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Research paper

qPCR based mRNA quality score show intact mRNA after heat stabilization

Oskar Karlsson[a], Lova Segerström[b], Robert Sjöback[c], Ingrid Nylander[b], Mats Borén[d,*]

[a] Department of Clinical Neuroscience, Karolinska Institutet, The Center for Molecular Medicine, Stockholm, Sweden
[b] Department of Pharmaceutical Biosciences/Pharmacology/Neuropharmacology, Addiction & Behavior, Uppsala University, Uppsala, Sweden
[c] TATAA Biocenter, Göteborg, Sweden
[d] Denator AB, Uppsala, Sweden

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ABSTRACT

Analysis of multiple analytes from biological samples can be challenging as different analytes require different preservation measures. Heat induced enzymatic inactivation is an efficient way to preserve proteins and their modifications in biological samples but RNA quality, as measured by RIN value, has been a concern in such samples. Here, we investigate the effect of heat stabilization compared with standard snap freezing on RNA quality using two RNA extraction protocols, QiaZol with and without urea pre-solubilization, and two RNA quality measurements: RIN value, as defined by the Agilent Bioanalyzer, and an alternative qPCR based method. DNA extraction from heat stabilized brain samples was also examined. The snap frozen samples had RIN values about 1 unit higher than heat stabilized samples for the direct QiaZol extraction but equal with stabilized samples using urea pre-solubilization. qPCR based RNA quality measurement showed no difference in quality between snap frozen and heat inactivated samples. The probable explanation for this discrepancy is that the RIN value is an indirect measure based on rRNA, while the qPCR score is based on actual measurement of mRNA quality. The DNA yield from heat stabilized brain tissue samples was significantly increased, compared to the snap frozen tissue, without any effects on purity or quality. Hence, heat stabilization of tissues opens up the possibility for a two step preservation protocol, where proteins and their modifications can be preserved in the first heat based step, while in a second step, using standard RNA preservation strategies, mRNA be preserved. This collection strategy will enable biobanking of samples where the ultimate analysis is not determined without loss of sample quality.

1. Introduction

The removal of a biological sample from its *in vivo* environment initiates a series of signaling cascades as the cells in the sample adapt to their new environment [14]. Analyte levels, *e.g.,* peptides, protein phosphorylation, lipids and metabolites, will start to change from their actual *in vivo* levels, distorting analysis results and making interpretation of analytical results difficult or even impossible [5,15]. A range of preservation techniques are used to deal with post-sampling change but they are usually optimized to preserve a specific analyte or class of analytes while not preserving other analytes. This approach is generally satisfactory as long as only analytes from a single analyte class are analysed. However, analysis of multiple analytes from precious biological samples can be challenging as different analytes require different preservation measures. Analysis of both nucleic acids, *e.g.,* RNA and DNA, as well as protein phosphorylation from a single sample is an example of such a situation. This is particularly challenging when selecting collection strategies for biobanking sample where the ultimate analysis is not known at the time of sampling. Classical RNA preservation strategies, *e.g.,* RNA Later, while preserving RNA quality do not prevent the more rapid changes to protein phosphorylation that occur post-sampling. Heat induced enzymatic inactivation on the other hand have been shown to be an efficient way to preserve protein phosphorylation but RNA quality has been a concern in such samples [7,12]. RNA quality as measured using electrophoretic analysis of RNA, *e.g.,* RIN value using an Agilent Bioanalyzer assay, has indicated a reduction in quality in samples after heat denaturation and a further reduction in RIN value when heat stabilized samples are incubated at room temperature indicating that RNases

* Corresponding author at: Denator, Dag Hammarskjöldsv. 10A, SE-751 83 Uppsala, Sweden. Fax: +46 18 50 81 00.
E-mail addresses: mats@denator.com, matsboren@hotmail.com (M. Borén).

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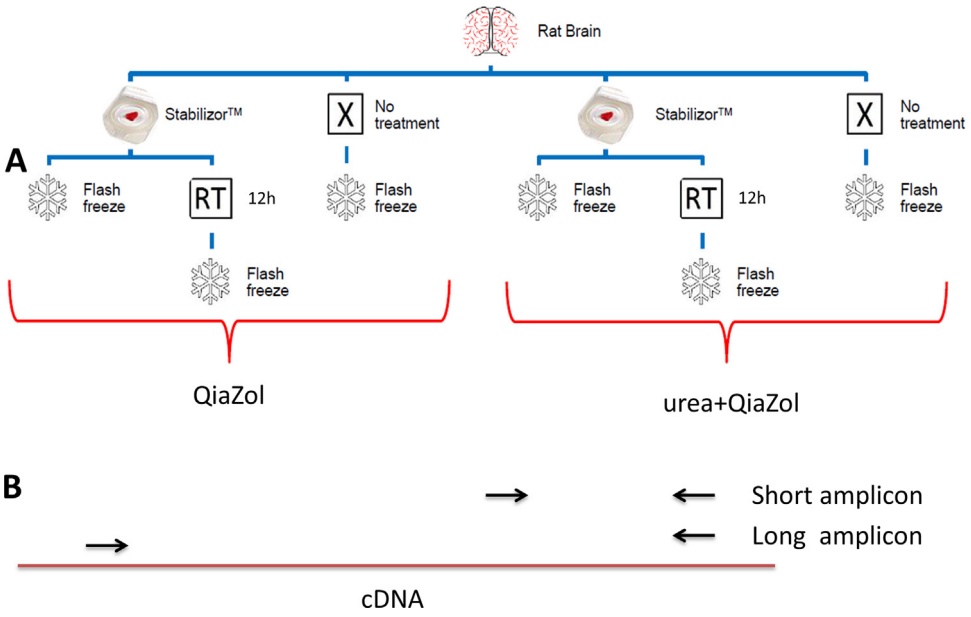

Fig. 1. Panel A: experimental layout. Sections of rat brain were either snap frozen or heat stabilized using the stabilizor T1. The heat stabilized samples were then either directly snap frozen or further incubated for 12 h at room temperature to determine stabilizing effect on RNA by heat stabilization. The samples were collected in duplicates to enable comparative extraction with two extraction protocols, either directly with QiaZol or with a pre-solubilization step with 8 M urea prior to QiaZol extraction. Each sample group consisted of four biological replicates, $N = 4$. Panel B: schematics of qPCR based RNA quality scoring. Two overlapping qPCR reactions, one short and one long, is amplified from the same cDNA. The difference in Ct between the long and the short will depend on cDNA fragmentation and becomes an indicator of RNA quality.

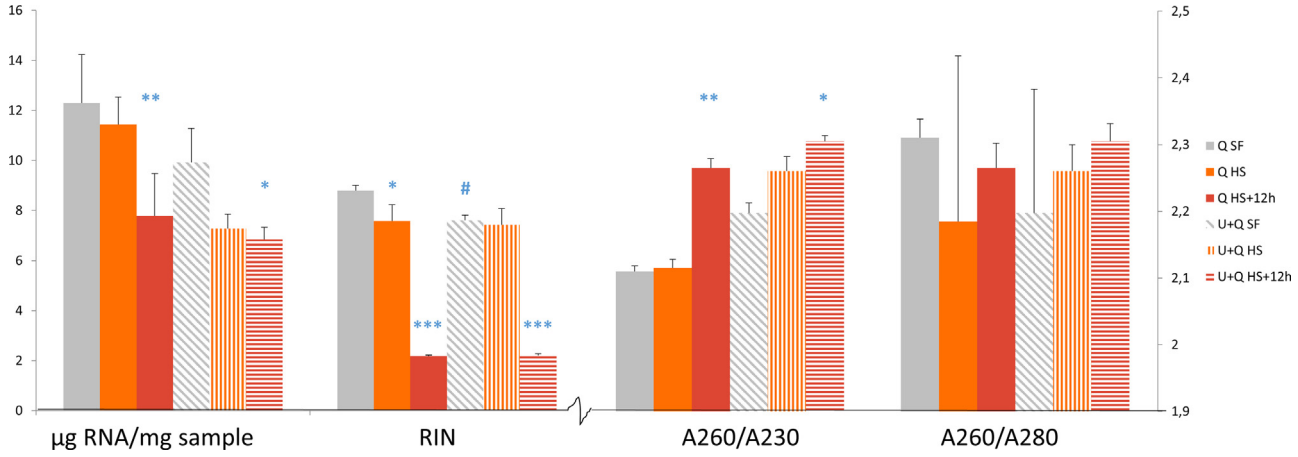

Fig. 2. Yield, quality and purity of extracted RNA (mean ± SD). Three different treatment groups, $n = 4$, (SF-snap frozen, HS-heat stabilized, HS + 12 h-heat stabilized with 12 h incubation at room temperature after heat stabilization), extracted with two different protocols (Q-QiaZol buffer and U + Q-Urea pre-solubilization prior to QiaZol extraction) were compared. µg RNA/mg tissue-yield of RNA from samples (left scale), RIN-RNA quality score as measured using the bioanalyzer (left scale), A260/A280-assesment of protein contamination (right scale), A260/A230-assesment of contaminants such as guanidine thiocynate and carbohydrates (right scale). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with snap frozen samples (SF) within respective extraction protocol, $^{\#}p < 0.05$, compared to corresponding group in non-urea pre-solubilized samples (ANOVA followed by Tukey HSD test; $n = 4$).

remain active after heat denaturation [12]. Heat stabilization can thus not be used alone as a way to preserve RNA but enable extraction of RNA as a secondary analyte from samples where proteins are the primary analyte of interest. The RIN value is primarily based on rRNA rather than mRNA, which is the RNA type typically analyzed, and give only a surrogate quality score for mRNA [13]. The ideal RNA quality score would rather be based on direct quantification of mRNA. Various approaches for using qPCR to measure mRNA quality have been proposed, *e.g.*, the use of a ratio Cq from 2 shorter qPCR assays [10] or comparing delta Cq of multiple 3′ amplicons of a single assay [16]. Another qPCR based mRNA quality score has been developed by TATAA Biocenter [1,6]. Their qPCR based RNA quality score measure the difference in amplification rate between a long and a short qPCR amplicon of a number of mRNA transcripts, selected based on their post-sampling degradation characteristics.

The aim of the present study was to investigate the effect of heat based sample stabilization through protein denaturation done using the Stabilizor system (Denator, Sweden) on RNA quality using Bioanalyzer (RIN value) and TATAAs RT-qPCR (mRNA based) quality scoring. If mRNA can be shown not to be degraded by heat stabilization in the Stabilizor system it would enable analysis of the primary analyte, *e.g.*, protein phosphorylation, as well as mRNA as a secondary analyte both with high quality. DNA extraction from heat stabilized brain samples was also examined to investigate the possibility to also analyze DNA from heat stabilized samples.