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Nucleic Acids Extraction from Formalin-Fixed and Paraffin-Embedded Tissues

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Abstract

Formalin-fixed paraffin-embedded (FFPE) tissues are an important sample source for retrospective studies. Despite its ability to preserve proteins and cell morphology, formalin hinders molecular biology tests since it fragments and chemically modifies nucleic acids, especially RNA. Although several studies describe techniques that allow extracting nucleic acids from FFPE tissues, so far there is no consensus in the literature about the best protocol to be used in this type of material. Thus, the current chapter aims to describe the factors affecting the FFPE tissue nucleic acid extracting process, compare the available protocols and to describe the modifications developed by our group in some protocols. Such modifications enable nucleic acids obtainment in satisfactory quantity and quality for molecular biology studies.

Keywords: DNA, RNA, FFPE, extraction

1. Introduction

Formalin-fixed paraffin-embedded (FFPE) tissues are of great importance to retrospective studies. Their main advantage lies on the possibility of correlating genetic and molecular biology analyses with data from patients' medical records and clinical outcomes [1].

Formalin fixation is the most widely used method for tissue fragment preservation. It is a low-cost and easy-to-handle method, which preserves good morphological cell quality. The method is compatible with the antibodies used in the immunohistochemistry technique [2]. However, although formalin fixation and routine histological processing techniques preserve tissue cellular morphology and protein integrity, they also impair the obtainment of

nucleic acids with the same quality, especially RNA, since they degrade and chemically modify such acids [3, 4].

Formalin replacement by other tissue fixatives such as Bouin, Carnoy, alcohol or HOPE (glutamic acid buffer Hepes-mediated organic solvent effect protection) may be an alternative to reverse the problem [4, 5]. However, since formalin is the fixative of choice in most pathology departments, several research groups have sought to reverse the chemical changes caused by this fixative type.

Although several papers describe techniques that allow FFPE tissue nucleic acid extracting process [6–8, 3, 1, 4], so far there is no consensus in the literature about the best protocol to be used in this type of material.

The studies based on such approach not often detail the used methodology. Besides, not all the published techniques were reproduced by our group. It took us approximately 8 months to standardize the DNA and RNA extraction process in our FFPE tissues research.

The current chapter aims to describe the factors affecting the process of nucleic acid extraction from FFPE tissue, compare the available protocols and to describe the modifications developed by our group in some protocols that enable nucleic acids obtainment in satisfactory quantity and quality for molecular biology studies.

2. Factors affecting nucleic acids extraction from FFPE tissues

Difficulties in obtaining quality nucleic acids, especially RNA, cause degradation and chemical modification, despite the fixation in formalin and the routine histological processing techniques used to preserve cellular morphology and tissues' protein integrity [3–4].

Nucleic acids extraction from FFPE tissue shows some critical issues that may affect the quality of the obtained DNA or RNA as well as these samples' subsequent amplification process. Such critical matters include tissue fixing and clamping as well as the post-fixing stage (tissue cutting preparations, deparaffinization and hydration processes, in addition to other stages of the extraction process itself, such as digestion and purification), which will be described below [9].

Pre-fixation is defined as the period between tissue collection and the beginning of the setting process. The material starts degrading shortly after its collection, right when the tissue is exposed to hypoxia and to the DNases and RNases found in the environment. The first biochemical modifications emerge after 10 min of anoxia. Thus, it is very important to reduce the pre-clamping time to seconds [4, 9].

The fixing conditions (time, temperature and fixative type) and, in some cases, the descaling processes alter material preservation and directly influence the quantity and quality of the obtained nucleic acid. Fixations kept in formalin solution for more than a week destroy the nucleic acids and lead to the cross-linking of all tissue components. It results in highly fragmented nucleic acids, which are more resistant to the extraction process [2, 3, 9].

Chemical studies have shown that formaldehyde breaks hydrogen bonds in double-stranded DNA adenine- and thymine-rich regions. It creates new chemical interactions in protein folding, thus resulting in bonds between DNA proteins and DNA fragmentation [10].

RNA messenger (mRNA) obtained from FFPE tissues is often not intact. It is usually degraded to less than 300 base pairs [1]. However, Hamatani et al. (2006) [11] found that 80% of the RNA samples presenting 60 base pairs may be sufficiently amplified by polymerase chain reactions (PCR). All post-fixation stages, such as the paraffin blocks attainment sections, are also essential to the obtainment of high-quality nucleic acids.

Contamination is one of the critical issues affecting the quality of the samples. Thus, it is necessary to decontaminate the workstation as well as to use DNases- and RNases-free tools. These DNases and RNases result from paraffin block cuts used to extract the nucleic acid of interest [2].

Although some authors state that deparaffinization is not a required step [2], most protocols suggest that the material must be deparaffinized before the extraction process in order to obtain nucleic acids in a more efficient way. Most protocols use solvent (usually xylene) to remove the paraffin cuts, and this procedure is followed by ethanol-based rehydration.

No matter the used protocol, digestion is the first step in the nucleic acid extraction process, and it aims to lyse membranes in order to release the cellular components. This step may be accomplished by several methods, such as elevated temperatures, enzymatic digestion, mechanical disruption or even by using other detergents or according to cell type solutions. In general, enzymatic digestion with proteinase K is used in most protocols; however, the concentrations and the incubation times are highly variable [9].

Nucleic acids purification is the next stage. Literature reports protocols using organic solvents (such as phenol-chloroform) [12–14], salt (salting out) [15] and other substances (Chelex-100) [16] as well as protocols using commercial kits available in the market [9].

Li et al. (2008) [1] observed that RNA extraction protocols based on proteinase K digestion followed by DNase, column purification and elution treatments led to good results in FFPE samples. These authors have shown that proteinase K is essential to degrade covalently linked proteins in order to release RNA from the cell array and to inactivate RNases, which tend to be stable.

Ribeiro-Silva and Garcia (2008) [4] have shown that proteinase K is used to degrade proteins bound to nucleic acids and that the incubation between 60°C and 70°C removes the methylol groups added by formalin. RNA isolation by denaturing agents prevents the RNases action. In addition, deoxyribonuclease (DNase) incubation is required to remove the deoxyribonucleic acid (DNA) sample. Finally, purification by precipitation with alcohol porous column removes any residue and contaminants.

All tested protocols will be detailed in the sections below, as well as the changes suggested to determine the protocols that would be viable to the obtainment of nucleic acids presenting adequate quality for molecular biology studies.

3. Preparing FFPE tissue sections for nucleic acids extraction

All samples included in the studies conducted by our team result from biopsies performed in diffuse large B cell lymphoma patients, from lymph node samples or from reaction amygdala samples stored in the Pathology Department of Hospital das Clínicas, Medical School of University of São Paulo. All the herein used samples were formalin-fixed and paraffin-embedded (FFPE) according to the standard methods described in the literature.

Four 20- μm thick cuts were performed in each sample using routine histological techniques. The sections were placed into 1.5 mL RNase- and DNase-free microtubes, and they were subsequently subjected to RNA and DNA extraction processes.

The first protocol used to prepare the cuts in the nucleic acid extraction process (suggested for the majority of commercial kits and protocols) consisted of deparaffinizing the sections with xylene and of rehydrating them with ethanol. In order to do so, 1 mL xylene PA was added to each sample (Synth® Diadema, SP, Brazil), and it was followed by homogenization using Vortex Genie 2T (Scientific Industries, Inc., Bohemia, NY, USA) and by incubation at 50°C, for 5 min, in digital thermomixer (Eppendorf AG, Hamburg, Germany). After incubation, the samples were centrifuged at maximum speed for 5 min in the R-5418 microfuge (Eppendorf AG, Hamburg, Germany). The xylene was discarded and the cell button was washed two times with absolute ethanol (Merck KGaA, Darmstadt, Germany). The supernatant was discarded after each wash. After the cell button was completely dried, the extraction process started, as it is described in the following sections.

Moreover, even after some RNA extraction methods that allowed finding some viable options were compared, it was observed that not all the extracted samples showed successful amplification in PCR reactions [17].

None of the previous studies available in the literature described the possible factors that could influence the amplification success. Therefore, the current study made the option of investigating some of the potential interferences in the nucleic acid obtainment process, namely: tissue fragment size, blocks' storage time, used fixative type, different cDNA synthesis primers and different primer sequences, among others [18].

After the aforementioned study, the tissue preparation protocol for RNA extraction process was modified by including a washing step. It consisted of using 1 ml phosphate buffer saline (PBS) with 5 min incubation at room temperature, followed by full speed centrifugation for 5 min in the R-5418 microfuge (Eppendorf AG, Hamburg, Germany). This procedure is done to remove possible fixative residues that could work as PCR inhibitors [18]. Next, the same process was used in the DNA extraction performed by our team.

Such protocol change was done under the assumption that the amplification failure in PCR reactions could be caused by the presence of contaminants such as fixative waste working as PCR inhibitors.

The results show that the PBS washing step inclusion in the samples' extraction preparation process led to some statistically significant advantages such as the obtainment of better

RNA concentration results ($p = 0.00025$), even when the same initial amount of tissue was used. In addition, the washing step allowed obtaining better sample purity levels ($p = 0.0000001$), increasing the samples amplification success ($p = 0.018$) both in the standard and in the real-time PCR reactions [18].

Two possible factors may have influenced the improved amplification efficiency of these samples. The first is based on the fact that formalin is water-soluble, thus the PBS washing step may have led to tissue-fixation residues' solubilization and removal, and it could possibly work as PCR inhibitors. Furthermore, previous studies suggested that pH values between 6.5 and 9.0 are optimal for amplification [11, 9]. Then, the PBS solution may have possibly altered pH levels, thus increasing the quality of the obtained RNA and the amplification success.

Despite the influence of the PBS-based washing step addition on the samples' successful amplification, we observed the fixative interference in this process too. Formalin-fixed samples showed more successful PCR amplification reactions than those fixed in formaldehyde or in Bouin's solution, even after further PBS washing ($p = 0.000018$) [18].

Another fact observed by our team refers to the paraffin-embedded tissue fragment size. All fragments size equals to or greater than 1.0 cm had the most successful samples' amplification ($p = 0.034$) [18]. This may be possibly due to the fact that smaller tissues may increase the fixative absorption and, consequently, cause greater nucleic acids degradation and chemical modification in the full extent of the tissue.

No impact was found on the amplification success when the other variables suggested in our study were tested (tissue type, block age, different primers used in the cDNA synthesis or the endogenous genes used in the PCR reaction), regardless of the use (or not) of the PBS washing step [18].

This protocol change was kept in the preparation of samples subjected to DNA extraction, due to the statistically significant impact caused by the PBS washing step inclusion on either the obtainment of better RNA concentrations or purity relations.

It is possible to conclude that this sample preparation is an essential step to obtain better quality nucleic acids for molecular biology studies.

4. RNA extraction from FFPE tissues

Three different RNA extraction protocols were tested, as described below:

Protocol 1: Commercial kit RECOVERALL Total Nucleic Acid Isolation Optimized for FFPE Samples (Ambion, Inc., Austin, Texas, USA) [17–18]

Two hundred (200 μ l) of digestion buffer and 5 μ L protease were used for tissue lysis in each sample. It was followed by incubation for 15 min at 50°C and for 15 min at 80°C. Next, RNA isolation was held by the addition of 790 μ L buffer containing absolute ethanol and by

the subsequent passage through the separation column. After washing the column with two wash buffers, realized treatment with DNase and further washing with two buffers, according to the manufacturer's instructions. The eluted RNA was obtained by using 60 μL of the kit elution buffer at RT. After incubation for 5 min at room temperature, the samples were centrifuged at maximum speed and the obtained RNA was stored at -80°C until its use.

Protocol 2: Paradise®Whole Transcript RT Reagent kit System (Arcturus Bioscience, Inc., Mountain View, California, USA) [17]

Incubation with buffer containing proteinase K was held for 20 h, at 37°C , after the samples' preparation process, to digest the proteins. The RNA isolation was performed by two successive washes using buffer containing the ethanol kit. Then, the samples were purified by passing them through the column kit according to the manufacturer's instructions. Subsequently, samples were incubated with buffer containing DNase at 37°C for 15 min and at 4°C for 1 min. DNase inactivation was performed by incubating the samples at 70°C for 10 min and at 4°C for 1 min. The RNA samples were stored at -80°C until they were used.

Protocol 3: Trizol extraction method (Invitrogen, UK) [17]

The RNA extraction by Trizol method was performed as recommended by Körbler et al. (2003) [8] and Antica et al. (2010) [19]. The tissue was digested by incubating the samples in buffer containing 10 mM NaCl, 500 mM Tris pH 7.6, 20 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS) and 500 $\mu\text{g}/\text{mL}$ proteinase K. First, the sample was incubated at 55°C , for 3 h; then, it was incubated at 45°C overnight with proteinase K inactivation by elevating the temperature to 100°C for 7 min in the next day. Finally, all samples were subjected to RNA extraction process according to the classic Trizol method previously described by Chomczynski and Sacchi (1988) [20]. The obtained RNA was stored at -80°C until it was used.

4.1. Evaluating RNA concentration and quality

NanoDrop equipment (NanoDrop Technologies, Inc. Wilmington, DE) was used to evaluate the concentration and purity of RNA samples extracted according to the three protocols described above. RNA amounts above 50 $\text{ng}/\mu\text{L}$ with purity between 1.7 and 1.9 were considered to be suitable.

4.2. Results and comments on the herein described RNA extraction protocols

The purity levels and degrees obtained by the three protocols were satisfactory (over 50 $\text{ng}/\mu\text{L}$ and purity concentrations between 1.8 and 1.9). However, only samples obtained according to protocols 1 (Ambion) and 2 (Arcturus Bioscience) showed appropriate amplification in real-time PCR reactions [17].

Despite the RNA produced with appropriate concentrations and purity degrees, the samples obtained by the Trizol method showed no amplification in real-time PCR reactions. It corroborates the results found by Witchell et al. (2008) [5], but it did not confirm the data obtained by Körbler et al. (2003) [8] and Antica et al. (2010) [20].

After these results were published, it was decided to check the impact of the residuals and potential contaminants. Thus, the purification step using alcohol in a porous column of the QIAamp®Viral RNA Mini Kit for commercial extraction (Qiagen) was adopted. In brief, after isopropyl alcohol was added to the samples, DNA was filtered using the purification column, according to the manufacturer's recommendations. It was done by transferring the samples to the Kit's purification columns, which were centrifuged at 8,000 g for 1 min. Subsequently, the column products were moved to other tubes. Next, 500 µL buffer AW1 was added to column and it was once again centrifuged at 8,000 g for 1 min. After the filtrate was discarded and the column transferred from the column to another tube, 500 µL Buffer AW2 was added to the solution and a new centrifugation was performed at 14,000 g for 3 min. Then, the column was shifted into a sterile 1.5 mL tube and 60 µL elution buffer was added to it. After the reaction was incubated for 5 min at RT, the tubes were centrifuged at 8,000 g for 1 min and the obtained RNA was stored at -80°C.

A significant improvement was found in the quality of the samples as well as in their adequate amplification in real-time PCR reactions. Thus, RNA extraction from FFPE samples of the three tested protocols became feasible. However, protocol 1 (Ambion commercial Kit) was used as the standard method in current research by taking into account the protocol's practicality and cost.

5. DNA extraction from FFPE tissues

Two different RNA extraction protocols were tested (unpublished data) as described below.

Protocol 1: Phenol-chloroform method [21]

After the sections were prepared and the button cell was completely dried as described in Section 3, tissue digestion was performed by adding 480 µL Tris-EDTA buffer (TE) and 20 µL proteinase K (200 mg/ml) to each sample. Next, these samples were incubated at 37°C for approximately 16 h in the digital thermomixer (Eppendorf AG, Hamburg, Germany). The temperature was then raised up to 90°C for 10 min to inactivate the proteinase K.

One (1) ml of the mixture was added to phenol:chloroform:isoamyl alcohol (Invitrogen Corporation, Carlsbad, CA, USA) at the ratio 25:24:1, respectively. After homogenization using the Vortex Genie 2T (Scientific Industries, Inc., Bohemia, NY, USA), samples were centrifuged at 13,000 g for 15 min at the 5418-R microcentrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was transferred to a fresh 1.5 ml DNase- and RNase-free microfuge tube, and 1 mL of the phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added to it. Then, the centrifugation process was repeated under the same conditions.

The supernatant was transferred to a new 1.5 mL DNase- and RNase-free microtube and 20 µL of 3M sodium acetate and 900 µL of absolute ethanol (Merck KGaA, Darmstadt, Germany) were added to it. The samples were homogenized by inversion and incubated at 20°C for at least 30 min. The samples were centrifuged at 13,000 g for 15 min. The supernatant

was discarded and the pellet was completely dried. Samples were resuspended in 50 mL TE buffer and stored at -20°C , until they were used.

Protocol 2: Phenol-chloroform method (modified by our team)

By following the same reasoning used to modify the RNA extraction protocol by Trizol method, the current study made the option to add a DNA purification step using the QIAamp DNA Blood Mini Kit commercial columns (Qiagen, Hilden, Germany) to check the impact from residual and potential contaminants.

As it was described in the previous protocol, the samples were transferred to the column commercial kit – QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), after being incubated with 3M sodium acetate and absolute ethanol. Subsequently, they were centrifuged at 8,000 rpm for 1 min in the 5418- R microfuge (Eppendorf AG, Hamburg, Germany). The filtrate was discarded, and the column was transferred to a new tube and 500 μL of buffer AW1 were added to it. Then, the samples were centrifuged at 8,000 rpm for 1 min in the 5418-R microcentrifuge (Eppendorf AG, Hamburg, Germany).

After the filtrate was discarded and the column transferred to a new tube, a second wash was performed using 500 μL buffer AW2. The samples were centrifuged at 14,000 rpm for 3 min in the 5418-R microcentrifuge (Eppendorf AG, Hamburg, Germany).

Columns were transferred to a 1.5 mL DNases- and RNases-free microtube and 200 μL of buffer AE were added to the center of the column. The samples were then incubated at room temperature for 5 min, and it was followed by centrifugation at 8,000 rpm for 1 min in the 5418-R microfuge (Eppendorf AG, Hamburg, Germany). The samples were stored at -20°C until they were used.

5.1. DNA quality quantification and analysis

The DNA samples' concentration and purity were set by spectrophotometry in NanoDrop®ND-2000 machine (Thermo Fisher Scientific, Wilmington, DE). Samples with absorbance ratios ($A_{280} / A_{260\text{nm}}$) between 1.7 and 1.9 were considered to be appropriate.

5.2. Results and comments on the herein described DNA extraction protocols

Despite the DNA produced with suitable concentrations and purity degrees, not all the samples obtained by the phenol-chloroform method showed amplification in real-time PCR reactions. It corroborated the results by Witchell et al. (2008) [5], but it did not confirm the data obtained by Körbler et al. (2003) [8] and Antica et al. (2010) [19].

A significant improvement was observed in the quality of the samples and adequate amplification in real-time PCR reactions performed according to method 2. Thus, DNA extraction from FFPE samples of the three tested protocols has become more viable. However, taking into account both the protocol practicality and the cost, the protocol 1 (Ambion commercial Kit) was used as the standard method for the current research.

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