Comparison of Analytical Characteristics of Commercial and in-House Methods for DNA Isolation from Paraffin Histology Blocks

Natalya Oskina[1,*], Ashot Avdalyan[2], Dmitriy Subbotin[3], Alexandr Lazarev[2], Alexandr Kel[1], Nikolay Kushlinskii[4] and Maxim Filipenko[1,3]

[1]Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences, Novosibirsk, Russia

[2]Altai Affiliated Department of Blokhin Cancer Research Center, Barnaul, Russia

[3]Novosibirsk State University, Novosibirsk, Russia

[4]Russian Blokhin Cancer Research Centre, Moscow, Russia

Abstract: One of the hotspots in clinical research today is molecular genetic analysis of structural DNA alterations. Working with DNA obtained from formalin-fixed and paraffin-embedded (FFPE) tissue specimens is particularly challenging, due to cross-linking and fragmentation of DNA. We performed a comparative analysis of DNA extraction methods from FFPE tissue using two in-house protocols and Qiagen (QIAamp DNA FFPE Tissue Kit) and Roche (High Pure FFPET DNA Isolation Kit) commercial kits presented in the Russian market and used in clinical practice. To assess the quantity and quality of the isolated DNA, we used the real-time PCR to rate DNA yield, the inhibited impurity content and the degree of fragmentation. Our findings may be useful for a medical laboratory that performs testing of somatic mutations for the targeted therapy selection, and researchers who specialized in tumor genome structure studies in respect to patient prognosis and prediction of the sensitivity or resistance of tumor cells to therapy.

Keywords: DNA extraction, formalin-fixed and paraffin-embedded (FFPE) tissue blocks, QIAamp DNA FFPE Tissue Kit, High Pure FFPET DNA Isolation Kit, PCR.

INTRODUCTION

Currently, molecular genetic tests have become frequent in clinical practice, including oncology. Any biological material containing nucleic acids can be used for such studies, in particular, formalin-fixed and paraffin-embedded (FFPE) tissue.

FFPE tissue preparation appeared, approximately 100 years ago, as a method permitting long-term tissue preservation. And certainly, the isolation of nucleic acids was not considered. The current personalized medicine needs analyzing of FFPE tissues when fresh clinical material is unavailable to study various biomarkers used for targeted therapies and prognosis. Today, using formalin (the most widely used fixative for routine histology) is the critical point of the isolation of DNA from FFPE tissues. Formaldehyde (the main component of formalin) is highly reactive with DNA bases and proteins, generating various types of damage: histone–DNA crosslinks, formaldehyde–DNA adducts, DNA–protein crosslinks, and DNA–DNA crosslinks; uracil and thymine, which result from deamination of cytosine and 5-mC; DNA bases are also lost, resulting in a basic sites, and DNA strands are broken, leading to fragmentation of DNA [1, 2]. Thus nucleic acids obtained from FFPE tissue are generally of poor quality. The degree of degradation of these samples limits a performance of analysis techniques involving amplification. Appropriate methods must reliably and sufficiently preserve nucleic acid integrity while eliminating impurities.

There are many different protocols and their modifications for DNA extraction from FFPE tissue. Most of them contain three steps: deparaffinization, digestion, and purification. Nevertheless, this division is conditional, for example, incubation of the samples at high temperatures for dewaxing also results in cell lysis [3, 4]. Each step of DNA extraction may include a series of centrifugations, washes, and transfers from tube to tube that could increase the likelihood of contamination. Thus, reduction of steps number results in a decrease in the risk of contamination and errors. For example there is a commercial kit (QuickExtract ™ FFPE DNA Extraction Kit from Epicentre Biotechnologies) that allows DNA extraction procedure using 1 step. However, this approach consistently requires high-quality FFPE tissues. Unfortunately, this single-step approach is often inapplicable in clinical practice because the FFPE tissues are heterogeneous in terms of processing and tissue type.

Originally, deparaffinization was performed by dissolution of the wax in xylene followed by rehydration

*Address correspondence to this author at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, 8 Lavrentiev Avenue, Novosibirsk, 630090, Russia; Tel: +79139825039; Fax: (383) 363-51-53; E-mail: nattasha.o@gmail.com

ISSN: 1927-7210 / E-ISSN: 1927-7229/17 © 2017 Lifescience Global

with alcohols [5]. Currently, there are many different deparaffinization protocols: using microwaves [6], heating with Chelex-100 and Tween-20 [7], etc. Heating of the samples at dewaxing step improves DNA extraction [7, 8]. Boiling FFPE tissue sections in alkaline solution with detergents may significantly improve quality of extracted DNA [9, 10]. However, DNA yields are lower than those obtained by the classical enzymatic method [9]. Commercial DNA extraction kits commonly combine proteinase K digestion with solid-phase DNA purification and uses xylene for deparaffinization step [11]. Of course, the key disadvantage of all commercial kits for DNA extraction from FFPE is their high cost. In-house methods for DNA extraction from FFPE represent good alternative to commercial ones. We have been successfully using in-house” method based on slightly modified protocol described by Shen-Rong Shi *et al*. [9, 10].

In this work, we performed a comparative analysis of DNA extraction methods from FFPE tissue using two in-house protocols and two commercial kits presented on the Russian market and used in clinical practice.

MATERIALS AND METHODS

A total of 10 randomly chosen archival tissue blocks from patients with colorectal adenocarcinoma were included in this study. None of the blocks were older than 4 years. All tissues had been fixed in 10% neutral buffered formalin. Two dry sections, 5 nm thick, were sliced using a microtome and placed in a 1.5-mL microfuge tube three times per sample. The first cut, sections (2-3), was discarded to avoid contamination. The microtome and blade were carefully cleaned between each block to prevent sample-to-sample contamination.

DNA from each FFPE sample was isolated in four different ways: by two in-house methods and two commercial kits - Qiagen (QIAamp DNA FFPE Tissue Kit), and Roche (High Pure FFPET DNA Isolation Kit). The negative control was performed for each method (empty tube without sample) to exclude contamination. The manufacturer's instructions were followed for DNA isolation by the QIAamp DNA FFPE Tissue Kit (Qiagen) and High Pure FFPET DNA Isolation Kit (Roche). The sections were deparaffinized with xylene. The final elution volume was 50 µl as well as for in-house methods.

For the in-house protocol, dewaxing was performed by incubating samples at 98°C for 20 min in 0.1M NaOH and 0.5% Tween-20 solution (in an incubator Eppendorf ™Thermomixer™R). After centrifugation at 14000 rpm (15400xg) during 5 minutes, the aqueous layer was transferred to a new tube for DNA isolation by in-house method H1, DNA from the remaining precipitate was isolated by in-house method H2.

In-house method H1. Equal volumes of phenol and chloroform (200 µl) were added to the result, after dewaxing aqueous layer, and thoroughly vortexed. After centrifugation at 14000 rpm (15400xg) for 10 minutes, the aqueous layer was transferred to a new tube and he 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol with linear polyacrylamide as carrier (5µl 0.5%) (no comma)were added. After a thorough mixing, tubes were incubated at -20°C for 1 hour. Then the tubes were centrifuged at maximum speed for 10 minutes. The supernatant was discarded and the pellet washed with 1 ml 70% ethanol twice and dried at 37C 20 minutes. The pellet was dissolved in 50 µl 10мM Tris-HCl pH 8.0.

In-house method H2. All pellets (after dewaxing) were digested with proteinase K (3 µ g/µl) in 300 µl digestion buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, and 1% SDS), by incubating at 56°C for 12 hours (or until the samples were completely lysed). Equal volumes of phenol and chloroform (200 µl) were added and vortexed. After centrifugation at 14000 rpm (15400xg) for 10 minutes, the aqueous layer was transferred to a new tube. The 0.1 volume of 3M sodium acetate and 1 volume of isopropanol with linear polyacrylamide as carrier (5µl 0.5%)(no comma) were added. After a thorough mixing, it was incubated at -20°C for 1 hour. Then the tubes were centrifuged at maximum speed for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol twice dried at 37°C for 20 minutes. The pellet was dissolved in of 50 µl 10мM Tris-HCl pH 8.0.

The obtained DNA was analyzed by quantitate real-time PCR. We used two PCR systems to amplify fragments of KRAS gene - different length (95bp and 217bp). PCR mixture (20 µl) contained 300 nM primers, 100 nM of TaqMan probes, 65 mM Tris-HCl (pH 8.9), 16 mM $(NH_4)_2SO_4$, 2.5 mM MgCl2, 0.05% Tween-20, 0.2 mM dNTPs, 2 µl DNA and 0.5 units Taq polymerase (hot-start, ICBFM SB RAS). Primers and probes are shown in Table **1**. All samples were tested in duplicates.

The control DNA was obtained from leukocytes of venous blood using the standard procedure including

Table 1: Oligonucleotide Primers and Probes for PCR

Amplicon length, bp	Oligonucleotide primers	TaqMan probes
95	F 5'-GACTGAATATAAACTTGTGGTAGTTGGA -3' R 5'-CATATTCGTCCACAAAATGATTCTG -3'	FAM-CTGTATCGTCAAGGCACTCTTGC-BHQ1
217	F 5'- GTGTGACATGTTCTAATATAGTCACATTTTCA-3' R 5'-AAAGAATGGTCCTGCACCAGTA-3'	FAM- CTGTATCGTCAAGGCACTCTTGC-BHQ1

blood cell analysis, protein digestion with proteinase K, DNA purification by phenol-chloroform extraction following DNA precipitation with ethanol.

RESULTS AND DISCUSSION

There are no generally accepted recommendations for the selection of nucleic acid extraction methods for laboratories. We compared four DNA extraction methods (2 in-house protocols and 2 commercial kits, which were widely spread in the Russian market) from ten identically processed FFPE colorectal adenocarcinoma tissues. We used real-time PCR to assess the quantity and quality of isolated DNA, since the spectrophotometric and the fluorometric methods, both commonly used for DNA quantification, have several disadvantages. Spectrophotometric analysis may be inaccurate in FFPE samples because it can overestimate the quantity due to the presence of degraded DNA and RNA. Fluorometric DNA assessment only detects double-stranded DNA, but even double-stranded DNA may not equate an amplifiable template due to crosslink and fragmentation during formalin fixation.

To estimate the quantity of released DNA and the presence of PCR inhibitory impurities, we performed the quantitative PCR (qPCR) for amplifying 95bp fragment of the KRAS gene. To assess the presence of inhibitory impurities we carried out a comparative analysis of changes in the threshold cycle (Ct) at the

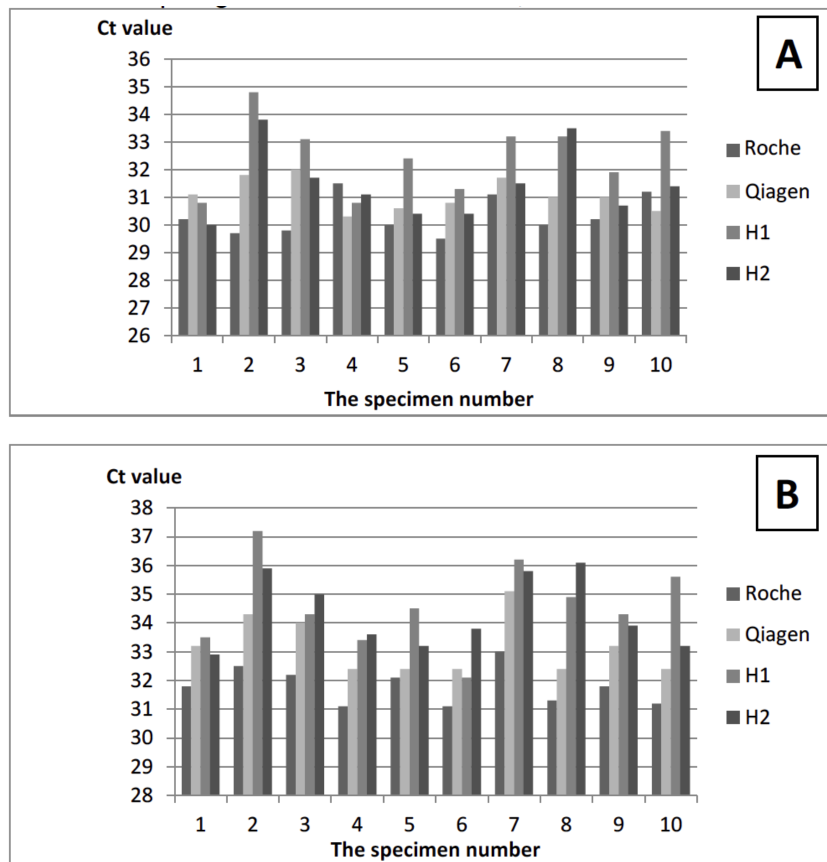

Figure 1: The Ct values of the individual DNA samples obtained by quantitative PCR for 95bp fragment. A - for stock DNA, B - for 1/4 dilution DNA.

amplification of the stock and 1/4 dilution DNA. Same procedure was performed for control high purity DNA. The obtained results are shown in Figure **1** and Table **2**.

Table 2: Mean Ct Values with Standard Deviation (SD), Obtained by qPCR for 95bp Fragment

Method	Stock DNA	
	Ct	SD(Ct)
Roche	30.32	0.69
Qiagen	31.08	0.58
H1	32.49	1.29
H2	31.45	1.28

Linear regression models were used to construct calibration curves. The efficiency of PCR in the absence of inhibitors (it was assumed that the highly purified control DNA does not contain them) was 84%, while the shift Ct (dCt) DNA by 4-fold dilution was 2.31±0.11. We supposed that the DNA dilution could reduce the effect of impurities, so the dCt should be smaller (compared to dCt by diluting control DNA). The obtained results of Ct for specimens with and without dilution are shown in Figure **2**. The mean dCt values and standard deviation (SD) are shown in Table **3**. Finally, to assess the DNA fragmentation, we used the qPCR for amplifying 217 bp fragment of the KRAS gene. The obtained Ct values are shown in Table **4**.

Table 3: The Mean dCt Values and Standard Deviation (SD) for 95bp Fragment Obtained by 4-Fold Diluction of DNA Samples

Method	dCt	SD(dCt)
Roche	1.49	1.00
Qiagen	2.1	0.55
H1	2.11	0.69
H2	2.89	0.71
The control DNA	2.31	0.11

According to the results, the commercial kits (Roche and Qiagen) showed higher DNA yield (lower Ct) and less variability (Table **2**). Despite lowest Ct value shown with DNA samples isolated with the Roche kit (30.32 +0.69) there were no statistical significant differences with the Qiagen ones (Paired Wilcoxon Signed Rank test, two-tailed, $p<0.05$). The DNA obtained by the Roche kit had the most amounts of PCR inhibitory impurities that was characterized by

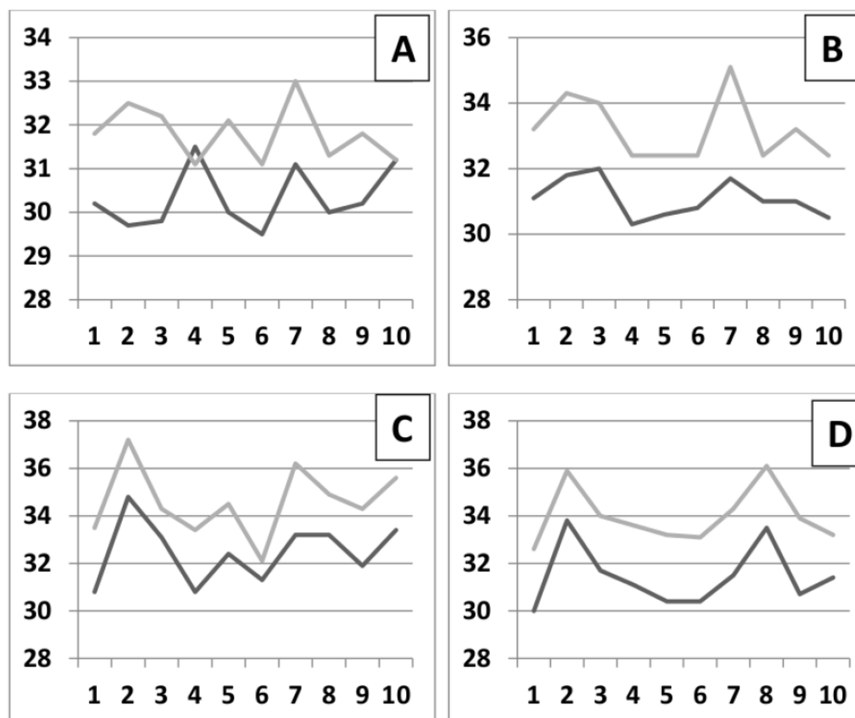

Figure 2: The graphical representation of Ct values for specimens with and without dilution (95bp fragment).

A - Roche, **B** - Qiagen, **C** - H1, **D** - H2.

The light line - 4-fold diluted DNA samples; the dark line - stock DNA.

On the horizontal axis - the specimen number. On the vertical axis – the Ct value.

Table 4: Ct Values in Duplicate, Obtained by qPCR for Amplifying 217bp Fragment of Gene KRAS (Ct Value of Control DNA was 29.5)

Method	The specimen number									
	1	2	3	4	5	6	7	8	9	10
Roche	-	41.4	41.9	-	41.5	-	43.3	-	-	-
	-	42.4	42.5	-	41.1	-	43.5	-	-	-
Qiagen	-	-	42.1	-	43.6	-	-	-	-	44.4
	-	-	41.3	-	43.2	-	-	-	-	43.6
H1	44.5	-	-	42.1	43.4	-	-	44.0	41.6	-
	45.0	-	-	40.4	43.4	-	-	43.2	41.8	-
H2	-	39.3	39.9	39.6	44.6	-	44.2	41	42.8	39.8
	-	38.4	39.1	40.8	45.0	-	44.9	39.6	43.5	39.2

lowest dCt of four folds diluted DNAQs (1.49+1.00) (Table **3**). It should be noted that the presence of PCR inhibitory impurities could be especially problematic for degraded or low amounts of template DNA when a simple resolving dilution of the template would be undesirable.

The most significant differences between the used methods were observed for amplification of 217 bp fragments. The Qiagen set had the worst result (4 positive results from 10) and in-house method H2 had the best one (8 positive results from 10) (Table **4**). Yet there are not statistically significant differences between abovementioned method, possibly because of the small size of the studied groups of samples (Fisher's exact test, p=0.17). In general, no one method had an acceptable result for amplification the long fragment. Some important characteristics for selection DNA extraction method are shown in Table **5**. The eventual decision in choosing a particular method will of course also depend on the price and required accompanying lab equipment. For example in the Russian Federation the cost for one DNA sample isolation will be about 8 and 6.2 $ using Qiagen and Roche kits and not to exceed half a dollar for in-house methods described in present paper.

It is important to note the quality of nucleic acids from FFPE is highly influenced by pre-analytical tissue processing. It is well known that the level of degradation of DNA in FFPE depends on tissue composition, prefixation conditions, fixative solution, fixation time and storage period [12-14]. Despite of publication of few guidelines tissue-handling processes, we steel meet very different quality of specimens for molecular diagnostic tests. This problem can be solved in part by choosing reliable and robust method for DNA extraction. At the present time, DNA isolated from FFPE tissues are also used for the next-generation sequencing (NGS) analysis. This method required high quality and more accurate DNA assessment for fragmentation [15]. Our findings may

Table 5: Some Important Characteristics of DNA Extraction Methods were Considered in this Paper. From "A" to "D" - from Best to Worst Result

Method	Time spent on job (hours)	DNA yield	Fragmentation of DNA	Presence of PCR inhibitory impurities	Special requirements	Cost (per specimen)
Qiagen (QIAamp DNA FFPE Tissue Kit)	3 - 4	B	C	B	no	High
Roche (High Pure FFPET DNA Isolation Kit)	3 - 4	A	C	D	no	High
In-house H1	2 - 3	D	B	C	Fume Hood (to work with a phenol)	Low
In-house H2	3 - 4	C	C	A	Fume Hood (to work with a phenol)	Low

be useful to medical laboratories that perform testing of somatic mutations for the targeted therapy selection, and to researchers who specialized in tumor genome structure studies with respect to patients' prognosis and prediction of the sensitivity or resistance of tumor cells to therapy.

FUNDING

Targeted Program 'Research and development on priority directions of science and technology in Russia, 2014–2020' [contract No. 14.604.21.0101, code 2014-14-576-0109, a unique identifier of the applied scientific project: RFMEFI60414X0101].

REFERENCES

[1] Rait VK, Zhang Q, Fabris D, Mason JT, O'Leary TJ. Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society [Internet] 2006; 54(3): 301-10. https://doi.org/10.1369/jhc.5A6725.2005

[2] Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. American journal of clinical pathology [Internet] 1991; 95(2): 117-24. https://doi.org/10.1093/ajcp/95.2.117

[3] Stein A, Raoult D. A simple method for amplification of DNA from paraffin-embedded tissues. Nucleic acids research [Internet] 1992; 20(19): 5237-8. https://doi.org/10.1093/nar/20.19.5237

[4] Coates PJ, d'Ardenne AJ, Khan G, Kangro HO, Slavin G. Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. Journal of clinical pathology [Internet] 1991; 44(2): 115-8. https://doi.org/10.1136/jcp.44.2.115

[5] Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochemical and biophysical research communications [Internet] 1985; 130(1): 118-26. https://doi.org/10.1016/0006-291X(85)90390-0

[6] Banerjee SK, Makdisi WF, Weston AP, Mitchell SM, Campbell DR. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. BioTechniques [Internet] 1995; 18(5): 768-70, 772-3.

[7] Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic acids research [Internet] 1999; 27(16): e12. https://doi.org/10.1093/nar/27.16.e12

[8] Frank TS, Svoboda-Newman SM, Hsi ED. Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. Diagnostic molecular pathology : the American journal of surgical pathology, part B [Internet] 1996; 5(3): 220-4. https://doi.org/10.1097/00019606-199609000-00012

[9] Shi S-R, Cote RJ, Wu L, Liu C, Datar R, Shi Y, *et al.* DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society [Internet] 2002; 50(8): 1005-11. https://doi.org/10.1177/002215540205000802

[10] Shi S-R, Datar R, Liu C, Wu L, Zhang Z, Cote RJ, *et al.* DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochemistry and cell biology [Internet] 2004; 122(3): 211-8. https://doi.org/10.1007/s00418-004-0693-x

[11] Kocjan BJ, Hošnjak L, Poljak M. Commercially available kits for manual and automatic extraction of nucleic acids from formalin-fixed, paraffin-embedded (FFPE) tissues. Acta dermatovenerologica Alpina, Pannonica, et Adriatica [Internet] 2015; 24(3): 47-53. https://doi.org/10.15570/actaapa.2015.12

[12] Bonin S, Hlubek F, Benhattar J, Denkert C, Dietel M, Fernandez PL, *et al.* Multicentre validation study of nucleic acids extraction from FFPE tissues. Virchows Archiv : an international journal of pathology [Internet] 2010; 457(3): 309-17. https://doi.org/10.1007/s00428-010-0917-5

[13] Ludyga N, Grünwald B, Azimzadeh O, Englert S, Höfler H, Tapio S, *et al.* Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Archiv : an international journal of pathology [Internet] 2012; 460(2): 131-40. https://doi.org/10.1007/s00428-011-1184-9

[14] Funabashi KS, Barcelos D, Visoná I, e Silva MS, e Sousa MLAPO, de Franco MF, *et al.* DNA extraction and molecular analysis of non-tumoral liver, spleen, and brain from autopsy samples: the effect of formalin fixation and paraffin embedding. Pathology, research and practice [Internet] 2012; 208(10): 584-91. https://doi.org/10.1016/j.prp.2012.07.001

[15] Yu B. Setting up next-generation sequencing in the medical laboratory. Methods in molecular biology (Clifton, NJ) [Internet] 2014; 1168: 195206. https://doi.org/10.1007/978-1-4939-0847-9_11

Received on 08-12-2016 Accepted on 02-01-2017 Published on 26-01-2017

https://doi.org/10.6000/1927-7229.2017.06.01.1