies have been published to our knowledge that compare -80°C with liquid nitrogen (-196°C) storage for RNA quality. In the first, storage temperature had no effect in RNA extracted from buffy coat (from four donors) that had been in storage for 13–17 years then analysed using a microarray.¹³ In the second study, biospecimens stored in -80°C freezers were counterintuitively found to be better preserved (with higher RNA yields and RINs) than when stored in vapour phase liquid nitrogen.¹⁴ The authors could not offer an explanation for this, but it is noteworthy that, when quality assessed, the RNA was generally found to be of poor quality and the assay acceptance criteria thresholds for the downstream assays were correspondingly low. So, preanalytical factors relating to sample collection and processing protocols could be significant factors explaining their results. However, regardless of the reasons for the findings of Auer *et al*, we currently see no evidence from the (albeit sparse) literature that liquid nitrogen-based storage systems more effectively preserve RNA than -80°C freezers.

The average RINs of 7.7 from the CTB biospecimens are higher than those found in cryopreserved anaplastic thyroid tumours by Andreasson *et al* (RIN 5.3).¹² It is likely that tumour type and presurgical treatment explain the differences. CTB thyroid cancers are all differentiated, so were collected without presurgical treatment. However, presurgical

neoadjuvant radio and chemotherapy (that would cause necrosis and drive RINs down) is commonplace in anaplastic thyroid cancer in order to shrink the tumours to make them operable.

We are unsure why we found tumour tissue to have a slightly lower RIN than normal tissue taken from the same thyroid. Although statistically significant, the difference was inconsequential in magnitude (0.2 RIN units). This finding was also seen with statistical significance in the Russian and Ukrainian cohorts singularly. A logical suggestion would be that, in general, tumours usually contain more apoptotic cells. However, high growth rates are not generally a feature of differentiated thyroid tumours and the histological FFPE sections from the CTB samples rarely showed apoptotic tissue. The lower tumour RINs may relate to the tissue preparation protocol because the tumour is sliced more and takes longer to sample as larger numbers of FFPE blocks are taken from it. In addition, RNases are more highly expressed in thyroid follicular epithelium and interfollicular tissue than in thyroid colloid tissue, the latter of which is much more prevalent in normal tissue compared with tumour tissue.¹⁵ The opposite result (ie, better quality RNA in tumour compared with normal tissue) has been reported in other tissue types, with differences in morphology (eg, connective or fatty tissue content) and higher resilience to anoxia (and therefore ischaemia during tissue procurement) in tumour tissue being cited as reasons.¹⁰¹⁶

Our comparison between the degraded cryopreserved and FFPE samples demonstrated that although having comparably poor RINs, the cryopreserved samples were more amenable to RT-PCR than the FFPE samples. It is possible that residual formaldehyde adducts covalently bound to RNA extracted from FFPE sections inhibited the reverse transcription reaction, thus making FFPE-RNA less amenable to RT-PCR than cryopreserved RNA independently of transcript length (and therefore RIN). However, we do not think this is the sole explanation here, because RT-PCR amplicon lengths in the FFPE samples were not improved when a heating step previously shown to improve RT efficiency in FFPE samples was carried out (data not shown).¹⁷ An alternative explanation is that at the low end of the RIN scale the correlation between RIN and mRNA quality is unreliable. This is feasible because the RIN algorithm significantly uses the relative degradative states of the 18S and 28S rRNA peaks, neither of which are present at such an advanced state of RNA degradation. However, regardless of the reasons, the important point is that for suboptimal biospecimens yielding highly degraded RNA, a sample's RIN is not a reliable measure of its amenability to RT-PCR (and presumably other applications as well), and therefore, 'fitness for purpose' decisions should not be taken using RIN alone. Although RINs have been shown to correlate to sample amenability in several analytical platforms, these evaluations necessarily included samples that were of good quality, rather than focusing only on samples with very low RINs as we have done here.⁵⁶⁸

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Take home messages

- ▶ RNA in frozen thyroid biospecimens is stable for at least 11 years at -80°C .
- ▶ Frozen biospecimens deemed to be extremely degraded using RNA integrity numbers (RINs) are still fit for purpose for RT-PCR.
- ▶ Degraded frozen and FFPE samples with equally low RINs are not equally amenable to RT-PCR.
- ▶ The use of RIN alone to determine a degraded sample's 'fitness for purpose' is questionable.

Figure 1. RNA quality (RIN) does not correlate with time at -80°C storage. The boxes represent the first and third quartile intersected by the median and the whiskers are the range. The numbers above each year's data denote the number of samples. RIN, RNA integrity number.

Figure 2.

Relationship between RNA integrity number (RIN) and cDNA amplicon length. Amplicons of 942 bp were obtainable in all cryopreserved samples with RIN >8.0 and 87% of samples with RIN 5–7. Amplicons of 534 bp were obtainable in all remaining cryopreserved samples including those with RIN <3.0. In contrast, no 534 bp amplicons were obtained from FFPE samples although their RINs were also <3; maximum amplicon length in FFPE samples was 265 bp (58% of samples) with only 65 bp amplicons obtainable from the remaining 42% samples.