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Comparison of DNA extraction using proteinase K and extraction kit: analysis of the quality of the genetic material

Comparação da extração de DNA utilizando proteinase K e kit *de extração: análise da qualidade do material genético*

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ABSTRACT

Introduction: Deoxyribonucleic acid (DNA) is the raw material for genetic studies, and therefore laboratory techniques are developed to obtain it with appropriate concentration and integrity. **Objective:** To compare two methods of DNA extraction regarding sample concentration and integrity. **Methods:** DNA was extracted from the tail end (2 mm long) of mice, stored at -20°C. The proteinase K method (PKM) and the Kappa Express Extract® kit were used for extraction. The concentrations and ratios 260/280 and 260/230 were determined by spectrophotometry. DNA integrity was checked on 2% agarose gel with ethidium bromide. For the final test of the extracted samples, a multiplex polymerase chain reaction (PCR) was performed, with primers for the Large gene. **Results:** Samples extracted by the PKM presented mean concentration value of 59.4 ± 18.5 ng/µl (260/280 = 1.74 ± 0.04 and 260/230 + 1.85 ± 0.14) and the samples extracted by the commercial kit presented mean concentration value of 178.8 ± 42.0 ng/µl (260/280 = 1.09 ± 0.04 and 260/230 = 0.62 ± 0.66). PCR amplified the Large gene in the DNA extracted, regardless of the methodology used. **Conclusion:** Both methodologies studied can be used, and the PKM is cheap but a time-consuming method, while the commercial kit is more expensive, however DNA extraction is faster.

Key words: DNA; mice; genotyping techniques.

INTRODUCTION

Deoxyribonucleic acid (DNA) was discovered in 1869 by the Swiss Johann Friedrich Miescher (1844-1895). In analyzing the nuclei, Miescher was able to discover that nucleic acids are macromolecules of a chemical nature, composed of nucleotides with a phosphoric grouping (phosphate), one sugar and nitrogenous base (adenine, guanine, thymine and cytosine), constituting the genetic material of living beings. In 1953, Francis Crick and James Watson concluded that the DNA molecule structure has two helical chains, which are coiled around the same axis, and are held together by hydrogen bonds. This discovery was very important for science, but was not immediately recognized[1]. The paper "Molecular structure of the nucleic acids" by Francis Crick and James Watson, published on April 25, 1953 in the journal Nature, radically changed the direction of science in the search for the origins of life. With less than a thousand words and a simplified graphic, they described the DNA structure[2].

DNA is the raw material for many genetic studies. The different techniques for its study are important, since some genetic characteristics may be of economic interest and/or related to health, therefore, basic studies on DNA extraction techniques with adequate and complete concentration are crucial for the development of research in the field of molecular biology[3].

The techniques used in the molecular analyzes require good quality nucleic acids, that is, not fragmented and in adequate concentration. Therefore, special attention should be paid to the extraction and storing of samples. It is also essential to use reliable methods to determine their concentration/integrity[4].

Isolated DNA should be free of contaminants such as sugars, proteins and phenol, and careful specimen handling is essential.

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Most tissue DNA extraction protocols use sodium dodecyl sulfate (SDS) detergent, and there is the possibility of adaptations in the protocols to extract the DNA faster, however not losing the quality of the sample[5].

OBJECTIVE

The objectives of this work were to evaluate and compare the quality of the DNA extracted from animal tissue by different methods.

METHODS

All procedures were performed in accordance with the international principles of animal welfare, and the work was approved by the Ethics Committee on Animal use of the Institute of Biosciences of the Universidade de São Paulo, CEUA no. 199/2014.

Obtaining the material and methodology for DNA extraction

The present work used 40 mice of the Large^{myd} lineage, from the Project "Evaluation of reproductive performance growth curve of murine models for muscular dystrophies" ("Avaliação do desempenho reprodutivo curva de crescimento de modelos murinos para distrofias musculares"). The animals Large^{myd} affected are recessive homozygous for the mutation in the Large gene and have to be genotyped to be distinguished from heterozygote and the wild type. After completion of the aforementioned project, the animals were euthanized in CO_2, chamber and then, 2 mm from the tail end were taken and stored at -20°C for two months until DNA extraction.

Two extraction methods were used: 1) the extraction by the proteinase K method (PKM), which uses proteinase K (20 ng/ml, Sigma®) and lysis buffer [12.2 g of Tris; 1.9 g of ethylenediaminetetraacetic acid (EDTA); 2 g of SDS and 11.7 g of NaCl to 1 l solution, with pH 8.5]; and 2) the Kappa Express Extract® kit (KappaBiosystem®). Twenty samples were used for each method, identified as 1-20 (PKM) and 1k-20k (kit).

In the PKM, 2.5 µl of proteinase K (0.0001 ng/µl in the reaction) were used, and the tail fragments were incubated in 500 µl of lysis buffer at 55°C, over night. After incubation, the DNA was precipitated with isopropanol (500 µl, Synth®) and centrifuged at 14,000 g, for 15 minutes; after that centrifugation, the supernatant was discarded and 100 µl of Tris-EDTA buffer (0.5 ml of Tris-HCl, 1 M, pH 8.0 and 0.1 ml EDTA; 0.5 M, pH 8.0) was added. The sample was subjected to a new centrifugation (14,000 g, for 10 minutes) and the supernatant was maintained[6]. In the extraction using the kit, 10 µl of 10× KAPA Express Extract Buffer®, 2 µl of KAPA Express Extract Enzyme® (1 U/µl) and 88 µl of ultrapure water were added to the kit. The samples were conditioned in the thermocycler so that the lysis step was performed (incubation at 75°C, for 10 minutes). After tail lysis, there was another stage in which the sample was heated at 95°C, for 5 minutes, for inactivation of the KAPA Express Extract® enzyme. After the stage of lysis and inactivation, the samples were centrifuged for one minute at the rate of 14,000 g, so that the debris from the extraction may precipitate. The supernatant, with the extracted DNA, was transferred to a new tube.

Determination of concentration and integrity of samples

After obtaining the DNA samples, their concentrations were measured by spectrophotometry in the NanoDrop 1000® device. To determine the purity of the sample, the ratios 260/280 and 260/230 were calculated. The integrity of the sample was established using a 2% agarose gel (2 g of agarose dissolved in 90 ml Tris/borate/EDTA (TBE) 1×, buffer with 3 µl de ethidium bromide). For the electrophoresis, 5 µl of the sample was used together with the same volume of bromophenol blue (BPB). The run was performed at a voltage of 40 mV, for 60 minutes[6].

PCR test

To test the extracted DNA and compare the two extraction methods, a multiplex polymerase chain reaction (PCR) was performed, which amplifies the normal and mutated alleles of the Large gene, simultaneously. The PCR amplification was performed in a volume of 25 µl, containing 20 µM of each primer, 40 ng of DNA and 0.5 unit of Taq DNA polymerase recombinant® (GE Healthcare®). The reaction conditions were: 2 min pre-incubation at 96°C, followed by 35 cycles of 96°C for 30 seconds, 59°C for 30 seconds and 72°C also for 30 seconds. The PCR products were analyzed on 2% agarose gel, using 5 µl of sample, together with the same volume of BPB; running was performed at 120 mV, for 30 minutes[7]. In homozygous individuals, there is amplification of the mutated allele (421 pb), whereas in normal individuals there is amplification only of the normal allele (162 pb). In the heterozygote, the two alleles are amplified. The sequence of the oligonucleotides (primers) used in the reaction are in **Table 1**[7].

Statistical analysis

The descriptive statistics were performed in the Microsoft Excel® software, while the inferential analysis was carried out in the Past3® software. The comparison test between the methods

used the non-parametric Mann-Whitney test, with a significance level of 5%.

TABLE 1 – Sequence of primers used in PCR for genotyping

Primer name	Sequence
MWTF	5'-GGC CGT GTT CCA TAA GTT CAA-3'
MWTR	5'-GGC ATA CGC CTC TGT GAA AAC-3'
MUTF	5'-ATC TCA GTC CCA AAG GGT GAA G-3'
MUTR	5'-GCC AAT GTA AAA TGA GGG GAA A-3'

PCR: polymerase chain reaction; MWTF: mouse wild type forward; MWTR: mouse wild type reverse; MUTF: mutation forward; MUTR: mutation reverse.

RESULTS

Samples extracted by the PKM showed a mean concentration of 59.4 ± 18.5 ng/µl with 260/280 and 260/230 ratios of 1.74 ± 0.04 and 1.85 ± 0.14, respectively (**Table 2** and **Figure 1**).

Samples extracted by the kit had a mean concentration of 178.8 ± 42 ng/µl with 260/280 and 260/230 ratios of 1.09 ± 0.04 and 0.62 ± 0.66, respectively (**Table 3** and Figure 1).

TABLE 2 – Concentration and ratios 260/280 and 260/230 of the samples extracted by the PKM

Sample	Concentration (ng/µl)	260/280	260/230
1	151.5	1.1	0.52
2	159.8	1.15	0.66
3	209.5	1.14	0.72
4	209	1.14	0.69
5	289.4	1.1	0.73
6	154.5	1.05	0.54
7	242.1	1.05	0.62
8	192.6	1.08	0.61
9	150.8	1.09	0.57
10	234.5	1.03	0.62
11	114.8	1.05	0.59
12	167.7	1.14	0.62
13	188.7	1.15	0.68
14	181.6	1.15	0.68
15	174.9	1.01	0.48
16	157.4	1.07	0.65
17	136.1	1.1	0.64
18	186.7	1.13	0.61
19	148.5	1.07	0.58
20	134.4	1.08	0.58
Median	171.3	1.1	0.62
Average	178.8	1.09	0.62
Standard deviation	42	0.04	0.06
Coefficient of variation (%)	23.5	3.96	10.47

PKM: proteinase K method.

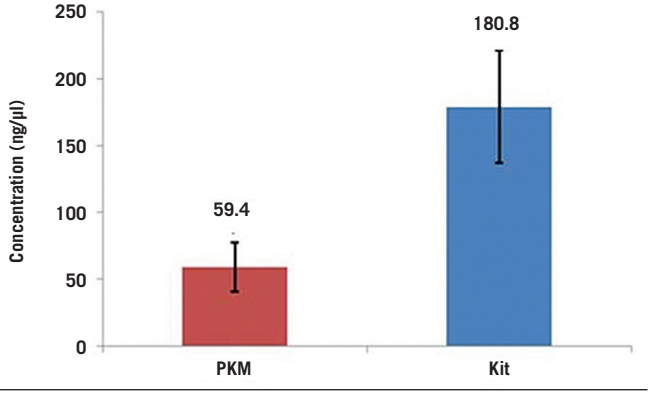

FIGURE 1 – *Mean values of the concentrations of samples extracted by the two methods PKM: proteinase K method.*

Although the concentration of the samples extracted by the PKM was on average lower (59.4 versus 178.8 ng/µl; $p < 0.05$), it was also observed that they had lower standard deviation. In both methods, there are two distinct concentration values: in extraction by the PKM, the concentration ranges, approximately, from 35.3-151.5 ng/µl, while with the kit, from 114.8-289.4 ng/µl (Figure 1).

Figure 2 shows the ratios 260/280 (Figure 2A) and 260/230 (Figure 2B) in the samples extracted by the PKM (260/280 =

TABLE 3 – Concentration and ratios 260/280 and 260/230 of the samples extracted by the kit

Sample	Concentration (ng/µl)	260/280	260/230
1	115.6	1.69	1.36
2	58.8	1.76	1.95
3	65.4	1.77	1.91
4	37.1	1.7	1.83
5	52.1	1.7	2
6	77.8	1.74	1.77
7	52.2	1.74	1.88
8	48.4	1.78	1.97
9	75.1	1.78	1.85
10	49.5	1.67	1.96
11	67.9	1.78	1.91
12	71.2	1.84	1.96
13	78.7	1.72	1.79
14	49	1.74	1.86
15	46	1.71	1.83
16	56.5	1.7	2
17	38.2	1.76	1.75
18	59.3	1.79	1.83
19	35.3	1.75	1.69
20	56.4	1.76	1.79
Median	56.5	1.75	1.86
Average	59.4	1.74	1.85
Standard deviation	18.5	0.04	0.14
Coefficient of variation (%)	31.2	2.38	7.78

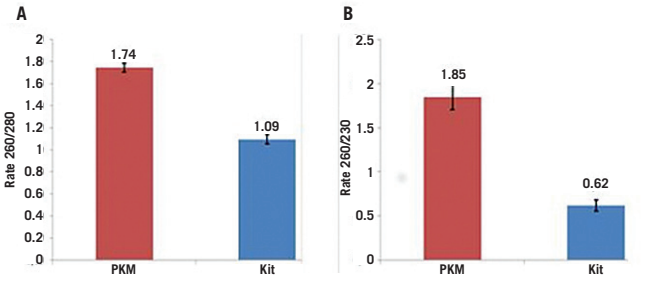

FIGURE 2 – *Ratios 260/280 (A) and 260/230 (B) in the methods used for extraction (*p < 0.05)*

PKM: proteinase K method.

1.74 ± 0.04; 260/230 = 1.85 ± 0.14) and by the kit (260/280 = 1.09 ± 0.04; 260/230 = 0.62 ± 0.06). The values observed in the samples extracted by the PKM are higher than those found in the extraction with the kit ($p < 0.05$).

As observed in **Figure 3A** (PKM), the bands are well defined in the gel, therefore presenting a greater amount of undegraded DNA. A 2% and 40 mV agarose gel was used to perform the electrophoresis. **Figure 3B** (kit) shows clear, slightly visible bands, demonstrating a possible degradation of the DNA contained therein. It is possible to observe that the bands have moved a lot compared to Figure 3A.

After the DNA extraction, PCR was performed for the Large gene, with the samples extracted by the PKM (1, 2 and 3) and by the kit (1k, 2k and 3k). In the homozygous (affected) individuals there is the amplification of the mutated allele (421 pb), while in the wild type, there is the amplification only of the normal allele (162 pb). In the heterozygote, the two alleles are amplified. Based on **Figure 4**, it is possible to observe that the PCR reaction worked. The control amplified properly and the blank did not show amplification, showing that there was no contamination during the reaction.

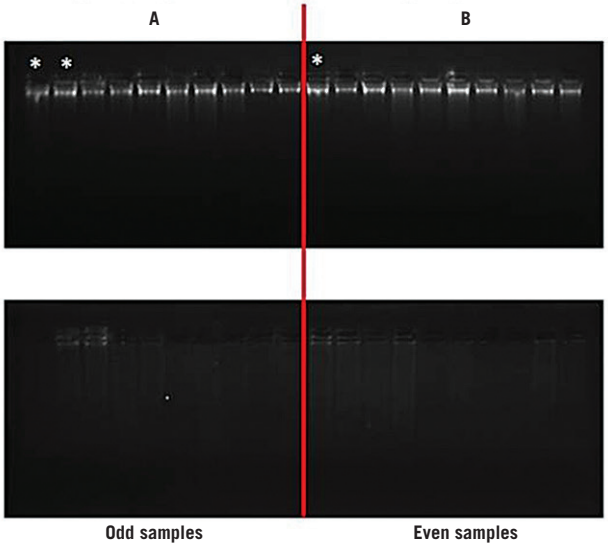

FIGURE 3 – *DNA bands run on 2% agarose gel*

A) PKM; B) kit.

DNA: deoxyribonucleic acid; PKM: proteinase K method.

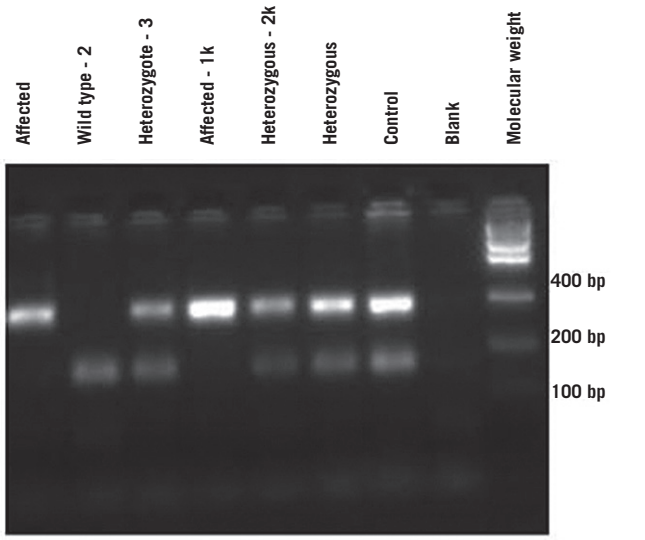

FIGURE 4 – *Genotyping in mouse tails, for the Large gene*

DISCUSSION

The use of fast, effective and manageable cost-effective rate protocols for genomic DNA extraction is important in the area of molecular biology. The present study aimed to compare the traditional method (PKM) and the kit (Kappa Extract Buffer®) regarding DNA integrity and concentration, which may influence many reactions, such as PCR. The extraction kits are usually expensive or not readily available, especially for researchers in developing and underdeveloped countries[8].

Most of the techniques used in molecular analyzes require good quality DNA, that is, not fragmented. Thus, the efficiency of these analyzes is essentially dependent on the quality and quantity of the extracted DNA, requiring a special consideration to the storing of the samples intended for use[4]. The tails in our study were duly stored at -20°C, ideal temperature for preservation according to Borges *et al*. (2015)[9], who investigated the interference of storage temperature in DNA degradation, quoting as ideal temperature -20°C or less. DNA isolation is significantly affected by the condition of the tissue prior to extraction. Therefore, it is recommended to use a material as fresh as possible[10]. Based on

this information, although the samples were collected in animals that were immediately euthanized, they were correctly stored for two months, which ensured good tissue quality, similar to the study developed by, Sollero *et al*. (2004)[11], in which they maintained the samples in absolute ethanol during the same timeframe.

The formulation of the reagents from the extraction kit is not available because it is an industrial secret. However, it is possible to infer that the reagents that compose it, probably a detergent solution and a proteinase enzyme, must be in high concentrations to extract the DNA of the sample quickly, however without the risk of degrading it to the point that it can not be used in a PCR. It is important to highlight that the kit was designed for genotyping in mice, a reaction of an exclusively qualitative nature. Silva *et al*. (2013)[12] extracted peripheral blood DNA from bovines using protocols with increasing amount of proteinase K in the reaction (0.04 ng/µl to 0.08 ng/µl); they observed that high concentrations of proteinase K in the reaction increase DNA degradation, that is, negatively influence its integrity. These results are similar to those in this study found in the samples obtained with the commercial extraction kit.

CONCLUSION

It was concluded that the two methods can be used for DNA extraction, since the PCR reactions worked perfectly. The factors that will influence directly in the choice are the binomial cost and time, which is related to the genotyping routine of the institution. However, it should be noted that a purest DNA is obtained by the PKM.

RESUMO

Introdução: *O ácido desoxirribonucleico (DNA) é a matéria-prima para os estudos genéticos, por isso são desenvolvidas técnicas laboratoriais para sua obtenção, com concentração e integridades adequadas.* ***Objetivo:*** *Comparar dois métodos de extração de DNA em relação à concentração e à integridade da amostra.* ***Métodos:*** *O DNA foi extraído da extremidade das caudas (2 mm de comprimento) de camundongos, as quais foram estocadas a -20°C. Utilizou-se a metodologia de extração com proteinase K (MPK) e o* kit *Kappa Express Extract®. As concentrações e as relações 260/280 e 260/230 foram determinadas por espectrofotometria. A integridade do DNA foi verificada em gel de agarose a 2%, com brometo de etídeo. Para o teste final das amostras extraídas, realizou-se reação em cadeia da polimerase (PCR)* multiplex*, com* primers *para o gene Large.* ***Resultados****: As amostras extraídas pela MPK apresentaram concentração média de 59,4 ± 18,5 ng/µl (260/280 = 1,74 ± 0,04 e 260/230 + 1,85 ± 0,14) e as extraídas pelo* kit *comercial, concentração média de 178,8 ± 42 ng/µl (260/280 = 1,09 ± 0,04 e 260/230 = 0,62 ± 0,66). A PCR amplificou o gene Large no DNA extraído, independente da metodologia utilizada.* ***Conclusão:*** *As metodologias estudadas podem ser utilizadas, sendo a MPK uma metodologia barata, porém demorada, enquanto o* kit *comercial é mais oneroso, contudo a extração do DNA é célere.*

Unitermos: *DNA; camundongos; técnicas de genotipagem.*

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