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From Cell Biology to Tissue Engineering

An optimized xylene-free protein extraction method adapted to formalin-fixed paraffin embedded tissue sections for western blot analysis

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Summary. Deparaffinization of formalin-fixed paraffin embedded (FFPE) tissues with xylene currently remains a major challenge to the biomedical community. We developed an efficient xylene-free protocol to isolate proteins from archived FFPE human tissue sections. A total of 79 different types of FFPE tissue sections of 8 μm thickness were obtained from various archived FFPE specimens. Deparaffinization was conducted by gently washing each section with around 1 ml of hot distilled water (≈80°C). The deparaffinized tissues were homogenized in lysis buffer, and the isolated proteins were quantified and efficiently resolved using western blot analysis for the presence of Protein kinase B (PKB/AKT) and β-actin. Moreover, a significant amount of proteins was successfully isolated with an average of 2.31 μg/μl. The migration pattern of AKT and β-actin obtained from the specimens was similar to the positive control obtained from protein lysates prepared from *in vitro* cultured MDA231 cancer cell lines. AKT was successfully identified in all specimens, and β-actin protein was resolved with an efficiency higher than 80%. The entire extraction procedure requires only 20 minutes. This newly developed technique is an efficient, safe, cost-effective, and rapid method to isolate proteins from FFPE tissue sections adequate for molecular analysis.

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Introduction

Formalin fixed paraffin embedding of tissues is the most common procedure adopted by health institutions for the storage of clinical samples. While formalin plays an important role in the long term preservation of the sample's architecture and stability for later pathological analysis, paraffin facilitates the microtome slicing of the samples (Fox et al., 1985). FFPE tissues are widely available and easily accessible. Also, they can be stored under ambient conditions for longer periods of time with no noticed reduction in tissue integrity during staining procedures. Billions of archived FFPE tissue samples are available all over the world along with their clinical data providing an important source for molecular diagnosis. However, the lack of optimized molecular tools to efficiently isolate proteins limits the number of tests that can done using FFPE. Although a small number of trials succeeded in extracting RNA and DNA from FFPE tissues, protein extraction from FFPE remains a major challenge due to the crosslinks formed between the proteins following formalin fixation and lengthy storage time of the blocks (Sutherland et al., 2008). The first method of protein extraction that was described by Shi et al. in 1991, applied heat for antigen retrieval (Shi et al., 1991). Several other methods have been developed to extract proteins from FFPE tissues, but the disadvantage associated with these protocols is the routine use of

"xylene" as a deparaffinization agent. Moreover, they are time consuming and associated with a low protein yield and quality. Also, other attempts to isolate protein from FFPE tissues without the use of xylene have been described (Ikeda et al., 1998; Kunkle et al., 2008; Loiacono et al., 2010; Nicholson, 2011; Rodriguez-Rigueiro et al., 2011). The protein extraction methods described in these studies did not involve xylene as a deparaffinization agent, but they required multiple snap-freeze (ethanol on dry ice) in addition to multiple boiling steps of the samples with lysis followed by a series of centrifugation in order to separate the paraffin while pelleting the tissue. The development of these techniques is based on the assumption that not all the proteins can be extracted using the same conditions, and the use of xylene may affect the stability or the optimal conditions needed to extract other important proteins. But all these technique faced the challenge of removing paraffin without impairing the extraction yield as well as the integrity of the isolated proteins (Kunkle et al., 2008; Loiacono et al., 2010; Nicholson, 2011; Rodriguez-Rigueiro et al., 2011). Interestingly, none of these procedures used distilled water as a deparaffinization agent. We previously established a novel xylene-free method to deparaffinize FFPE tissue blocks using hot distilled water, and succeeded in the extraction of proteins in high yield and quality good enough to be used for western blot analysis (Mansour et al., 2014). One of the disadvantages encountered was the use of the entire block to perform the extraction procedure. To address this issue, we established an optimized, fast, simple and reproducible procedure for the extraction of proteins from FFPE tissue sections that are up to 7 years old and of only 8 μm thickness that can be efficiently used for western blot analysis. We have shown in our previous study that protein extraction can be successfully extracted from FFPE tissue blocks using hot distilled water that replaced xylene as a deparaffinization agent (Mansour et al., 2014). The present study further confirms this basic principle of using hot distilled water as a powerful deparaffinization agent. The novelty of the protocol is in its efficient application on tissue sections, its reproducibility, cost-effectiveness, as well as the high yield and quality of the extracted proteins. A significant finding in this study was the minimum amount of tissue required, which is equivalent to an 8 μm section from each type of tissue. In conclusion, we believe that this new method will enable efficient protein isolation from FFPE sections suitable for routine molecular use, including western blot analysis.

Materials and methods

Tissue specimens

Formalin fixed paraffin embedded tissue sections from different organs were obtained from the pathology department at the Lebanese American University – Medical Center – Rizk Hospital (LAU-MC-RH). A total of 79 archived tissue specimens, which were stored in paraffin for the indicated time, were obtained and include: 36 colorectal cancer (5-7 years), 12 breast cancer (3 years), 3 thyroid cancer (2 years), 4 ovarian cancer (2 years), 1 ganglion (4 years), 10 uterine cancer (1 year) and 2 uterus (2 days), 1 kidney (1 day), bone (5-7 years), 1 stomach (5-6 years), 3 pancreatic (2 years) and 5 prostate cancer (1 year). The tissue specimens' cross sections were of 8 μm. The research project was approved by the Institutional Review Board (IRB) at the Lebanese American University.

Lysis buffer

Lysis buffers used in the experiment were prepared as follow: 10 ml of lysis buffer containing the following components: 50 mM Tris–HCl adjusted to a pH=7.4 (0.5 ml of 1 M), 1% Triton-X (10 ml of stock), 0.2% Sodium deoxycholate (50 mg), 1 mM disodium EDTA (20 ml 0.5 M), 0.2% SDS (100 ml of SDS 20%), Adjust the final volume with H_2O to obtain a final volume of 10 ml. PMSF buffer: Prepare 100 mM of PMSF (phenylmethylsulfonyl fluoride) by adding 174 mg in 10 ml of ethanol, isopropanol or methanol and directly store at -20°C. 10 ml of 2X Laemmli sample buffer was prepared by mixing the following components: 1.2 ml of 1 M Tris–HCl (pH=6.8), 4 ml of glycerol (50%), 4 ml of 10% SDS and 0.8 ml H_2O. β-mercaptoethanol 3%, Bromophenol blue 0.2% (Sigma-Aldrich) (Mansour et al., 2014).

Deparaffinization

Tissues are sectioned using a microtome. Slides with paraffin sections are placed on a warming block, which melt the wax and thus allows the adherence of the tissue on the slide. The deparaffinization process was performed with hot distilled water (approx. 80°C). Hot distilled water was used to replace both the toxic organic solvent xylene as well as the serial ethanol washes done thereafter. The paraffin was washed off the slide by adding 1ml of hot distilled water dropwise. Then, the tissue specimen was scraped off the slide using a clean cell scraper and transferred into a clean 1.5 ml tube thereafter.

Tissue lysis, protein extraction and quantification

A volume of 200 μl of lysis buffer containing the PMSF solution was added to the 1.5 ml microcentrifuge tube containing the specimen along with 200 μl of Laemmli 2x buffer (volume ratio of 1:1). Then, 3% (v/v) of β-mercaptoethanol was added to the final mixture. The samples were then incubated at 100°C for 8-10 minutes with intermittent vortexing every 2-3 minutes. Finally, the samples were centrifuged at high speed in microcentrifuge at 13000 rpm for 5 minutes at room temperature, and the supernatant was collected into a new clean 1.5 ml microcentrifuge tube. Total protein

concentration was quantified using the Thermo Scientific NanoDrop Spectrophotometers (NanoDrop 2000c) at 280 nm A graphical presentation presented in Fig. 1 summarizes the procedure of protein extraction. Measurement of protein concentration was achieved using both NanoDrop and the colorimetric Bio Rad DC protein assay (similar to Lowry assay) according to the manufacturer's protocol, and results are summarized in (Table 1).

Western blot

Western blot was performed as described previously (Mansour et al., 2014). Briefly, each well was loaded with 35 μg of the protein lysates and were separated onto 10% denaturing SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF) thereafter. The membranes, blocked with 5% skim milk in TBST for 1 hour, were washed and then incubated at 4°C overnight with primary antibody buffers that contained 5% TBST (1X), skimmed milk and anti-β-Actin polyclonal antibody (1:1500) or anti-Akt-1 polyclonal antibody (1:4000). Anti-β-actin and anti-Akt antibodies were purchased from Abcam. The membranes were washed 3 times with 50ml TBST (1X) every 5 minutes and then incubated with HRP-coupled secondary anti-rabbit antibody (1:2000) for 1 hour at room temperature. Finally, the membranes were washed 3 times every 5 minutes with 50 ml TBST (1X). Protein detection was performed using the ECL kit (Abcam plc, 330 Cambridge Science Park, Cambridge UK). Blot images were obtained with the Image Lab Software (BioRad, Chemidoc imaging instrument).

Results

In this study, we performed protein isolation using hot distilled water for deparaffinization of FFPE tissue sections from archived specimens for up to 7 years. To evaluate the efficiency of this improved protocol we measured the protein concentrations and tested the lysate using western blot analysis. First, the yield of the protein extraction was evaluated by measuring the total amount of protein in each sample using the NanoDrop. We extracted proteins from a single section of FFPE with a thickness 8 μm tissue section with an approximate area of 150 mm^2. As expected, the tissue sections yielded a high concentration of proteins with an average amount of 2.31 $\mu g/\mu l \pm 0.83$ or 924 μg/tissue section. The yields ranged from 0.51 mg/ml to 4.46 mg/ml for all specimens. The different amounts of proteins obtained from each type of tissue are presented in (Table 2). Further on, we performed western blot analysis to ensure the successful extraction of proteins of high quality. The results of the western blot analysis of β-actin and Akt-1 obtained from sections of archived specimens revealed clear bands with a migration pattern similar to positive control samples (Fig. 2). Control samples were obtained from protein lysates prepared from *in vitro* cultured MDA 231 cells in a 6-wells plate. Interestingly, the bands were clearer and more intense compared to the control samples. Protein kinase B (PKB/AKT) was

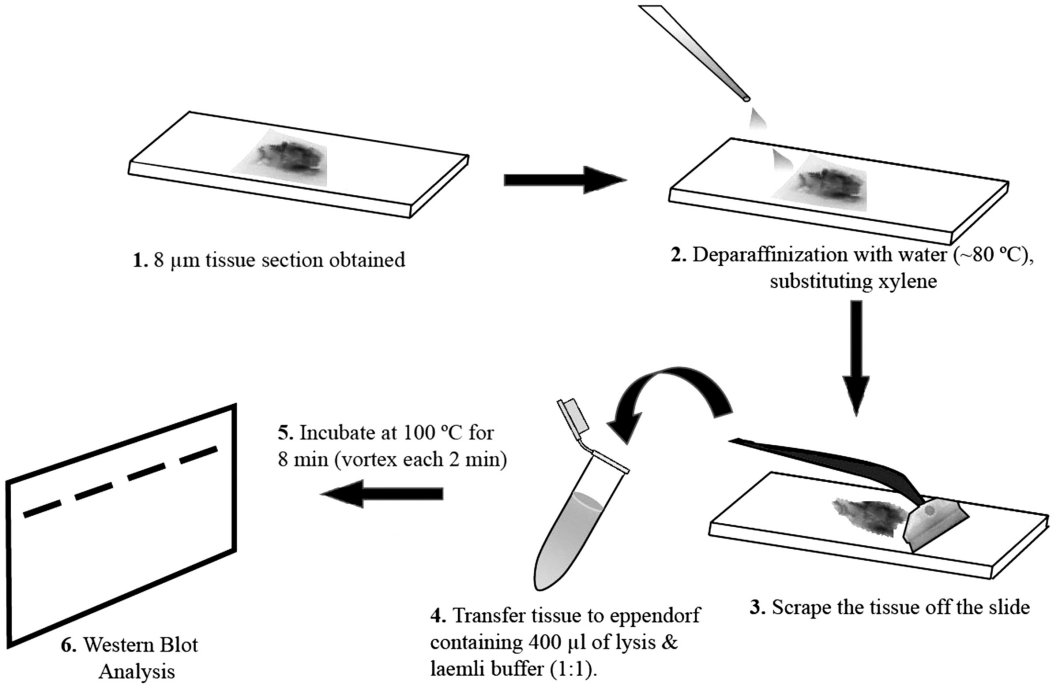

Fig. 1. Graphical presentation of the procedure for the extraction of proteins from FFPE tissue sections.

successfully identified in all specimens (38/38), while beta-actin protein was resolved with an efficiency higher than 80% (56/66). Alternatively, Figure 3 shows the results of western blot analysis for β-actin using protein lysates obtained from processing 2 fresh specimens embedded in paraffin for no more than 2 days. In Table 1 we present the protein concentration values prepared at the same time of fresh (3 tissues) and archived (10

Table 1. Protein concentrations of both fresh and archived 8 μm FFPE tissue sections (150-200 mm^2) using 2 different protein quantification methods: Nanodrop and Bio Rad DC Protein Assay (similar to Lowry Assay).

Type of tissue	Protein conc. NanoDrop (mg/ml)	Protein conc. DC Protein Assay (mg/ml)	Fresh embedded tissues	Archived tissues
Uterus	0.82	1.35	X	
Uterus	0.81	1.01	X	
Kidney	0.83	0.66	X	
Stomach	0.89	0.46		X
Colon	0.76	1.70		X
Colon	0.93	0.49		X
Colon	1.08	3.09		X
Uterus	0.90	1.53		X
Uterus	0.86	1.35		X
Breast	0.21	0.14		X
Bone	0.92	2.91		X
Breast	0.87	0.83		X
Ganglion	1.06	2.74		X

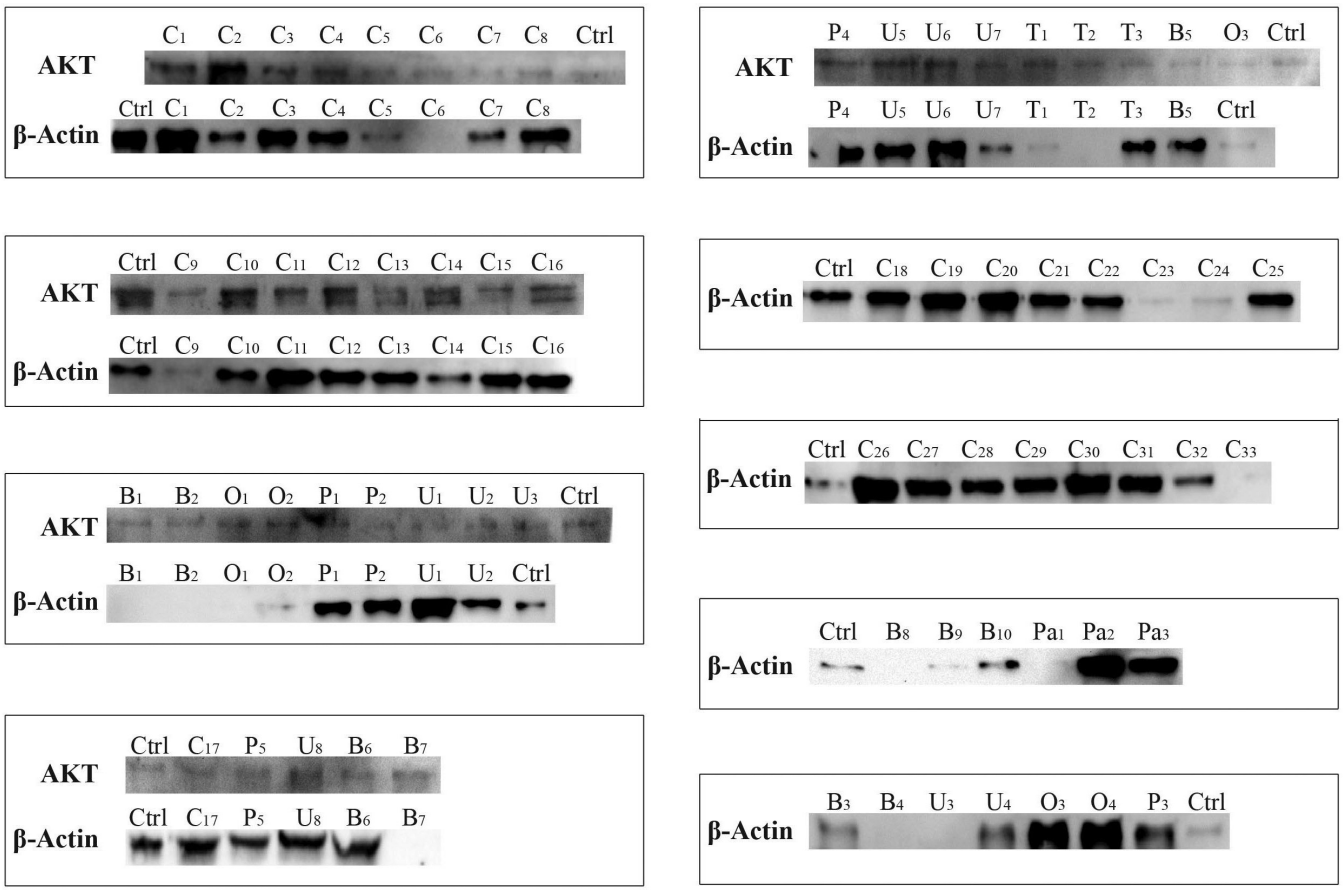

Fig. 2. Protein expression of β-Actin and Akt in different FFPE cancer tissue sections of 8 μm as compared to control (Ctrl) protein isolated from MDA 231 cell lines. C: Colorectal Cancer; B: Breast Cancer; O: Ovarian Cancer; P: Prostate Cancer; U: Uterine Cancer; T: Thyroid; Cancer Pa: Pancreatic Cancer.

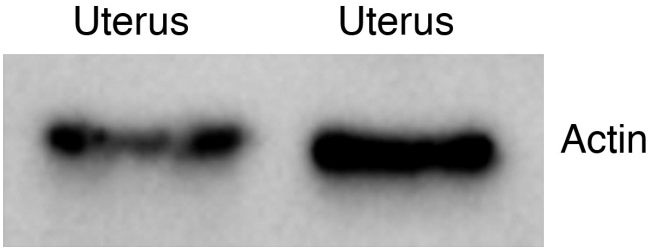

Fig. 3. Protein expression of β-Actin in 2 samples obtained from 2 different sections of fresh uterus specimens and embedded in paraffin for 1-2 days.

Table 2. Protein yield from various 8 μm FFPE tissue sections (150 mm^2).

Tissue	Protein Conc. (mg/ml)	Protein yield μg/tissue section
Colon	2.12±0.41	848 μg
Thyroid	2.58±0.41	1032 μg
Ovarian	2.92±0.39	1168 μg
Uterus	2.66±0.72	1064 μg
Breast	2.17±1.00	868 μg
Prostate	2.95±0.68	1180 μg
Pancreas	2.03±0.36	812 μg
Average	2.31±0.83	924 μg

Table 3. Summary of different methods for extracting proteins from FFPE tissue and their yield.

Qproteome kit: 105 μg/10 μm section of Colon cancer (500 mm^2)
Qproteome kit: 25-80 μg/10 μm section of breast cancer (100 mm^2)
Becker et al. 2006: 150 μg/10 μm tissue section (100 mm^2)
Wolff et al. 2007: range: 52 -170 μg/10 μm tissue section (50 mm^2)
Nirmalan et al. 2011: 33 μg/10 μm kidney tissues (25 mm^2)
Chung et al. 2008: 250-300 μg/20 μm prostate tissue section

tissues) samples measured using both NanoDrop and colorimetric DC Protein Assay (similar to Lowry assay). Interestingly, around 60% of the sample concentrations obtained using DC protein assay were higher than the concentrations calculated by NanoDrop.

Discussion

Isolation of proteins from FFPE sections suitable for various molecular biology applications remains a major challenge. Several studies have shown that sample lysis at high temperature with the presence of a strong detergent such as sodium dodecyl sulfate (SDS) provided better results with respect to efficient protein recovery (Fowler et al., 2007; Rodriguez- Rigueiro et al., 2011). In this study, we demonstrated that the amount of protein that can be isolated using our protocol (Tables 1, 2) is at least 3-4 times higher than the amount described in previously described procedures (Becker et al., 2007; Chung et al., 2008; Nirmalan et al., 2009; Wolff et al., 2011) (Table 3). The concept of using hot distilled water was adopted due to the fact that paraffin melts at around 70°C (Abhat, 1983) along with the simplicity of washing the paraffin off the slide. Very brief exposure of the sample to high temperature can help break down most of the bonds, as well as crosslinks formed during the preservation procedure, in addition to melting the paraffin wax (Sompuram et al., 2004; Yamashita and Okada, 2005). Considering the fact that using single tissue sections will contribute to decrease the amount of tissue needed for the experiment, the remaining block can be preserved and used for additional molecular and clinical investigations such as DNA, RNA or proteomic analysis. Furthermore, the relatively high amount of isolated proteins will offer the advantage of analyzing various proteins of interest. One of the major advantages of our technique relies upon its applicability on archived tissues. As such we were able to extract proteins from FFPE tissue sections that were stored for up to 7 years. Moreover, the quality of the isolated proteins was good enough to be evaluated with western blot, a routine molecular technique used worldwide. The western blot results of the old samples shown in (Fig. 2) showed similar intensity and clarity when compared to the β-actin bands obtained from the lysates of the recently stored tissues (Fig. 3). Efficient protein isolation is a key step toward successful protein analysis. As such, we used DC Protein Assay (similar to Lowry colorimetric assay) for protein quantification as an additional method to assess the protein isolation efficiency of our technique. Importantly, when the protein concentrations were measured with DC Protein assay the concentration values of 8 samples out of a total of 13 were found to be higher than the concentration values measured with NanoDrop. Also, 2 samples from the DC group had concentration values around 3 times higher than the values obtained with NanoDrop. We believe that the NanoDrop values are more relevant considering that the volume of lysate used for DC Protein assay is 100 μl compared to 1-1.5 μl for NanoDrop. Thus, it is evident that the amount of paraffin as well as other remaining fixative buffers will exist in higher proportion in the sample dilutions used for DC Protein assay. One limitation of the technique is the absolute requirement of addition of β-mercaptoethanol to the prepared samples in order to prevent protein degradation during long-term storage. Based on our preliminary observations, samples prepared with lysis buffer lacking PMSF and β-mercaptoethanol will show fast degradation and the protein content and quality can be significantly affected. The latter mechanism was seen when we used an antibody that specifically recognizes PERK (Protein kinase RNA-like endoplasmic reticulum kinase) a protein of 140 KD. While the antibody apparently recognized the protein, multiple bands were resolved on the membrane which may be due primarily to protein degradation (data not shown). Therefore, we believe

using this technique, proteins with low (e.g., actin) to moderate (e.g., AKT) molecular weight can be isolated with a good quality compared to proteins with higher molecular weight, which can be much more susceptible to protein degradation and fragmentation. Protein degradation can significantly alter protein content and impair proper protein recovery by western blot. Knowing that tissues are not homogenous and multiple types of cells exist in the same section, efficient detection of a protein of interest necessitates that this protein should be expressed in sufficient amount in at least one cell type. For the reasons described above, western blot analysis using antibodies with reduced specificity or targeting proteins with low expression level can be challenging and may not provide satisfactory results. Finally, several additional factors affect the molecular analysis of FFPE tissues including postmortem interval, size of the specimen, fixation temperature and duration, and storage time (Bass et al., 2014). We believe that the low yield obtained with certain samples can be attributed to these previously described factors, which can only be controlled on site when performing biopsy procedures. It is noteworthy to mention that the levels of β-actin were not uniform as expected and differences existed between certain specimens. Such variability can be attributed to the previously described limitations, mostly protein degradation, since specimens with low levels of β-actin also showed low levels of AKT as well. Therefore, we propose using at least 2 loading controls, including β-actin. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as the total non-phosphorylated forms of particular signaling kinases that exist at relatively abundant levels, including extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), could be interesting candidates as an additional loading control. On the other hand, xylene is broadly used in tissue processing, staining and deparaffinization in the pathology lab (Kandyala et al., 2010). Although other successful xylene-free protein isolation attempts are documented in the literature, these protocols are time consuming and include the use of other types of toxic solvents such as phenol and chloroform (Pena-Liopis and Brugarolas, 2013). Moreover, other available protocols exist but are focused on nucleic acid isolation for genomic analysis and other nucleic acid manipulations (Summersgill et al., 2008; de Planell-Saguer et al., 2010; Casadonte and Caprioli, 2011; Fanelli et al., 2011). Therefore, it is of considerable clinical importance to replace xylene with a bio-friendly solvent such as distilled water. Hence, this development will have a great impact in decreasing exposure to xylene as well as the disposal cost of such a toxic compound. Also, immunohistochemistry and pathology are considered the gold standard for the diagnosis of most tumors. Therefore, efficient extraction of proteins from FFPE can help identify much needed biomarkers for the diagnosis of tumors, selecting the most convenient therapy as well as predicting prognosis and clinical outcome. Finally, this technique showed results promising enough to warrant further investigation to render the extraction procedure suitable for nucleic acid isolation, as well as proteomic analysis.

Conclusion

In conclusion we believe that replacing xylene with a biofriendly solvent such as water is an important step toward reducing the exposure of lab technicians to such a hazardous organic solvent and, interestingly, without reducing the effectiveness of the experiment. Additionally, it is possible that this technique which can be used effectively in research can also be adapted in the future as a diagnostic tool in a clinical setting.

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