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Research Article

Treatment of long-term stored DNA—Comparison between different methods to obtain high-quality material

Long-term stored DNA can be sometimes the only source of genetic material of an organism that does not exist anymore, but a research interest still persists. However, there is a lack of information about useful methods to improve quality from such type of material. In this study, we compared four different protocols using DNA samples collected in 1998. Fresh DNA was also tested aiming to check the differences between these two material types. Sixteen samples of each DNA type treated with phenol-chloroform with PEG 5.0%, silica-gel membrane spin column, PEG 7.5%, and glass-fiber matrix spin column were submitted to spectrophotometer measurements, electrophoresis, PCR, and RFLP-PCR to assess the best method concerning yield, quality, and purity. Based on the results, purification with PEG 7.5% was considered the best method to treat aged DNA samples. In addition to the efficiency, this protocol has low cost. Analyzing the data, we also conclude that long-term stored DNA may be considered a reliable and potential resource for future molecular studies.

Keywords:
DNA purification / Long-term storage / Polymerase chain reaction
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1 Introduction

In earlier studies, the major challenges in molecular analysis were genotyping technology and cost. However, the accessibility of samples has become the biggest problem faced by researchers in molecular biology studies [1]. In cases when the access to fresh biological tissues is not possible, old DNA samples may be the only source of genetic material available. For some techniques in which high-quality material is required, such as PCR, samples stored for a long time might generate altered results. However, the main advantages of using aged samples are the elimination of two expensive and time-consuming steps: collection of biological material and DNA extraction.

Long-term storage of DNA at −20°C has a detrimental impact on yield and quality mainly due to two reasons: hydrolysis and oxidative damage caused by water and oxygen, and exposure to temperature fluctuations such as freeze-thaw cycles [2, 3]. Moreover, chemical degradation such as depurination hydrolysis may be responsible for the small recovery of DNA from aged samples [4].

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Material loss can also occur when microcentrifuge tubes retain the DNA with the amount of 5 ng/mm^2 of tube wall [5]. All these problems cited above not just have influence on the recovery, but they can also lead to differences in the ability of retesting the samples.

In this present work, we propose a useful and rapid purification method with PEG 8000, in a concentration of 7.5% v/v, to obtain high-quality DNA from aged samples, collected 14 years ago. This protocol is able to precipitate DNA molecules with appropriate sizes and is suitable for procedures sensitive to impurities or high salt concentrations, such as PCR and sequencing, due to the use of PEG. Higher molecular mass DNA precipitates at lower PEG concentrations than lower molecular mass DNA. Therefore, a concentration of 7.5% can precipitate DNA fragments from 700 base pairs (bp), retaining, thereby, species <50 bp in the supernatant. Furthermore, this proposed method is simple and cheap [6–8].

Thus, we performed a comparison between our PEG 7.5% purification protocol with a reextraction method that also uses PEG (phenol-chloroform with PEG 5.0%) and with two common genomic DNA extraction and purification kits (silica-gel membrane spin column and glass-fiber matrix spin column, respectively). A fresh DNA was also used as a control to compare the results with the old DNA samples.

Colour Online: See the article online to view Fig. 3 in colour.

2 Materials and methods

2.1 DNA samples

We selected 16 old DNA samples stored for 14 years and 16 fresh DNA samples, all of them kept at −20°C in microcentrifuge tubes (the fresh DNA samples were kept at −20°C for one week, until all the assays were done). Both DNA types were extracted from pig (*Sus scrofa domesticus*) blood using Sambrook et al. [9] protocol.

The old DNA samples were obtained through Pires et al. [10]. To make the comparison in a viable and accurate way, all the old and fresh DNA samples were diluted in water to100 ng/μL. Animal handling was done in accordance with regulations approved by the institutional animal welfare and ethics/protection commission of the Federal University of Viçosa.

2.2 Reextraction methods

2.2.1 Protocol 1: Phenol-chloroform with PEG 5.0%

This protocol was performed as described by Sambrook and Russell [11] with minor modifications. Briefly, 100 μL of DNA was added to a 1.5 mL microcentrifuge tube and then centrifuged at 20 000 × *g* for 5 min. Supernatant was eliminated and 0.8 μL of DTT 10% and 50 μL of DNA extraction buffer with RNAse (1 M Tris-HCL pH 8.0, 0.5 M EDTA pH 8.0, NaCl 5 M, SDS, and RNAse A 10 μg/μL) were added to the remaining pellet. After incubation at 37°C for 2 h, 1 μL of Proteinase K (10 mg/mL) was added and then the solution was incubated at 50°C for 12 h. The deproteinization phase was performed first with phenol and chloroform isoamyl (24:1) and then with just chloroform isoamyl (24:1). DNA present in the aqueous phase was precipitated with 0.1 volume of 3 M sodium acetate, 1 volume of isopropanol, NaCl, and PEG 8000 to a final concentration of 0.9 M and 5.0%, respectively. Samples were kept at 4°C for 2 h and then centrifuged at 20 000 × *g* for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol, dried, and resuspended with 50 μL of TE (1 M Tris-HCL and 0.5 M EDTA, pH 8.0).

2.2.2 Protocol 2: Silica-gel membrane spin column

DNA reextraction was performed following the protocol of DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, USA). Briefly, 100 μL of DNA were added to a 1.5 mL microcentrifuge tube with 20 μL of Proteinase K and 100 μL of PBS. After that, 200 μL of lysis buffer (AL) were added. Each tube was vortexed vigorously and then incubated at 56°C for 10 min. After incubation, the samples received 200 μL of ethanol (100%), were vortexed, loaded on a DNeasy Mini spin column (Qiagen) placed in a 2 mL collection tube and centrifuged at 8000 × *g* for 1 min. The silica membrane was washed in two steps: first with wash buffer 1 (AW1) and then with wash buffer 2 (AW2). The spin column was transferred to a new 1.5 mL microcentrifuge tube and received 50 μL of elution buffer (AE) to elute the DNA. Samples were then incubated for 1 min at room temperature and centrifuged at 8000 × *g* for 1 min.

2.3 Purification methods

2.3.1 Protocol 3: Purification with PEG 7.5%

An aliquot of 100 μL of each DNA sample was transferred to a 1.5 mL tube. To each tube, 36 μL of NaCl (5 M) and PEG 8000 to a final concentration of 7.5% were added, bringing the final volume to 200 μL. The samples were incubated at 4°C for 2 h, centrifuged at 17 000 × *g* for 20 min and then the supernatant was discarded. The pellet was washed with 70% ethanol, dried, and resuspended with 50 μL of TE (1 M Tris-HCL and 0.5 M EDTA, pH 8.0).

2.3.2 Protocol 4: Glass-fiber matrix spin column

DNA purification was performed according to the protocol of Illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Healthcare Life Sciences, Piscataway, NJ, USA). Briefly, 500 μL of capture buffer and 100 μL of DNA were transferred to a GFX column (GE Healthcare). The solution was mixed thoroughly by pipetting up and down six times and centrifuged at 18 000 × *g* for 30 s. Wash buffer (500 μL) was added and centrifuged at 18 000 × *g* for 30 s. The column was transferred to a fresh 1.5 mL microcentrifuge tube and received 50 μL of 10 mM Tris-HCL, pH 8.0. Samples were incubated at room temperature for 1 min and then centrifuged at 18 000 × *g* for 1 min to recover the purified DNA.

2.4 Spectrophotometer measurements and integrity assay

Yield and quality of DNA reextracted or purified by the different methods were assessed using the spectrophotometer NanoDrop 2000 (Thermo Scientific). The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. Samples with a ratio between 1.8 and 2.0 were accepted as pure, and a value appreciably <1.8, might indicate presence of protein. The ratio of absorbance at 260 and 230 nm was used as a secondary measure of DNA purity. To be considered pure, the material must present a value between 2.0 and 2.2. If the ratio is lower than expected, it may indicate contamination with organic compounds.

The integrity of DNA treated by each method was assessed by electrophoresis performed with DNA 7500 kit of Agilent 2100 Bioanalyzer (Agilent Technologies) following the manufacturer's protocol.

2.5 PCR and genotyping

PCR targeting a pig leptin gene fragment (*LEP*; GenBank: NM_213840.1) was used to assess whether the products of each method were amplifiable. PCR mixture was

composed of PCR 2× *Taq* Green Master Mix (Fermentas), $MgCl_2$ 3.0 mM, 0.2 μM of each primer (forward and reverse), and 25 ng of genomic DNA. The forward primer sequence was 5′-TGTGAGAAACAGACAGTCGTGG-3′, and the reverse primer sequence was 5′-TGAGGAT CTGTTGGTAGATCGC-3′. The amplification conditions were according to those described by Peixoto et al. [12]. The PCR products were visualized on 1% agarose gel electrophoresis containing ethidium bromide (Promega) and were photographed. The fragment amplified had 423 bp.

For genotyping analysis was tested ryanodine receptor 1 (*RYR1*; GenBank: NM_001001534.1) gene. PCR was performed with a 5′-CCACACCCTCCCCGCAAGTGC-3′ forward primer and with a 5′-GCCAGGGAGCAAGTTCT CAGTAAT-3′ reverse primer. The PCR conditions used were described by Luerce et al. [13]. PCR products (144 bp) were digested at 37°C for 3 h with *HhaI* (Promega). The normal genotype results in two fragments, 95 and 49 bp. The homozygote-mutated genotype is not cut by the restriction enzyme and the heterozygote produces three fragments of 144, 95, and 49 bp. The digestion products were visualized on 2% agarose gel electrophoresis with ethidium bromide staining.

Real-time PCR was performed as an additional assessment of old-treated DNA. The gene 18S ribosomal RNA (GenBank: NR_046261.1) was used as a target to check for amplification inhibition. PCR mixture was composed of Go *Taq*® qPCR Master Mix 2× (Promega), 10 μM of each primer (forward and reverse), CXR Reference Dye (Promega), and three amounts of genomic DNA, 10, 5, and 2.5 ng. The forward primer sequence was 5′-ACGGACAGGATTGACAGATT-3′ and the reverse primer sequence was 5′-TCGCTCCACCAACTAAGAA-3′. The amplification conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The reactions were conducted in ABI Prism 7300 Sequence Detection Thermocylcer (Applied Biosystems). The threshold cycle (Ct) numbers of each treated sample were used to build a linear regression and then the efficiency could be calculated with its slope. All the reactions were performed in duplicate.

2.6 Statistical analysis

Data were submitted to a variance analysis (ANOVA) to detect interaction between DNA type (fresh and old) and the methods used to improve its quality. Interaction tests were performed for DNA yield and purity (260/280 and 260/230 nm ratios). Results are expressed as mean ± SD. For these analyses, R Program 2.14.2 (http://www.R-project.org) was utilized. Interactions were considered statistically significant at $p < 0.05$.

3 Results

3.1 Spectrophotometer measurements

According to ANOVA for yield, the factors type of DNA and method are not independent. There are appropriate methods

Table 1. Comparison of four extraction and purification methods between two DNA types

Purification method	Old DNA			Fresh DNA			Process duration (h)	Cost per sample (US$)
	DNA yield (ng/μL)	260/280 nm	260/230 nm	DNA yield (ng/μL)	260/280 nm	260/230 nm		
Phenol-chloroform with PEG 5.0%	18.98 ± 4.37c	1.88 ± 0.05a	0.44 ± 0.08c	52.70 ± 7.70c	1.90 ± 0.02a	0.97 ± 0.11c	17.50	0.40
Silica-gel membrane spin column	123.90 ± 20.14a	1.87 ± 0.02ab	2.09 ± 0.55a	141.15 ± 14.76a	1.86 ± 0.02ab	1.85 ± 0.50a	0.50	10.00
PEG 7.5%	95.40 ± 12.06b	1.84 ± 0.07ab	1.14 ± 0.15b	112.45 ± 6.67b	1.85 ± 0.05ab	1.36 ± 0.13b	3.00	0.15
Glass-fiber matrix spin column	73.05 ± 10.18b	1.80 ± 0.04b	0.91 ± 0.31b	122.40 ± 12.46ab	1.84 ± 0.04b	1.40 ± 0.31b	0.25	2.70
Interaction DNA type and method	DNA yield (ng/μL)			260/280 nm		260/230 nm		
ANOVA *p*-value	0.04			0.67		0.08		

Average ± SD values are represented for DNA yield, 260/280 and 260/230 nm. Cost per sample includes only materials. The letters a–c in the columns represent the order of the methods according to DNA yield, 260/280 nm and 260/230 nm. The letter "a" means the best and "c" the worst method. Same letters mean that the numbers are statistically equal.

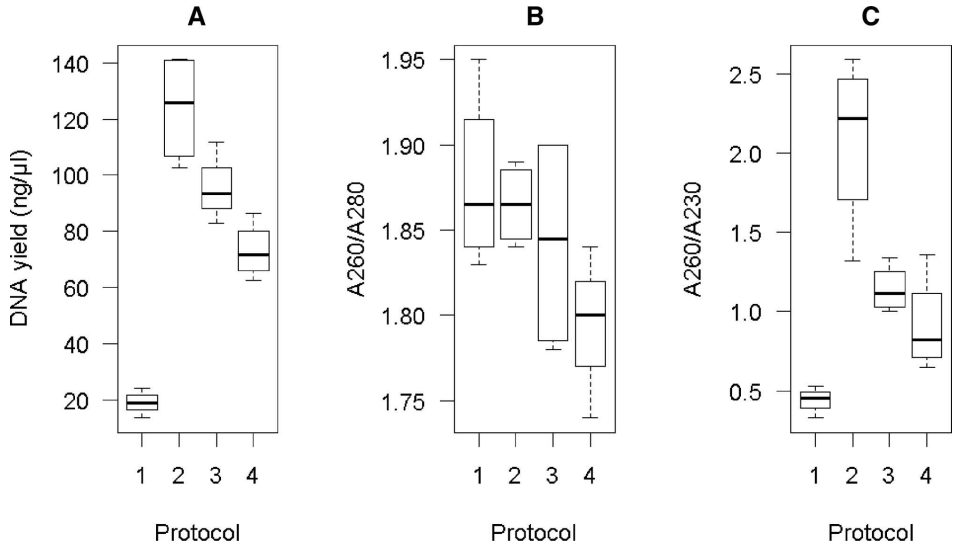

Figure 1. Old DNA concentration and purity. (A) DNA yield (ng/μL), (B) A260/A280 ratio assessing protein contamination, (C) A260/A230 ratio assessing contamination for organic compounds. The boxes represent the average ± SD values; maximum, and minimum values are also demonstrated.

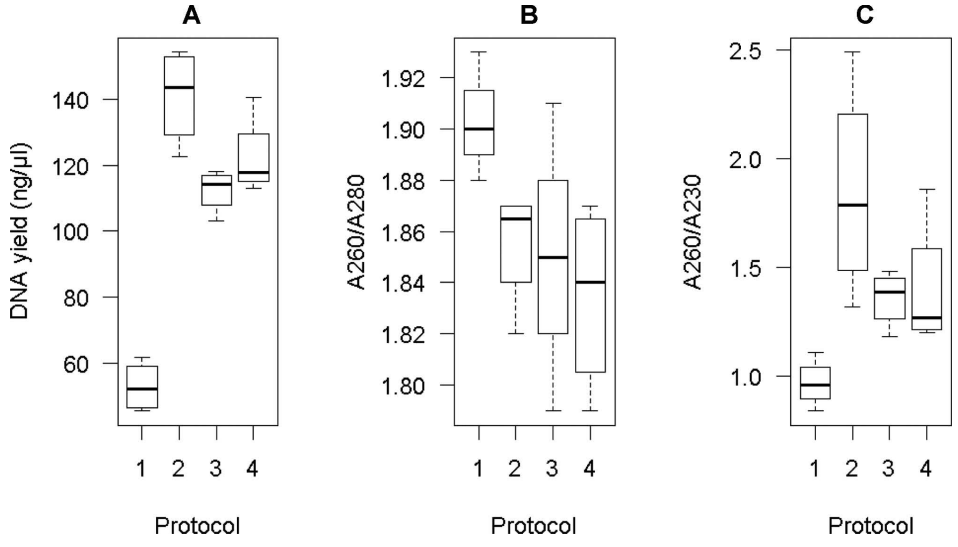

Figure 2. Fresh DNA concentration and purity. (A) DNA yield (ng/μL), (B) A260/A280 ratio assessing protein contamination, (C) A260/A230 ratio assessing contamination for organic compounds. The boxes represent the average ± SD values; maximum, and minimum values are also demonstrated.

for each DNA type ($p = 0.04$). For old DNA, the best method was silica-gel membrane spin column (123.90 ± 20.14 ng/μL, 62% recovery), then PEG 7.5% (95.40 ± 12.06 ng/μL, 48% recovery), and glass-fiber matrix spin column (73.05 ± 10.18 ng/μL, 36% recovery). For fresh DNA, silica-gel membrane spin column (141.15 ± 14.76 ng/μL, 70% recovery) and glass-fiber matrix spin column (122.40 ± 12.46 ng/μL, 61% recovery) were the best methods, followed by PEG 7.5% (112.45 ± 6.67 ng/μL, 56% recovery). Regardless the age of the sample, old or fresh, phenol-chloroform with PEG 5.0% had the worst recovering of DNA (10 and 26%, respectively; Table 1; Figs. 1 and 2).

Concerning the 260/280 and 260/230 absorbance ratios, the ANOVA showed that DNA type and methods are independent ($p = 0.67$ and 0.08, respectively). In other words, the best methods for purity are the same for old and fresh DNA. Phenol-chloroform with PEG 5.0%, silica-gel membrane spin column, and PEG 7.5% had the lowest protein contamination. Silica-gel membrane spin column had the lowest organic compounds contamination, followed by PEG 7.5% and glass-fiber matrix spin column (Table 1; Figs. 1 and 2).

3.2 Genomic DNA integrity

Integrity of treated DNA was assessed by electrophoresis with DNA 7500 kit of Agilent 2100 Bioanalyzer (Agilent Technologies; Fig. 3). All the methods showed DNA degradation, which is known to occur when DNA samples are frozen and thawed. For old DNA, phenol-chloroform with PEG 5.0% had the worst quality level, with the most of fragments <500 bp. For fresh DNA, purification with PEG 7.5% showed the smallest fragments with length around 3500 bp. The other three methods performed for old and fresh DNA presented fragments around 6000–10 000 and 8000–14 000 bp, respectively. These data confirm that old DNA samples tend to be more degraded than recently collected samples.

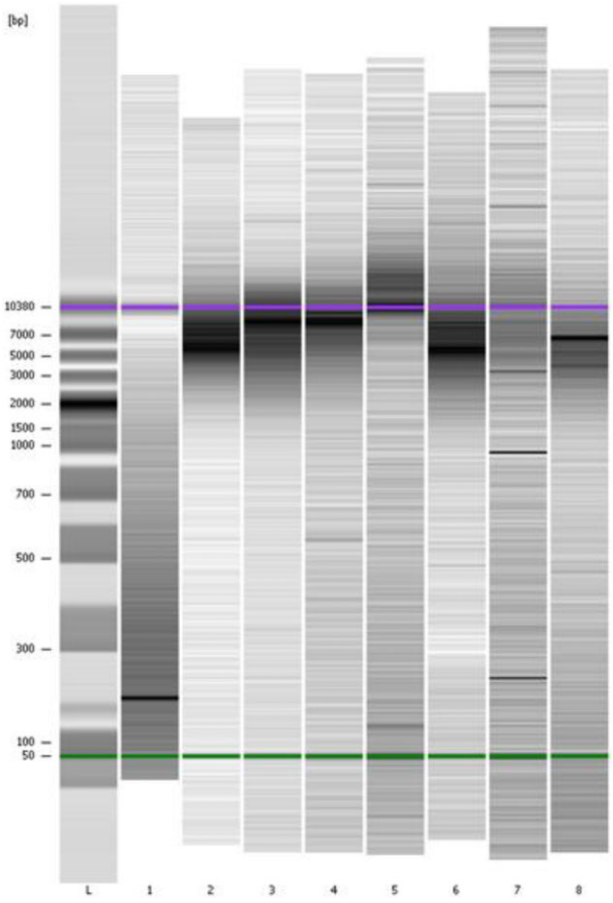

Figure 3. Electrophoresis analysis results of old and fresh DNA treated with four different protocols. The first four lanes represent old DNA and the last four fresh DNA. Phenol-chloroform with PEG 5.0% (lanes 1 and 5), silica-gel membrane spin column (lanes 2 and 6), PEG 7.5% (lanes 3 and 7), glass-fiber matrix spin column (lanes 4 and 8), and molecular weight marker (lane L). Electrophoresis performed with DNA 7500 kit of Agilent 2100 Bioanalyzer (Agilent Technologies).

3.3 Time, labor, and cost analysis

The method that spent less time was glass-fiber matrix spin column (0.25 h) while the most time consuming was phenol-chloroform with PEG 5.0% (17.50 h). Purification with PEG 7.5% was the cheapest treatment per sample (US$ 0.15; Table 1). Considering that the objective of this work is to report a method to improve old DNA quality and analyzing all these data of yield, purity, labor, time, and cost, purification with PEG 7.5% can be considered the best protocol to achieve it.

3.4 Amplification and genotyping efficiency

To assess if all treated DNA with the four methods are amplifiable, the samples were submitted to a PCR targeting the *LEP* gene (Fig. 4). All samples tested were amplified, showing no signs of PCR inhibition. Contradicting the expectations, old DNA had stronger bands than fresh material, even without signs of inhibition.

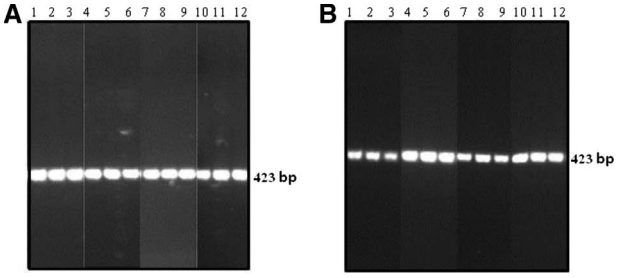

Figure 4. Representative electrophoresis gel of leptin gene for old DNA (A) and fresh DNA (B). Phenol-chloroform with PEG 5.0% (lanes 1–3), silica-gel membrane spin column (lanes 4–6), PEG 7.5% (lanes 7–9), and glass-fiber matrix spin column (lanes10–12).

For genotyping test, the treated samples were submitted to an RFLP-PCR for *RYR1* gene (Fig. 5). The four methods had similar results, having no differences between old and fresh DNA, despite the reextraction and purification methods. All the samples were from *RYR1* nonmutated animals, since two bands of 95 and 49 bp were seen in the gel.

All the old DNA samples treated with the four methods were also submitted to a real-time PCR as an additional method for amplification inhibition assessment (Fig. 6). A desirable reaction should have an efficiency ranging from 90 and 110%, which corresponds to slopes values around −3.58 and −3.10. A 100% efficiency means that the template doubles after each cycle during the exponential amplification. Silica-gel membrane spin column and PEG 7.5% presented positive results, 93 and 94% efficiency, respectively, showing no signals of inhibition. However, phenol-chloroform with PEG 5.0% and glass-fiber matrix spin column presented higher efficiencies with values of 251 and 150%, respectively, which might indicate a presence of inhibitors.

4 Discussion

The exposure to temperature fluctuations such as freeze-thaw cycles, hydrolysis, and oxidative damage caused by water and oxygen, decrease DNA yield and purity, negatively affecting PCR protocols. Researchers have to choose not only a method that recovers high amounts of nucleic acids but also a method that provides a high-quality, pure, and amplifiable material at a reasonable cost of labor and expenses.

As we have observed, the method of purification with PEG 7.5% has given the best results among the other methods evaluated. Over the satisfactory yield and the best amplification efficiency, the quality and purity can be considered as good as those obtained with commercial purification kit. The silica-gel membrane spin column protocol gives the best DNA yield and quicker process, however, it is a costly method. Besides the high cost, the main limitation of glass-fiber matrix spin column method is the poor amplification efficiency.

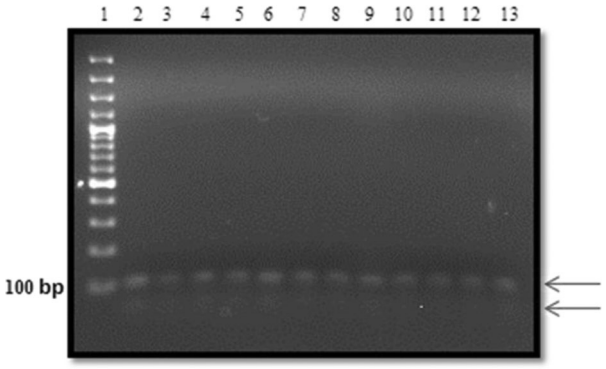

Figure 5. Representative electrophoresis gel of *RYR1* RFLP-PCR for old (lanes 2–9) and fresh DNA (lanes 10–13). Phenol-chloroform with PEG 5.0% (lanes 2, 3, and 10), silica-gel membrane spin column (lanes 4, 5, and 11), PEG 7.5% (lanes 6, 7, and 12), glass-fiber matrix spin column (lanes 8, 9, and 13), and 100 bp DNA ladder (lane 1). The arrows indicate the 95 and 49 bp fragments.

The method of phenol-chloroform with PEG 5.0% led to a low yield, high organic compounds contamination, poor amplification efficiency, and has the longest process duration; therefore, is the worst protocol evaluated in this present study.

The use of phenol to purify nucleic acids can be problematic. If a small amount of it remains in the sample, its oxidation products may damage the DNA. To avoid this, chloroform can be applied to remove the phenol, however, residual chloroform can also be harmful. These two problems can explain the poor yield of DNA presented by the phenol-chloroform with PEG 5.0% protocol. Furthermore, the many vortexing and pipetting steps may have sheared some of the DNA molecules, which also explain the unsatisfactory results. Purification with PEG allows selectively precipitation of high molecular weight DNA, resulting in a high-quality material with a short time and low cost. The ability to precipitate high molecular weight DNA can be extremely important for some assays where fragment length is a limiting factor. Although the commercial kit, silica-gel membrane spin column, results in a high-quality DNA and has short process duration, it is a costly method, especially if a large number of samples must be processed; while glass-fiber matrix spin column, besides cost, has poor amplification efficiency as a limiting factor.

Di Pietro et al. (2010) in a similar work searching for an appropriate extraction method for long-term stored blood, reported a revised phenol-chloroform protocol as the best choice to obtain high-quality DNA. However, our phenol-chloroform method presented the worst results concerning yield and organic compounds contamination among the methods evaluated. Therefore, the sample origin is considered to be of crucial importance to choose the best method, since there are appropriate protocols to extract DNA from long-term stored blood and appropriate protocols to purify DNA from long-term stored samples.

Based on these results, we concluded that purification with PEG 7.5% is a useful method to improve yield, quality, and purity of long-term stored DNA samples, which may be considered a reliable and potential resource for futures research, providing real and valuable information. This simple and low-cost method is suitable for PCR and genotyping analysis.

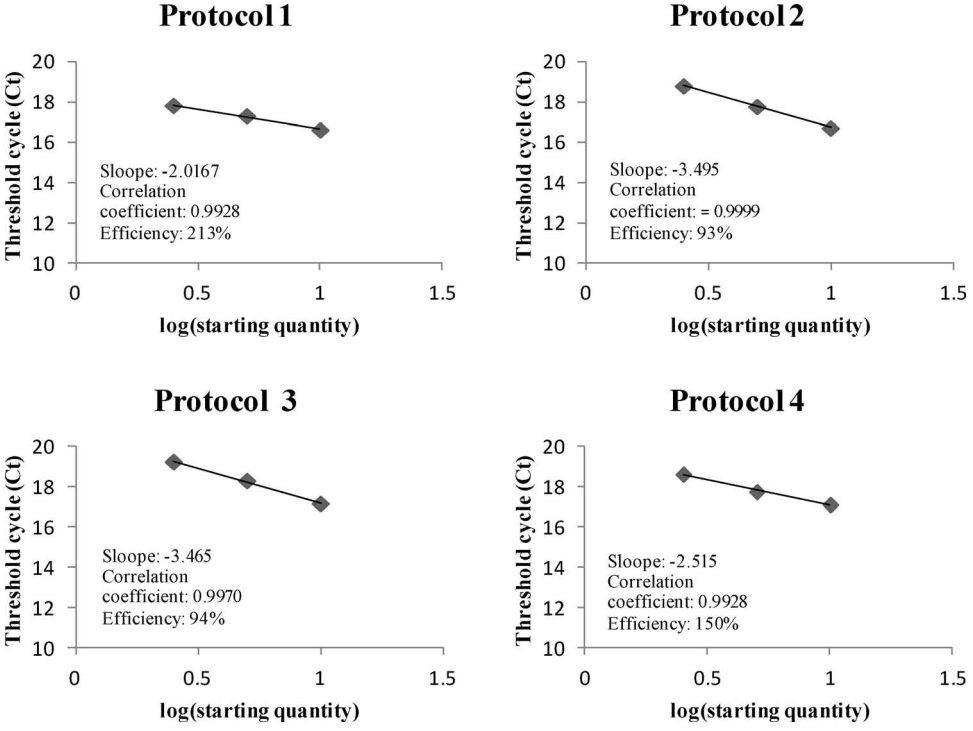

Figure 6. Linear regression showing threshold cycle (Ct) on the y-axis and the logarithm of the DNA starting quantity on the *x*-axis. The spots mean the amounts of 2.5, 5, and 10 ng. Slope, amplification efficiency, and correlation coefficient values are demonstrated to provide information about the performance of the reaction. Phenol-chloroform with PEG 5.0%, silica-gel membrane spin column, PEG 7.5%, and glass-fiber matrix spin column are presented as protocols 1, 2, 3, and 4, respectively.

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