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Reliability of RT-qPCR from degraded RNA samples: An *in vitro* model

R. Palladino[a], S. Sorçaburo Cigliero[a], S. Bonin[a], P. Pitacco[a], V. Nicolin[a], C. Previderè[b], P. Vatta[c], G. Marrubini[d], P. Fattorini[a,*]

[a] Department of Medicine, Surgery and Health, University of Trieste, Italy
[b] Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Italy
[c] Neurogenomics Lab, Neurosciences Area, SISSA, Trieste, Italy
[d] Department of Drug Sciences, University of Pavia, Italy

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ABSTRACT

A rapid and reliable protocol was developed to produce chemical degradation of the RNA *in vitro*. Cell line MDA-MB-453 was used as source of RNA and hydrolysis in water at 70 °C was performed. The outcome was monitored by RT-qPCR of two housekeeping targets and a significant correlation between the Ct and the extent of molecular damage of the template was found. A molecular degradation index of the RNA is proposed.

1. Introduction

RNA is an instable molecule and suboptimal storage conditions promote its degradation [1,2]. Therefore, accurate assessment of its integrity is required for accurate gene expression studies [3]. The analysis of mRNA for the identification of the source of body fluids is a challenging task in Forensic Genetics. To this aim, after RNA extraction from the stain, the cDNA obtained from the reverse transcription (RT) is analyzed by either end point PCR or quantitative PCR (qPCR) [4–6]. Surprisingly, reliable RNA profiles were achieved from years-old stains by the employment of these approaches while even transcriptomic analysis was successfully performed from swabs stored sex weeks at room temperature [7]. No data exists, however, on the level of RNA degradation that still allows a reliable RNA typing.

Virtually infinite are the environmental conditions that promote RNA degradation [2]. In addition, the employment of sets of naturally degraded RNA samples is quite expensive and time consuming. Therefore, a method to produce partially degraded samples was developed *in vitro*. The outcome of such procedure was then monitored by RT-qPCR analysis of two housekeeping targets.

2. Materials and methods

RNA was extracted from about 2×10^7 cells of the cell line MDA-MB-453 by Trizol method following standard procedures. After DN*aseI* treatment and ethanol precipitation, the sample was resuspended in water, quantified by Nanodrop (70 ng/μL) and stored at −70 °C until use. Five micrograms aliquots each were then hydrolyzed in duplicate at 70 °C for 0–11.5 h. After purification through Micron 3K columns, to visualize the outcome of the treatment 300 ng of each sample were run through agarose 1.2% gel for 20 min at 90 V. RT was carried out on 420 ng of each sample in a final volume of 20 μL at standard condition using Random Examers (IDT®). cDNA synthesis were then assessed (in triplicate) by two duplex-qPCR formats using the Jump Start 2x Ready Mix Taq (Sigma–Aldrich®). The short amplicon format (SAF) allows the simultaneous amplification of 60 bp fragments of GAPDH and PBGD targets while the long amplicon format (LAF) allows the amplification of 260 fragments of the same targets. TaqMan chemistry was employed for the detection of the syntheses (the probe of the GAPDH was labeled with 6FAM while that of the PDGD was labeled with VIC). PCR primers and TaqMan probes were designed by using the data of http://www.ncbi.nlm.nih.gov/ and the Primer Express software (Version 2.0 Applied Biosystems). Calibration data were obtained from scalar amounts (420–1.6 ng) of untreated control RNA. All the tests were performed by using a CFX96 Real-Time System (Biorad) thermocycler.

The relative quantification (RQ) of the two targets in the treated samples was calculate by the formula $RQ = E^{(Ct,ctrl - Ct,sample)}$ [8],

* Corresponding author. Fax: +39 40 3996265.
E-mail address: fattorin@univ.trieste.it (P. Fattorini).

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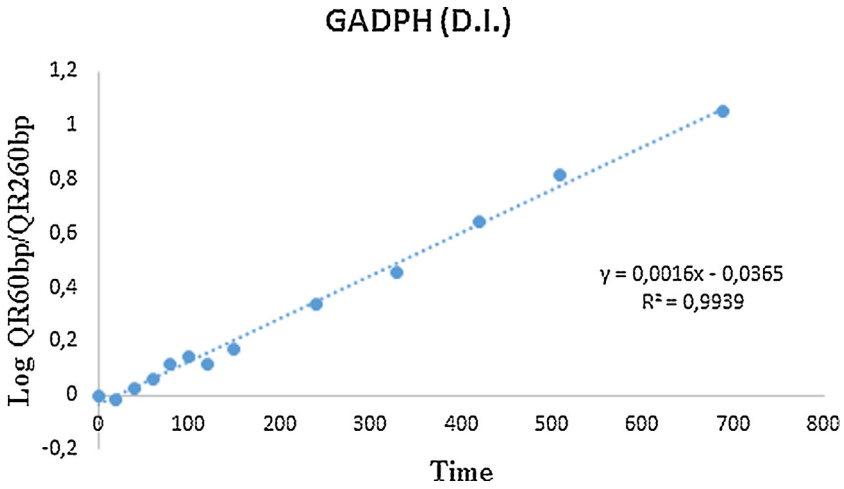

Fig. 1. Degradation index (D.I.) as assessed by the ratio between the QR of 60 *vs* 260 bp targets (*Y* axis) and the length of incubation at 70 °C (*X* axis; in minutes). Analysis performed on the housekeeping target GAPDH.

where *E* is the experimental efficiency of the qPCRs. Least, to calculate the degradation index (D.I.) of the samples, the following formula was used: D.I. = log [$QR_{GAPDH(60bp)}/QR_{GAPDH(260bp)}$].

3. Results and discussion

RT-qPCR calibration data showed a LOQ of 420–1.6 ng for the SAF ($r^2 = 0.998$ for GAPDH and $r^2 = 0.999$ for PDGD) and a LOQ of 420–6.4 ng for the LAF ($r^2 = 0.986$ for GAPDH and $r^2 = 0.943$ for PDGD). Below 6.4 ng of template, no Ct could be observed for the PDGD of 260 bp, in agreement both with the molecular weight of this housekeeping target [8–10] and its minor expression in the cell line here used. The Ct_{GAPDH}/Ct_{PBGD} ratio was 0.745 ± 0.023 for SAF and 0.760 ± 0.016 for LAF, with a clear trend to their increment ($r^2 = 0.9267$ and, respectively, $r^2 = 0.9591$) inversely related to the amount of template.

The treated samples exhibited a reduced molecular weight with the loss of the 18S and 28S bands after 60 min of incubation, as assessed by agarose gel electrophoresis. The RT-qPCR analysis of 420 ng of these degraded samples showed that both targets were always detected, in both amplicon formats. However, a clear increment of the Ct, related to the extent of the hydrolysis ($r^2 = 0.887–0.998$), was found for each of the four targets. As expected, this behavior was more marked in the LAF whose slopes were 0.641 and 0.663 for GAPDH and, respectively, for PDGD (*vs* 0.218 and 0.237 of the SAF). The Ct_{GAPDH}/Ct_{PBGD} ratios were, in mean, 0.719 ± 0.008 in the SAF and 0.755 ± 0.026 in the LAF with a direct correlation between their increments and the length of the degradation treatment ($r^2 = 0.795$ for SAF and, respectively 0.721 for LAF).

The increment of the Ct explains the low amount of targets that could be quantified in these samples. No more than 16.4% of the GAPDH and 7.1% of the PDGD could be quantified by the SAF after 11.5 h of the damaging treatment while, in agreement with the major length of their PCR products, only 1% of the original targets could be assessed by the LAF after the same time of degradation. In addition, a linear correlation ($r^2 = 0.904–0.988$) between the RQ of the samples and the length of the hydrolysis was found.

The qPCR data were also used to calculate the degradation index (D.I.) of these samples. As shown in Fig. 1, a strong correlation ($r^2 = 0.994$) with the length of the hydrolysis was found.

4. Conclusions

The aim of this study was to set up a rapid and reliable protocol that allows the controlled degradation of the RNA. The outcome of the aqueous hydrolysis at 70 °C was than assessed by RT and duplex-qPCR analysis of two housekeeping targets (GAPDH and the PBGD). The procedure proved to produce a time-depending outcome as assessed by the increment of the Ct. As expected [8–10], RT-qPCR of longer targets was more sensitive to the degradation as the less expressed PDGB. In addition, even if both targets were always detected, a direct correlation between the increment of the Ct_{GAPDH}/Ct_{PBGD} and the length of the degradation treatment ($r^2 = 0.795$ for SAF and, respectively 0.721 for LAF) was found. Therefore, the drop out of the less represented target PBGD is expected from massively degraded RNA. However, it is also true that the high Ct of the GAPDH should alert on the risk of misleading conclusion in such cases.

Several methods, based on different analytical principles, are described to provide information on the level of RNA degradation [10,11]. In this work, the RQ data of two differently long fragments of the GAPDH transcript were employed to tentatively calculate a degradation index (D.I.) of the samples. Since a strong correlation ($r^2 = 0.994$) between the QRs of the two targets and the length of the degradation was found, this approach could be of practical interest when dealing with degraded RNA samples.

In Forensic Genetics, the RNA analysis is usually employed for a qualitative evaluation of tissue-specific transcripts [4–6]. Therefore, assuming that appropriate targets are studied, the data of this preliminary work indicate that misidentification of the source of body fluids is unlikely even in highly degraded samples.

Conflict of interest

None.

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