Analytical Biochemistry 549 (2018) 107–112

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Producing standard damaged DNA samples by heating: pitfalls and suggestions

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ARTICLE INFO

Keywords:
qPCR
Degraded samples
DNA quantification
DNA hydrolysis

ABSTRACT

Heat-mediated hydrolysis of DNA is a simple and inexpensive method for producing damaged samples *in vitro*. Despite heat-mediated DNA hydrolysis is being widely used in forensic and clinical validation procedures, the lack of standardized procedures makes it impossible to compare the intra and inter-laboratory outcomes of the damaging treatments. In this work, a systematic approach to heat induced DNA hydrolysis was performed at 70 °C for 0–18 h to test the role both of the hydrolysis buffer and of the experimental conditions. Specifically, a trial DNA sample, resuspended in three different media (ultrapure water, 0.1% DEPC-water and, respectively, TE) was treated both in Eppendorf tubes ("Protocol P") and in Eppendorf tubes provided with screwcaps ("Protocol S").

The results of these comparative tests were assessed by normalization of the qPCR results. DEPC-water increased the degradation of the samples up to about 100 times when compared to the ultrapure water. Conversely, the TE protected the DNA from degradation whose level was about 1700 times lower than in samples treated in ultrapure water. Even the employment of the "Protocol S" affected the level of degradation, by consistently increasing it (up to about 180 times in DEPC-water). Thus, this comparative approach showed that even seemingly apparently trivial and often underestimated parameters modify the degradation level up to 2–3 orders of magnitude. The chemical-physical reasons of these findings are discussed together with the role of potential factors such as enhanced reactivity of CO_2, ROS, NO_x and pressure, which are likely to be involved.

Since the intra and inter-laboratory comparison of the outcomes of the hydrolytic procedure is the first step toward its standardization, the normalization of the qPCR data by the *UV/qPCR* ratio seems to be the simplest and most reliable way to allow this. Finally, the supplying (provided with the commercial qPCR kits) of a DNA sample whose degree of degradation is well documented could be helpful in ISO/IEC 17025 validation procedures and in proficiency testing.

Introduction

DNA degradation is a complex process [1] which can occur at low levels even in well preserved specimens [2]. High levels of DNA degradation are commonly observed in aged and forensic samples [2,3] leading, in some circumstances, to partial and/or inconclusive DNA typing. The study of PCR fidelity/processivity from naturally degraded samples is quite complicated and time-consuming because a multitude of enzymatic and non-enzymatic factors are involved in DNA degradation in *post mortem* tissues. Therefore, the employment of *in vitro* models, such as those represented by DN*ase*-digested, sonicated, UV-irradiated and heat-hydrolyzed DNA samples [3,4], is more convenient.

Heat-induced degradation is a complicated process since a multitude of factors are involved. The first factor to be considered is that heat-induced degradation causes different rates of hydrolysis of phosphodiesteric bonds and of *N*-glycosilic bonds. The heat-induced

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https://doi.org/10.1016/j.ab.2018.03.011
Received 11 January 2018; Received in revised form 8 March 2018; Accepted 12 March 2018
Available online 15 March 2018
0003-2697/

Table 1
UV/qPCR ratios of the set of samples.

L.o.H.	Medium A		Medium B		Medium C	
	PP	*PS*	*PP*	*PS*	*PP*	*PS*
6	19.5 ± 8.6	144 ± 33.6	876 ± 521	160489 ± 145755	1.15 ± 0.08	1.49 ± 0.10
12	314 ± 124	18119 ± 23665	31761 ± 9114	-	1.30 ± 0.13	1.62 ± 0.17
18	2298 ± 1066	–	–	-	1.37 ± 0.12	1.81 ± 0.40
24	91371 ± 38977	n.p.	n.p.	n.p.	n.p.	n.p.
36	–	n.p.	n.p.	n.p.	n.p.	n.p.
0	(1.16 ± 0.03)		(1.36 ± 0.24)		(1.18 ± 0.16)	

L.o.H.: length of hydrolysis (in hours); ***PP***: "Protocol P" (Eppendorf tubes sealed by Parafilm); ***PS***: "Protocol S" (Eppendorf tubes with screwcaps). The values in italics refer to DNA quantities below the LOQ (0.023 ng/μL); - indicates that no Cq was recorded in the course of six reactions; n.p.: not performed. The qPCR data were collected in three experimental sessions, which provided the following calibration data: r^2 from 0.996 to 0.999 (efficiency from 1.937 to 2.044). The Cq of the Internal Positive Control ranged from 25.8 to 26.3 (mean = 25.7 ± 0.5) for the samples while it ranged from 24.8 to 27.6 (mean = 25.9 ± 0.7) for the calibration standard.

aqueous hydrolysis of phosphodiesteric bonds occurs at very low rates (approximately $1 \times 10^{-15}\,s^{-1}$ at 25 °C) [5], while the hydrolysis of the *N*-glycosilic bond occurs at considerable higher rates (approximately $3 \times 10^{-11}\,s^{-1}$ at 37 °C) [6]. The hydrolysis of the *N*-glycosilic bond is enhanced by an acidic pH environment [6] and slowed by increased ionic stringency [6]. The hydrolysis of the *N*-glycosilic bond leads to the formation of A-P (Apurinic-Apyrimidinic) sites, with a preferential lack of the purinic moieties [6]. Thereafter, the presence of A-P sites promotes a further degradation of the nucleic chain via β-elimination [7]. Other factors involved in heat-induced degradation is that heating: induces the deamination of the bases [8,9]; promotes the formation of nitrogen oxides [10]; and induces the oxidation of the bases, which occurs through the formation of ROS [11], a process enhanced by heavy metal ions, such as Cu^{2+} and Fe^{3+} [12]. There are other factors that have been recently found to be involved in the heat-induced degradation of the nucleic acids, such as DNA concentration [13] and partial pressure of the gaseous phase present in the reaction vessels [14].

Due to the number of factors influencing the heat-induced degradation of DNA, the experimental design of the *in vitro* aqueous hydrolyses has to be based on the particular researcher's aims. When looking at previous protocols undertaken in various validation studies done in the field of forensic and clinical DNA analysis, it's been observed that a broad range of temperatures (from 37 to 99 °C) and incubation times (from minutes to hours) have been used (see Supplementary Table 2) [15–25]. Unfortunately, due to the fact that full details of the experimental conditions are rarely available, the inter-laboratory comparison of the results is impossible. All this adds to the uncertainty of the results obtained by the researchers when studying damaged DNA samples. It is thus of the utmost importance, in the authors' opinion, that are made available to the scientific community reference standard samples and controlled, well-understood, and basic protocols for the assessment of DNA damage in forensic and clinical investigations.

The aim of this study is to investigate the different levels of heat-induced degradation on one reference human DNA sample after incubation at 70 °C in three different conditions. The three conditions were achieved by using two different batches of water and one of the most used buffers for DNA resuspension (the so-called TE). In addition, the role of two different hydrolysis tubes was also investigated. The results of these comparative approaches show that even trivial parameters can modify the degradation level up to 2–3 orders of magnitude.

Materials and methods

Trial sample

The *trial DNA sample* (namely "*sample FM*") had already been used in previous work [17]. This sample had been extracted from the buffy coat of 500 mL of the peripheral blood of a 42- year-old male volunteer, who provided informed consent. After phenol/chloroform/isoamyl alcohol (25/24/1) purification, the sample was precipitated by the addition of 2.5 vol of ethanol and then was re-dissolved in 20 mL of 0.2 M Na-acetate pH 7.4. This sample was divided into 2 mL aliquots, which had 2.5 vol of ethanol added to them. These aliquots were then stored at −80 °C.

These samples were then used in the present study. Three original aliquots were centrifuged, washed twice with 70% ethanol and then each dissolved in 10.0 mL of medium A, B and C (see Hydrolysis medium). The resulting samples were then named sample FM-A, FM-B and FM-C, respectively. These three samples were then quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc.) apparatus. After adjusting the final volumes, six measurements for each sample showed that they had the following average concentrations (ng/μL): sample FM-A: 59.7 ± 2.6; sample FM-B: 58.7 ± 1.4; sample FM-C: 57.0 ± 1.5.

Hydrolysis medium

Three aqueous media were employed (named medium A, B and C) in this experiment. Medium A was sterile ultrapure water from Fresenius Kabi Italia (Italy). Medium B was 0.1% di-ethyl-pyro-carbonate (DEPC)-water. This medium was made following standard protocols [26] using Millipore water (18.2 MΩ/cm) plus DEPC (Aldrich; cat. n. 159220). The protocol involved the overnight dissolution of the DEPC and then the solution being autoclaved at 120 °C for 20 min and then stored at room temperature. Medium C was 10 mM Tris pH 7.5 and 0.1 mM Na_2EDTA re-dissolved in medium A. The resulting buffer is commonly known such as TE [27].

Hydrolysis of the samples

The three samples (FM-A, FM-B and FM-C) simultaneously underwent the same hydrolytic treatments. The same amount of each sample was used and each of the samples was subjected to the treatments for the lengths of time reported in Table 1. The following text uses sample FM-A as an example of the procedure done in the hydrolytic treatment applied to samples FM-B and FM-C, as well. During the hydrolytic treatments of sample FM-A, 500 μL aliquots of the sample, each containing about 30 μg of DNA, were incubated at 70 °C in a thermoblock for progressive intervals of time in duplicate. These samples were named FM-A-6, FM-A-12, FM-A-18, FM-A-24 and FM-A-36, respectively. To avoid evaporation of the samples during the hydrolytic treatments, the 1.7 mL Eppendor tubes were sealed with Parafilm (Whatman). This procedure was named "Protocol P" (PP). In addition to this hydrolytic procedure, the three samples were subjected to a further hydrolytic procedure. According to this protocol, 1.7 mL Eppendorf tubes (containing 500 μL of the sample) with screwcaps were employed in the course of the same incubations. This procedure was named "Protocol S" (PS).