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Quality Assessment of DNA Extraction Methods from Formalin-Fixed Paraffin-Embedded Tissues

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ABSTRACT DNA extraction from archived formalin-fixed paraffin-embedded (FFPE) tissues represent a great source of information. However, till date, it shows some challenges due to its fragmentation and degradation by formalin. The aim of this study was to assess 3 extraction methods used with 651 FFPE namely QIAamp® DNA FFPE Tissue Qiagen Kit (80%), MagaZorb® DNA Mini-Prep Kit Promega (17.8%) and phenol-chloroform classical extraction (2.2%). Extract concentration mean was 240.05 ng/µl (7-1985 ng/µl) and DNA purity (A260/A280) mean was 1.98 (1.2 - 4). The classical phenol-chloroform method had the most successful DNA concentration ($P < 0.001$) but had the worst DNA purity ratio while the Promega and Qiagen kits DNA purity ratio were within the norms ($P < 0.001$). This assessment showed that even with commercial kits, we should always adjust some parameters to have good DNA extracts which is the first step towards a successful molecular analysis.

INTRODUCTION

Currently, molecular pathology is widely used especially with the emergence of personalized medicine. Thereby, during the course of clinico-pathological diagnosis, millions of formalin-fixed paraffin-embedded (FFPE) tissues are collected and archived in different hospitals. These FFPE tissue banks represent a source of information, which can disclose genetic events involved in different clinical conditions. However, only high-quality nucleic acid can be used for downstream molecular application. Thereby many commercial kits for diagnostic molecular pathology testing are becoming available. Still there are some challenges with the DNA extraction due to its fragmentation and degradation into small fragments and cross-linking with protein in FFPE samples (Lindeman et al. 2013; Kresse et al. 2018). Thus, there is a need for quality assessment and evaluation in every laboratory that uses DNA extracted from FFPE in molecular analysis.

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Objectives

In this retrospective study, the researchers assessed the quality of 651 DNA extracts from FFPE tissues stocked in the researchers' archive department since 1990 and used in pyrosequencing assays during the period 2015-2017. Three methods of sampling were used as well as three methods of DNA extraction.

MATERIAL AND METHODS

Tumor Specimens and Tissue Processing

The researchers' cohort was composed of FFPE tissues taken made from colorectal tumors in 298 cases (45.8%), gallbladder tumors in 127 cases (19.5%), thyroid tumors in 116 cases (17.8%), pancreatic tumors in 89 cases (13.7%) and ampullary tumors in 21 cases (3.2%). Methods of sampling for the DNA extraction from 651 FFPE were as follows: films for FFPE of surgical specimens in 271 cases (41.6%), films for FFPE of biopsy specimens in 48 cases (7.4%) and films from recipient blocks after macrodissection in 332 cases (51%). Macrodissection and production of recipient blocks was explained in our previous study (2). DNA extraction was performed

by QIAamp® DNA FFPE Tissue Qiagen in 521 cases (80%), by MagaZorb® DNA Mini-Prep Kit Promega in 116 cases (17.8%) and by classical extraction using phenol-chloroform in 14 cases (2.2%). From each bloc, 20 sections of 3 µm thick were obtained to be used in the extraction.

Extraction Using QIAamp® DNA FFPE Tissue Qiagen

Genomic DNA extraction was performed according to QIAamp® DNA FFPE Tissue Kit from Qiagen, GmbH, Hilden, Germany manufacturer's handbook with prolongation in the lysis time. Briefly, sections of 3µm were deparaffinized using xylene and resuspended in an appropriate amount of tissue lysis buffer and proteinase K, then incubated at 56°C for 24h. The entire lysate was transferred to the QIAamp Minelute column. During centrifugation, the DNA binds to the membrane and contaminants flow through. Next, residual contaminants were eliminated with wash steps. After elution buffer addition, a full-speed centrifugation was performed to collect a pure and concentrated DNA.

Extraction Using MagaZorb® DNA Mini-Prep Kit Promega

Genomic DNA extraction was performed according to Kit (MagaZorb® DNA Mini-Prep Kit, Promega, Madison, Wisconsin, USA) manufacturer's handbook with prolongation in the lysis time. Briefly, sections of 3 µm were deparaffinized using xylene and resuspended in an appropriate amount of tissue lysis buffer and proteinase K, then incubated at 56 °C for 24h. Steps of DNA binding released from MagaZorb® paramagnetic beads, double wash and elution were subsequently performed.

Phenol-chloroform Classical DNA Extraction

Deparaffinization of FFPE samples was performed by xylene (1 ml of xylene, vortex and centrifugation at high speed for 3 min; supernatant discarded). DNA was extracted using the standard phenol-chloroform method. Briefly, 4 steps were performed as follows: Lysis: addition of 300ìl digestion solution (50 mM Tris-HL (pH: 7), 10 mM EDTA, 0.5% SDS and 50 mM NaCl) and 20ìl of proteinase K (300ìg/ml); then vortex solution was maintained at 65 °C for 48 to 72 h (FFPE; until complete lysis). Proteinase K was inactivated at 90 °C for an hour.

DNA Binding: addition of 300ìl phenol-chloroform solution (24ì phenol-chloroform (1:1) and 1ìl of the isoamyl alcohol); tubes were stirred gently for an hour. A 14000 rpm/30 min centrifugation was performed to facilitate aqueous phase transfer into a new sterile tube.

DNA Precipitation: addition of 1/10v sodium acetate (3M, pH: 5.2) and 2 percent cold absolute ethanol. Tubes were placed at -20 °C overnight. A 14000rpm/30 min centrifugation was performed to separate the pellet. An amount of 100 ìl of nuclease free-water was added to dilute the pellet.

Extraction Quality Assessment

After each extraction, whatever the method, quality control extraction was performed using a nanodrop (IMPLEN, Thermo Fisher Scientific).

Statistical Analysis

All data were analysed by SPSS 20.0 software (Chicago, IL, USA). P value less than 0.05 was considered statistically significant.

RESULTS

The mean of DNA extract was 240.05 ng/µl (7-1985 ng/µl) and the median was 125 ng/µl. For the DNA purity (A260/A280), the mean was 1.98 (1.2 - 4) and the median was 1.2. As shown in Tables 1 and 2, in terms of DNA extract concentration, the classical phenol-chloroform method was the most successful, followed by the Qiagen kit and the Promega Kit ($P < 0.001$). However, the classical phenol-chloroform method had the worst DNA purity ratio while the Promega and Qiagen kits DNA purity ratio were within the norms ($P < 0.001$).

Regarding the influence of the sampling method DNA purity, all means were within the norms (1.95, 2.02 and 2.00) ($P = 0.021$) while it strongly influenced on DNA extract concentration. In fact, films from recipient blocks after macrodissection seem to give the most valuable concentration followed by films of FFPE from

Table 1: Influence of DNA extraction method on DNA extract quality and quantity

Influence of DNA extraction method on DNA extract quality		*DNA extract concentration (ng/µl)*	*P*	*DNA purity (A260/A280)*	*P*
DNA extraction method	Kit Qiagen (521)	243.47 (±298.95)	< 0.001	2,02 (±0.22)	< 0.001
	Phenol-chloroform (14)	731.71 (±431.66)		1.44 (±0.04)	
	Kit Promega (116)	165.35 (±166.51)		1.88 (±0.33)	

Table 2: Influence of sampling method on DNA extract quality and quantity

Influence of sampling method on DNA extract		*DNA extract concentration (ng/µl)*	*P*	*DNA purity (A260/A280)*	*P*
Sampling method	Films of FFPE from surgical specimens (271)	271.00 (±368.28)	< 0.001	1.95 (±0.22)	0.021
	Films of FFPE from biopsy specimens (48)	202.05 (±173.82)		2.02 (±0.13)	
	Films from recipient blocks after macrodissection (332)	332.00 (±140.88)		2.00 (±0.29)	

surgical specimens and films of FFPE from biopsy specimens ($P < 0.001$).

DISCUSSION

In the great majority of pathology departments in the world, specimens are FFPE. Although nucleic acids of FFPE samples are degraded into small fragments, it is essential for us to extract high-quality nucleic acids with optimum concentration and suitable purity (Lindeman et al. 2013; Kresse et al. 2018).

Since DNA molecules of FFPE samples crosslink with each other and DNA is also crosslinking with RNA and protein cross-linking must be broken to release the DNA for subsequent purification. If possible, the modification caused by chemical crosslinking should also be reversed, as the chemically modified DNA cannot be recovered in the purification process, and it is also a poor substrate in other enzymatic or PCR analysis (Lindeman et al. 2013; Kresse et al. 2018).

As expected, in the researchers' quality assessment the classical phenol-chloroform technique was the most successful in terms of DNA concentration but this method has 2 essential drawbacks: its low DNA purity and its long time comparing with the 2 other kits.

For the 2 kits used in this study, they had a comparable DNA purity and concentration. But, even after prolongation of the lysis time to 24 hours, they were not still productive like the phenol-chloroform technique. In one study, authors found that boiling FFPE tissue sections in 0.1 M alkaline solution is a simple and more effective heat-induced retrieval protocol for DNA extraction. They also found that combination with some detergents may further significantly improve the efficiency of the heat-induced retrieval technique (Shi et al. 2004).

In the researchers' routine practice, they prolong the lysis time to have more concentrated DNA extracts. The same measure was recommended after a quality assessment in 13 European laboratories (Bonin et al. 2010) as modifications of the proteinase-K digestion time led to better results, even when commercial kits were applied.

It was proved that specimens with poor tumor cellularity may lead to false-negative results in molecular testing (Dudley et al. 2015). In addition to making the proper histologic diagnosis, the pathologist is responsible for requesting the necessary molecular testing, selecting appropriate tissue blocks and designating areas with an adequate amount of tumor cells for microdissection or macrodissection (Lindeman et al. 2013). In the researchers' laboratory, they performed macrodissection in most cases. In the other cases, the researchers selected the richest blocks in neoplastic tissue. In this assessment, the DNA extraction from recipient blocks after macrodissection was the most successful technique in terms of DNA extract concentration.

In the guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology, the fresh or frozen specimens are recommended as they are optimal for analysis of long DNA segments (>1000 bp). However, this seems unnecessary for the researchers' laboratory testing because the pyrosequencing requires small DNA segments (<200 bp) (www.qiagen.com). One drawback to testing fresh or frozen tissues is the need for correlative histologic examination, which may require cutting and staining frozen sections, flanking the portion of the specimen submitted to testing. In contrast, the use of FFPE material for DNA extraction allows a more convenient and accurate assessment of tumor content (Lindeman et al. 2013).

DNA extraction from FFPE tissues is then a robust procedure that relies on differential solubility to extract DNA. Extracted DNA quality and quantity and the success of subsequent DNA amplification does not only depend on the DNA extraction method but also on a number of parameters before, during and after extraction. These include, but are not limited to the type and amount of tissue, the type of fixative, the duration of fixation, the age of the paraffin block and on the storage conditions, as well as on the length of the desired DNA segment to be amplified. Removal of paraffin from the tissue is the most critical step towards a successful extraction for undissolved paraffin leads to poor sample quality and inhibits PCR amplification (Pikor et al. 2011).

However, one study that systematically examined the quality/quantity of genomic DNA, total RNA, and total protein in the FFPE blocks of malignant tumors of lung, thyroid, and salivary glands that had been stored over several years, showed that there was no significant difference between macromolecules extracted from blocks stored over 11–12 years, 5–7 years, or 1–2 years in comparison with the current year blocks (Kokkat et al. 2013).

CONCLUSION

Molecular pathology requires appropriate selection of neoplastic tissues, use of assays with high sensitivity and regular quality assessment. The first step towards a successful molecular analysis is a DNA extract with a good balance of quantity and quality. This assessment study showed that even with commercial kits, we should always adjust some parameters to have good DNA extracts. As a matter of fact, the researchers recommend the use of Phenol-chloroform classical extraction in laboratory on a routine practice as it is the Gold-standard.

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