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DETERMINATION OF RNA STABILITY USING REVERSE TRANSCRIPTION REAL-TIME PCR

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Abstract

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The aim of this study was to verify an effect of RNA storage in different laboratory conditions. Especially, this work was focused on the importance of using diethylpyrocarbonate (DEPC) treated water for storage of isolated RNA. The effect of storage of RNA samples in different temperatures was monitored according to various times as well. Isolated RNA was incubated at 20 °C, 4 °C, –20 °C and –80 °C, whereas the temperature –80 °C was used as a control. After incubation only mRNA was converted to cDNA by reverse transcription. The polymerase chain reaction in real time (real-time PCR) was used for a measurement of RNA degradation. No statistically significant interactions were found between RNA treatment conditions if analysis of variance (ANOVA) model was applied. The result showed that storage of isolated RNA in water treated with DEPC is not necessary. This approach prevents possible inhibition downstream reaction caused by DEPC. The results of this study can be used in all molecular applications based on RNA.

RNA, RNases, diethylpyrocarbonate, real-time RT-PCR

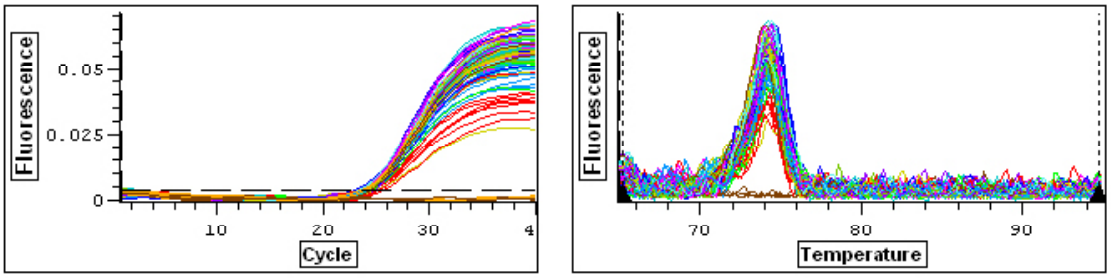
In the last century, there was fantastic progress and expansion of molecular biology in all biological fields. Nowadays, one of the major techniques in molecular genetics is polymerase chain reaction. This robust and sensitive method requires very careful preparation of samples and intact input material (e.g. Cankar *et al*., 2006; Fleige et al., 2006). Major problem of PCR processing is existence of enzymes (DNases and RNases) which lyse nucleic acids. The work with DNA is generally mastered because DNases are not ubiquitous and their activity is relative low. Every normal cell contains the same DNA sequence, thus in the event of partial DNA degradation in the sample, the genetic information is still saved. The situation with RNA is more complicated. The RNases are ubiquitous and more stabile than DNases (Rapley and Manning, 1998). The total RNA from samples includes different ratio of rRNA, tRNA, mRNA, siRNA etc. Degradation of each of these acids can cause various ratios of isolated RNAs. For that reason, the restriction of RNases is prerequisite for storage of intact RNA. Inactivation of RNases is performed by storage of isolated RNA in diethylpyrocarbonate-treated water (DEPC-treated water) or storage at temperature –80 °C. DEPC inactivates the RNases by the covalent modifications of their histidine residues. On the other hand, DEPC is generally an inhibitor of enzymes activity (e.g. Schultz and Baltscheffsky, 2004; Kuze *et al*., 1999) so it can inhibit subsequent reaction such as reverse transcription (RT) or PCR (Abu Al-Soud and Radstrom, 2000). RT is the first principal reaction which is used for characterizing or confirming of gene expression patterns in different samples, tissues or populations. Thus, the inhibition of RT can influence the process of all downstream reactions.

This work was focused on the investigation of the RNA stability in laboratory conditions. Especially, the effect of RNA storage in different elution solution (DEPC-treated water and RNase free water - PCR Ultra H_2O) was analysed, and the effect of storage of RNA samples in different temperatures was monitored according to various time as well.

MATERIALS AND METHODS

The skeletal muscle-specific RNA was isolated from *m. biceps femoris* of final hybrid pig (Large White × Landrase × Duroc). The sample homogenization was performed in FastPrep FP 120 (ThermoSa-

vant, Holbrook, New York, USA) and the total RNA was isolated from the sample using FastRNA Pro Green Kit (Q-BIOgene, Solon, Ohio, USA). The isolated RNA was eluted in DEPC-treated water (component of FastRNA Pro Green Kit) or PCR Ultra H_2O (Top-Bio s. r. o., Prague, Czech Republic). The RNAses were removed by DEPC in DEPC-treated water and by dual-afinity chromatography in PCR Ultra H_2O. Eluted RNA was incubated at 20 °C, 4 °C, –20 °C and –80 °C for various time 1, 2, 4, 8, 16, 32 or 64 hours (data not shown), whereas the temperature –80 °C was used as a control. There were analysed 44 samples in duplicate under these conditions. Each sample was overlayed with PCR oil (Top-Bio, s. r. o., Prague, Czech Republic) to provide against an evaporation. cDNA was synthesized from 1 µg of the total RNA solution with Omniscript RT (QIAGEN GmbH, Hilden, Germany) and Oligo$(dT)_{20}$ Primer (Invitrogen, Carlsbad, California, USA) in a total volume of 10 µl, following the manufacturer's instructions. The degradation of RNA and cycle threshold values (C(t)) were monitored by real-time PCR in the PTC-200 cycler and Opticon 4 Detector, and analysed by Opticon Monitor software (Bio-Rad Laboratories, Inc., California, USA). The C(t) is value when PCR product significantly grows up and passes the threshold value (Fig. 1; Left). The higher C(t) value indicates the degradation of RNA. The reactions were carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were cycled under following conditions: 50 °C for 2 min (uracil N-glycosylase, digestion); 95 °C for 10 min (initial denaturation) and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The degradation of RNA was monitored by real-time PCR amplification with primers for the *PPIA* (*cyclophilin*) gene described by Vallée *et al.*, 2003. Each sample was analysed in duplicate and no template control was included in the assay as well. The specificity of the PCR products was tested by dissociation analysis (Fig. 1) described by Ririe *et al.*, 1997. The statistical analysis was processed in SAS for Windows 9.1.3. (SAS Institute Inc., North California, USA) and ANOVA model was applied too.



1: *Left – PCR amplification plots of SYBR Green detector with different samples; Right – melting curves of amplification products from dissociation analysis, melting temperature of specific product was 74 °C.*

RESULTS AND DISCUSSION

The value of threshold cycle C(t) was exported from Opticon Monitor software to Excel platform. Two different groups of samples (DEPC water and PCR water) were studied under different conditions. In Tab. I there are shown average C(t) values for study of degradation of RNA stored in DEPC-treated water (DEPC water) or RNase free water – PCR Ultra H_2O (PCR water) at each studied temperature. There is small difference between C(t) values of the particular groups for the benefit of DEPC-treated water where the C(t) values are lower. Also, testing independently on storage temperature showed that the total average of C(t) values of RNA samples stored in DEPC-treated water was 24.9 ± 0.43 what was lower than the total average of C(t) values 24.99 ± 0.44 for samples stored in PCR Ultra H_2O. But no statistically significant interactions were found between RNA treatment conditions if ANOVA was applied. The results of this study correspond with Mabic and Kano (2003), who suggest that ultrafiltration of water is as efficient as DEPC treatment for suppressing RNase activity in water. Nevertheless, the degradation of RNA can be greater in PCR Ultra H_2O than in DEPC-treated water. But the DEPC inhibits the RT reaction (Edmands *et al.*, 1994). In Tab. II there are shown average C(t) values for study of RNA degradation depending only on temperature. The observed RNA degradation was low; what corresponds to Almeida et al. (2004) who monitored RNA degradation at temperature 25 °C when RNA was relatively stabile. No statistically significant influence of temperature and storage time (data not shown) on the RNA degradation was found. Despite commonly adviced storage of RNA in water treated with DEPC, this study establishes that RNase free water is very suitable for short time storage or storage of low concentrated RNA. By this way of RNA treatement there are not further applications as RT or real-time PCR inhibited. These results have implications for all molecular applications based on RNA. Good-quality of reaction components ensures the correct performing of RT and real-time PCR.

I: *Average C(t) values and standard deviations (SD) of RNA samples stored in DEPC water and in PCR Ultra H_2O (PCR water) at studied temperatures and total average of C(t) values for all analysed samples independently on temperature*

	Average C(t) ± SD	
Storage temperature	DEPC water	PCR water
–80 °C (Control)	24.67 ± 0.08	24.88 ± 0.15
–20 °C	24.80 ± 0.45	24.85 ± 0.37
4 °C	24.86 ± 0.31	25.13 ± 0.61
20 °C	25.05 ± 0.48	25.00 ± 0.16
Total average C(t) ± SD	24.90 ± 0.43	24.99 ± 0.44

II: *Average C(t) values and standard deviations (SD) of all RNA samples stored at studied temperatures independently on elution solution*

Set	Average C(t) ± SD
–80 °C (Control)	24.78 ± 0.16
–20 °C	24.83 ± 0.41
4 °C	24.99 ± 0.50
20 °C	25.03 ± 0.36

SUMMARY

The aim of this study was to verify the effect of RNA storage in different laboratory conditions. It was focused on the importance of storage of isolated RNA in DEPC-treated water and in different temperatures according to various times as well. For analysis the reverse transcription real-time PCR was used. If ANOVA model was applied, no statistically significant interactions were found between clustered groups. The results suggest that storage of isolated RNA in DEPC treatment is not necessary. This approach prevents possible inhibition of downstream reaction caused by DEPC. The results of this study can be applied to all molecular applications based on RNA.

SOUHRN

Ověření stability RNA pomocí reverzní transkripce a PCR v reálném čase

Cílem studie bylo zjistit stabilitu izolované RNA v různých laboratorních podmínkách. Studie byla zaměřena na možné inhibiční vlastnosti vody ošetřené diethylpyrocarbonátem (DEPC), dále byl zkoumán vliv času (1, 2, 4, 8, 16, 32 a 64 hodin inkubace) a teploty (20 °C, 4 °C, -20 °C a -80 °C) na degradaci izolované RNA. Inkubované vzorky byly následně převedeny pomocí reverzní transkripce na cDNA. Její množství bylo analyzováno pomocí polymerázové řetězové reakce v reálném čase (real-time PCR). Dále byl zkoumán pouze vliv teploty inkubace nebo elučního roztoku na degradaci získané RNA. Statistické zhodnocení výsledků bylo provedeno metodou analýzy variance (ANOVA) a nebyl zjištěn statisticky významný rozdíl mezi skupinami vzorků, respektive nedocházelo k významné degradaci RNA mezi pozorovanými skupinami vzorků. Zejména je důležité zjištění, že není významný rozdíl degradace RNA uchovávané ve vodě ošetřené DEPC a v RNáz prosté vodě. Výsledky této práce jsou použitelné pro veškeré molekulární aplikace, které pracují na bázi RNA.

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RNA, RNásy, diethylpyrocarbonát, real-time PCR

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