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Original Article

New tissue processing protocol for better long-fragment DNA preservation

Lixiong Ying, Ke Huang, Xiuming Zhang, Wei Ding

Department of Pathology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China

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Abstract: FFPE tissue blocks from pathology department are important resources for clinical diagnosis and scientific researches. Due to the chemical modification ability and fixation time of formalin, intact genetic materials such as the long-fragment DNA are difficult to achieve in the tissue blocks. How to properly process tissues especially during weekend and holidays remains complicated. Here we develop a new processing protocol in which the last step of paraffin immersion time is increased. Sections and DNA/RNA/protein from paired blocks (standard vs new protocol) were tested and compared for HE staining, IHC, FISH, RT-PCR, fragment analysis and Sanger sequencing. Our H&E, immunohistochemistry, FISH, RT-PCR and sequencing results suggested that tissues from the new method retained all the molecular features, and more importantly, fragment analysis showed the new method have better DNA fragment preservation. This new protocol can be easily applied during routine tissue processing including weekend and holidays to fulfill following elaborate molecular diagnostic requirements.

Keywords: Formalin-fixed, paraffin embedded (FFPE) tissue block, fragmentation, molecular diagnosis

Introduction

Formalin fixation and paraffin embedding has been the method of most diagnostic pathology archives for histological and pathological examination. Formalin as a reagent can preserve tissue morphology and cytological features as well as the immunoreactivity of many antigens. However, FFPE application in many molecular diagnostic techniques like quantitative PCR, RT-PCR, Sanger sequencing, next-generation sequencing (NGS), copy number analysis and whole transcriptome/genome shotgun have its limitations. Formalin fixates tissues by robust crosslinks formation between proteins and nucleic acids [1]. Harsh conditions for breaking these crosslinks will have big effects on the genetic material retrieval [2-5]. Additionally, with fixation time increasing, the DNA and RNA long size fragments start to break into shorter sizes and it is recommended that tissues should not fixed in formalin over 48 hours [6, 7]. After fixation, tissues will proceed into dehydration process which formalin is washed out and finally tissues are embedded in the paraffin. However, in many hospitals fixation longer than 48 hours cannot be avoided, for instance during the weekend and holidays. According to our knowledge, tissues during weekend and holidays are mainly kept in the formalin solution. And these samples usually have problem in long fragment analysis [8]. How to handle samples during weekend and holidays to fulfill both pathological diagnosis as well as molecular diagnosis? Here we develop a new tissue processing protocol which the tissues are immersed long-time in paraffin at the final step of dehydration process. In comparison with the standard protocol, our results revealed that this new protocol can preserve the pathological and molecular features of FFPE tissues.

Methods and materials

Tissue samples, fixation and processing

Samples of 10 surgically resected tissues including lung adenocarcinoma, liver cancer, colon adenocarcinoma, thyroid gland adenocarcinoma, non-Hodgkin B cell lymphoma (lymph

Table 1. Antibody clones, sources, dilutions and cellular distributions

Antibody	Clones	Sources	Dilutions	Cellular Distribution
ALK	D5F3	Ventana	Ready to use	Lung adenocarcinoma (C)
Cadherin-E	EP700Y	Epitomics	1:100	Breast carcinoma (M; M+C)
CD2	AB75	DAKO	1:100	T cell lymphoma (M)
CD3	PS1	Novocastra	1:100	T celllymphoma (M; M+C)
CD4	4B12	Novocastra	1:50	T cell lymphoma (M)
CD7	CBC37	Novocastra	1:50	T cell lymphoma (M)
CD8	C8/114B	Amsbio	1:300	T cell lymphoma (M)
CD20	L26	DAKO	1:200	B cell lymphoma (M)
CD79a	JCB117	LabVision	1:200	B cell lymphoma (M)
CD117/C-kit	Polyclonal	Invitrogen	1:200	GIST (C; M+C)
C-erb-B2	4B5	Ventana	Ready to use	Breast cancer (M)
Chromogranin A	Polyclonal	LabVision	1:50	Pheochromocytoma (C)
Cytokeratin	AE1/AE3	LabVision	1:100	Adenocarcinoma (C)
Cytokeratin 20	EP23	Epitomics	1:100	Colon adenocarcinoma (C)
DOG-1	SP31	Invitrogen	1:300	GIST (C; M+C)
Estrogen Receptor (ER)	1D5	DAKO	1:200	Breast cancer (N)
Ki-67	MIB-1	Invitrogen	1:500	Breast cancer (N)
Melan-A	A103	Invitrogen	1:100	Melanoma (C)
Melanoma	HMB45	DAKO	1:50	Melanoma (C)
Nestin	10C2	Invitrogen	1:200	Melanoma (C)
Progesterone Receptor (PR)	PgR636	LabVision	1:200	Breast cancer (N)
P53	DO-7	DAKO	1:150	Breast carcinoma (N)
P120 Catenin	EP66	Epitomics	1:200	Breast carcinoma (M; M+C)
Synaptophysin	Polyclonal	LabVision	1:100	Pheochromocytoma (C)
S-100	Polyclonal	DAKO	1:5000	Melanoma (N+C)
TTF-1	8G7G3/1	LabVision	1:500	Lung adenocarcinoma (N)

Abbreviations: N = Nuclear; M = Membranous; C = Cytoplasmic.

Table 2. Paired-FFPE tissue processing protocol 1 and 2

Dehydration Process	Protocol 1	Protocol 2
Formalin	50 h	2 h
Water	0.5 h	0.5 h
75% ethanol	2 h	2 h
85% ethanol	2 h	2 h
95% ethanol I	1.5 h	1.5 h
95% ethanol II	2 h	2 h
100% ethanol I	1.5 h	1.5 h
100% ethanol II	2 h	2 h
Xylene I	1 h	1 h
Xylene II	1.5 h	1.5 h
Paraffin I	1 h	12
Paraffin II	1.5 h	20
Paraffin III	1.5 h	20

node), non-Hodgkin T cell lymphoma (lymph node), breast invasive lobular carcinoma, gastrointestinal stromal tumor (GIST), melanoma and glioblastoma and 5 small tissues including lung biopsy, abdomen biopsy, breast biopsy, gastroscopic tissue and colonoscopic were selected from the Department of Pathology, the First Affiliated Hospital, College of Medicine, Zhejiang University. All above tissues were divided into 2 equal portions at <3 mm in thickness (for surgical samples) to have sections with similar sizes. Each portion was fixed in 4% neutral paraffin (Tongsheng Technology, Ningbo) for 8 h and went through the following two dehydration protocols: protocol 1 (the standard weekend protocol in our department). 50 h in Formalin, 0.5 h in water, 2 h in 75% ethanol, 2 h in 85% ethanol, 1.5 h in 95% ethanol I, 2 h in 95% ethanol II, 1.5 h in 100% ethanol I, 2 h in 100% ethanol II, 1 h in xylene I, 1.5 h in xylene II, 1 h in paraffin I, 1.5 h in paraffin II and 1.5 h in paraffin III (Chaser, Fujian). Protocol 2 (the new developed paraffin weekend protocol), 2 h in Formalin, 0.5 h in water, 2 h in 75% ethanol,

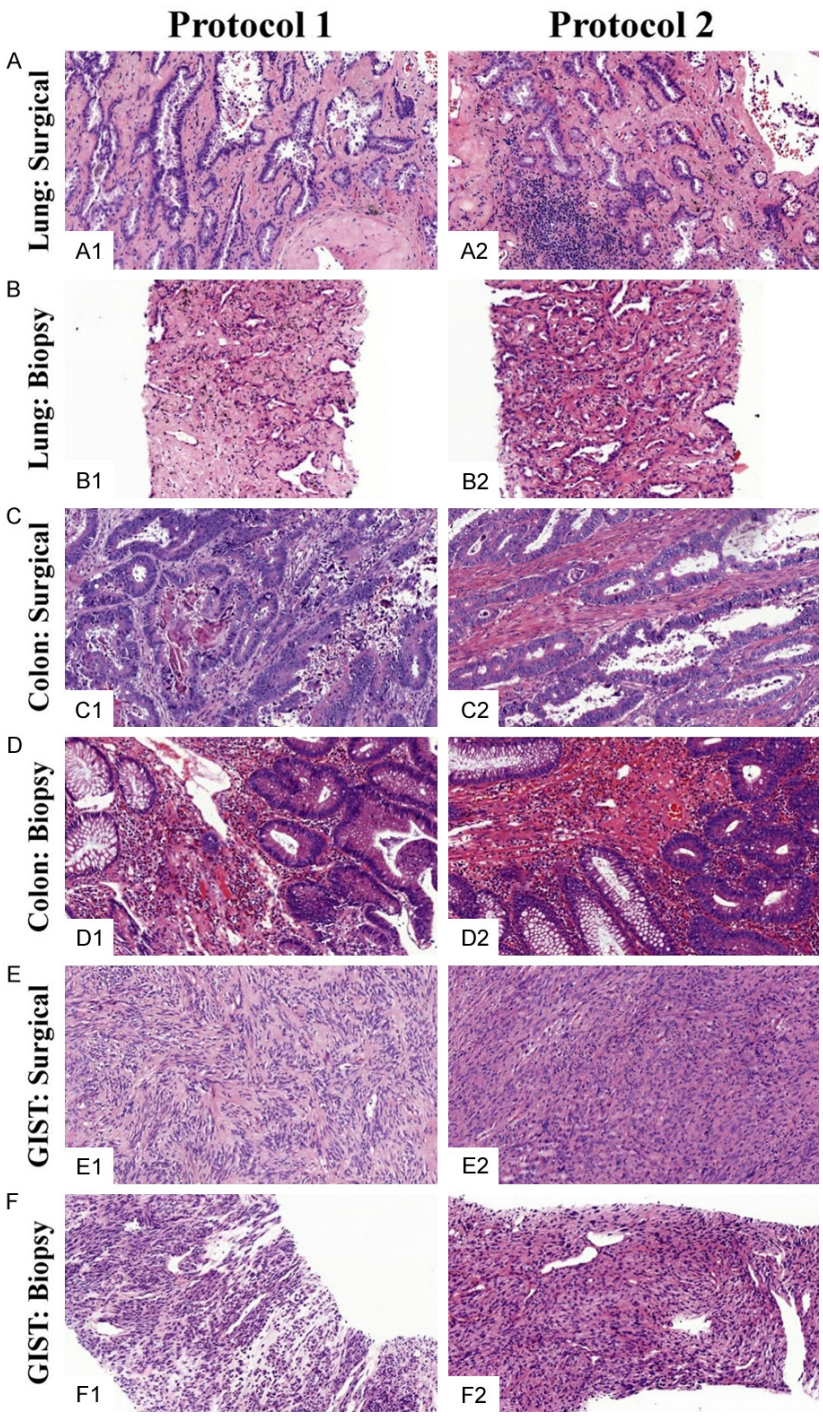

Figure 1. Representative hematoxylin and eosin stained sections. Surgical lung adenocarcinoma (A), lung biopsy (B), surgical colon adenocarcinoma (C), colonoscopic (D), surgical gastrointestinal stromal tumor (GIST) (E), abdomen biopsy (F) tissue samples treated with dehydrationprotocol 1 (A1-F1) and protocol 2 (A2-F2). The magnification for all images are ×200.

2 h in 85% ethanol, 1.5 h in 95% ethanol I, 2 h in 95% ethanol II, 1.5 h in 100% ethanol I, 2 h in 100% ethanol II, 1 h in xylene I, 1.5 h in xylene II, 12 h in paraffin I, 20 h in paraffin II and 20 h in paraffin III. The flowchart representation of two protocols was shown in **Table 2**. The study protocol was approved by the Hospital Ethics Committee. 4 μm sections from paired Formalin-fixed, paraffin embedded (FFPE) tissue blocks were cut for hematoxylin and eosin (H&E) staining and following analysis.

Immunohistochemistry

The slides were air-dried at room temperature (20°C) for 20 seconds. The details of 28 primary antibodies used are listed in **Table 1**. They are routinely used in our institution to test specific antigen expression on tissue blocks. All IHC procedures were carried out in a fully-automatic IHC machine (VENTANA BenchMark ULTRA, America; VENTANA BenchMark XT, America) by using a two-step En Vision system (DAKO, Denmark) with DAB colorization.

Fluorescent in situ hybridization (FISH)

FISH test was used to determine the HER-2/neu status. Sections were baked overnight at 60°C, deparaffinized two times in the xylene for 10 min, transferred twice through 100% ethanol, one time in 85% ethanol, one time in 70% ethanol, each for 3 min. Then the slides were immersed for 25 min in distilled water at 90°C, followed by 10 min incubation in protease solution at 37°C.

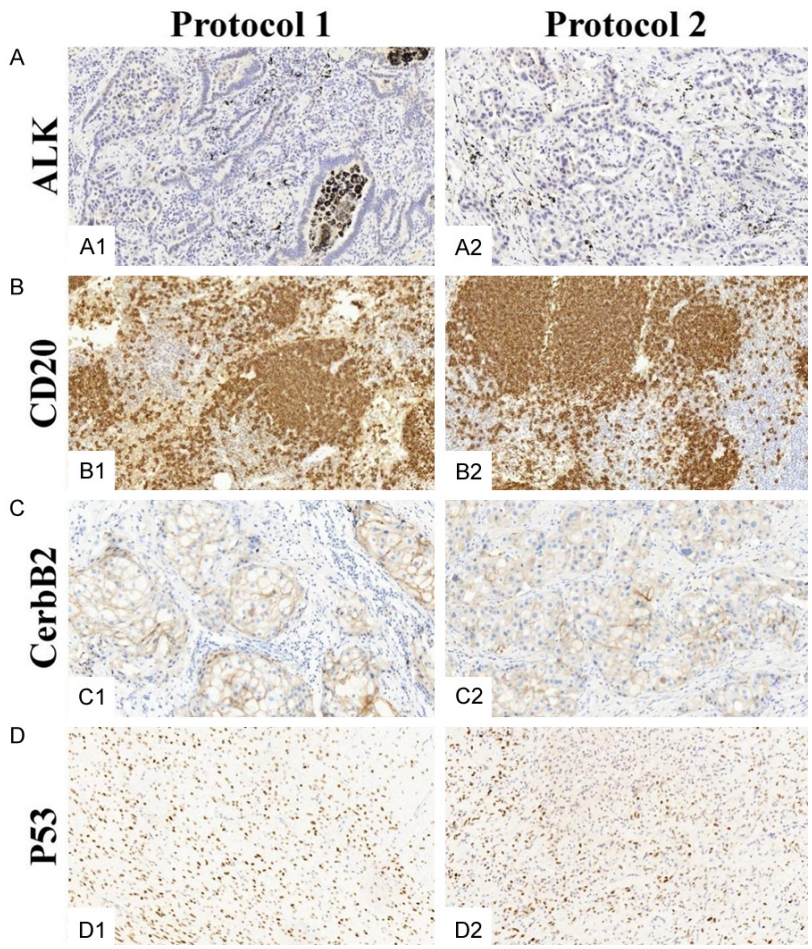

Figure 2. Immunohistochemical staining. Lung adenocarcinoma with ALK (A), B lymphoma with CD20 (B), breast cancer with CerbB2 (C), glioblastoma with P53 (D). (A1-D1) were tissues from dehydrationprotocol 1 and (A2-D2) were from protocol 2. The magnification for all images are ×200.

After that, the slides were briefly washed in sodium saline citrate (SSC, pH 7.2) at room temperature, dehydrated through 70%, 85%, 100% ethanol and acetone. After drying in the open-air, 10 μl of probe (Zytovision, Germany) was applied onto each slide, cover slip was placed and sealed with rubber cement, and then the slides were transferred to the hybridization oven (S500-24, Abbott molecular, USA). The procedure was as follows: denature at 83°C for 5 min, and hybridized overnight at 42°C. After that, the slides were washed in 46°C preheated post-hybridization buffer (2XSSC/0.1% sodium dodecyl sulfate) for 5 min and rinsed in 70% ethanol. After air-drying, the slides were counterstained with 15 μl DAPI and cover slip applied.

Thirty randomly selected invasive tumor nuclei in each of two separate, distinct microscopic areas were evaluated. Positive for *HER-2/neu* is defined as *HER-2/CEP 17* ratio ≥2.0 or *HER-2/CEP17* ratio <2.0 with an average *HER-2* copy number ≥6.0. Equivocal for *HER-2* is defined as *HER-2/CEP17* ratio <2.0 with an average *HER-2* copy number ≥4.0 and <6.0 signals/cell. Negative for *HER-2* is defined as *HER-2/CEP17* ratio <2.0 with an average *HER-2* copy number <4.0 signals/cell.

Nucleic acid extraction

5 slices were deparaffinized by two 5-min incubations in xylene at room temperature. The deparaffinized tissue was washed with two 100% ethanol and heated to 37°C for 10 minutes to remove excess ethanol. For DNA and RNA isolation, all extractions were carried out using DNA/RNA extraction kit according to the manufacturer's instruction (AmoyDx, Xiamen), surgical tissue with 100 μl elution buffer and biopsy samples with 50 μl elution buffer. DNA/RNA concentrations were determined by Nanodrop. DNA fragment length was assessed using the DNA quality control tube in Invivoscribe kit (Invivoscribe, USA), with amplicon lengths of 100, 200, 300, 400 and 600. The test was carried out in ABI3500Dx.

PCR and RT-PCR

Mutations of *EGFR*exon 18, 19, 20, 21, *ROS1* and *EML4-ALK* fusion were tested in lung adenocarcinoma and lung biopsy blocks using ARMS Detection Kit (AmoyDx, Xiamen). Mutations of *KRAS* exon 2, 3 and 4, *NRAS* exon 2, 3 and 4, BRAF V600E mutations were also tested for colon cancer and gastroscopic tissue blocks using ARMS Detection Kit (AmoyDx, Xiamen).

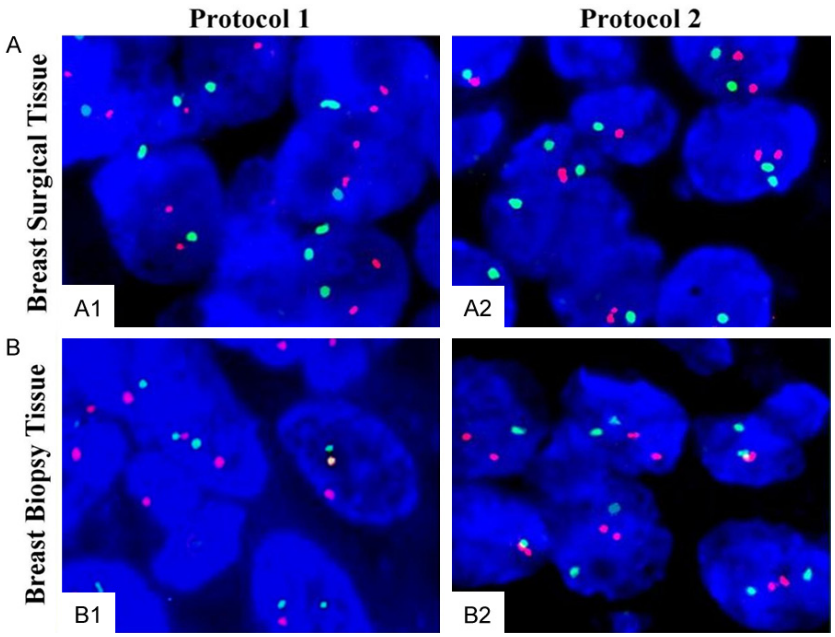

Figure 3. Fluorescent in situ hybridization (FISH) visualization of paired-breast samples.dehydration protocol 1 (A) and protocol 2 (B). Green dot: HER-2 gene. Red dot: CEP17 gene, blue background: DAPI staining. The magnification for both images are ×1000.

Sanger sequencing

DNA from the paired GIST FFPE blocks were tested for *KIT* exon 9, 11, 13, 17 and *PDGFRa* exon 12 and 18 mutations using Sanger Sequencing Kit (Yuanqi, Shanghai). Sequencing was carried out in ABI3500Dx.

Results

Histological presentation

Representative H&E stained images (4 paired-tumor tissues) of dehydration protocol 1 and 2 are illustrated in **Figure 1**. Overall, the HE staining of all sections from paired-FFPE tissue blocks with dehydration protocol 1 and 2 showed no significant differences in normal tissue architecture, cytological and nuclear details. We also noticed that sections of blocking processing protocol 2 tended to have a slight better eosin stain.

Immunohistochemical examination

The amount of tissue from protocol 2 did not decrease obviously after IHC stains including heating antigen retrieval. The comparison between protocol 1 and 2 showed the totally identical IHC staining pattern and intensity in all paired samples. The representative selected sequential stains for ALK, CD20, CerbB2 and P53 were illustrated in **Figure 2**.

Fluorescent in situ hybridization (FISH)

Surgical breast invasive lobular carcinoma and breast biopsy tissues were chosen for FISH analysis. Two sets of paired samples went through same protocol and showed almost same intensity of DAPI, *CEP17* and *HER-2* staining (**Figure 3**). The fluorescent signals of *HER-2* gene for both paired samples were negative. Result proved that long time in the paraffin had no influence on the probe binding and fluorescent visualization.

DNA yields

DNA from total 30 FFPE blocks was ranging from 13.8-976.3 ng/ul. The variation of DNA yields between paired blocks was from 6.0 ng/ulto 86.2 ng/ul. Moreover, the quality of all the blocks were very high (260/280 ratio ≥1.8 and 260/230 ration ≥1.6). There was no evidence that protocol 2 will influence the DNA yield.

PCR results

In our paired lung adenocarcinoma FFPE blocks, we detected an *EGFR* 19-DEL mutation using ARMS PCR (**Figure 4A**). All the Ct values were listed in **Table 3**. The Ct value of housekeeping gene and EGFR 19-DEL between two blocks were only 0.21 and 0.18. No *ROS1* or *EML4-ALK* fusion were found in the lung adenocarcinoma samples (**Figure 4B** and **4C**). In addition, *KRAS* exon 2, 3, 4, *NRAS* exon 2, 3, 4, *BRAF* V600E mutations were not found in the paired colon cancer blocks. The Ct values of the housekeeping genes for *KRAS*, *NRAS* and BRAF between two blocks were also very close

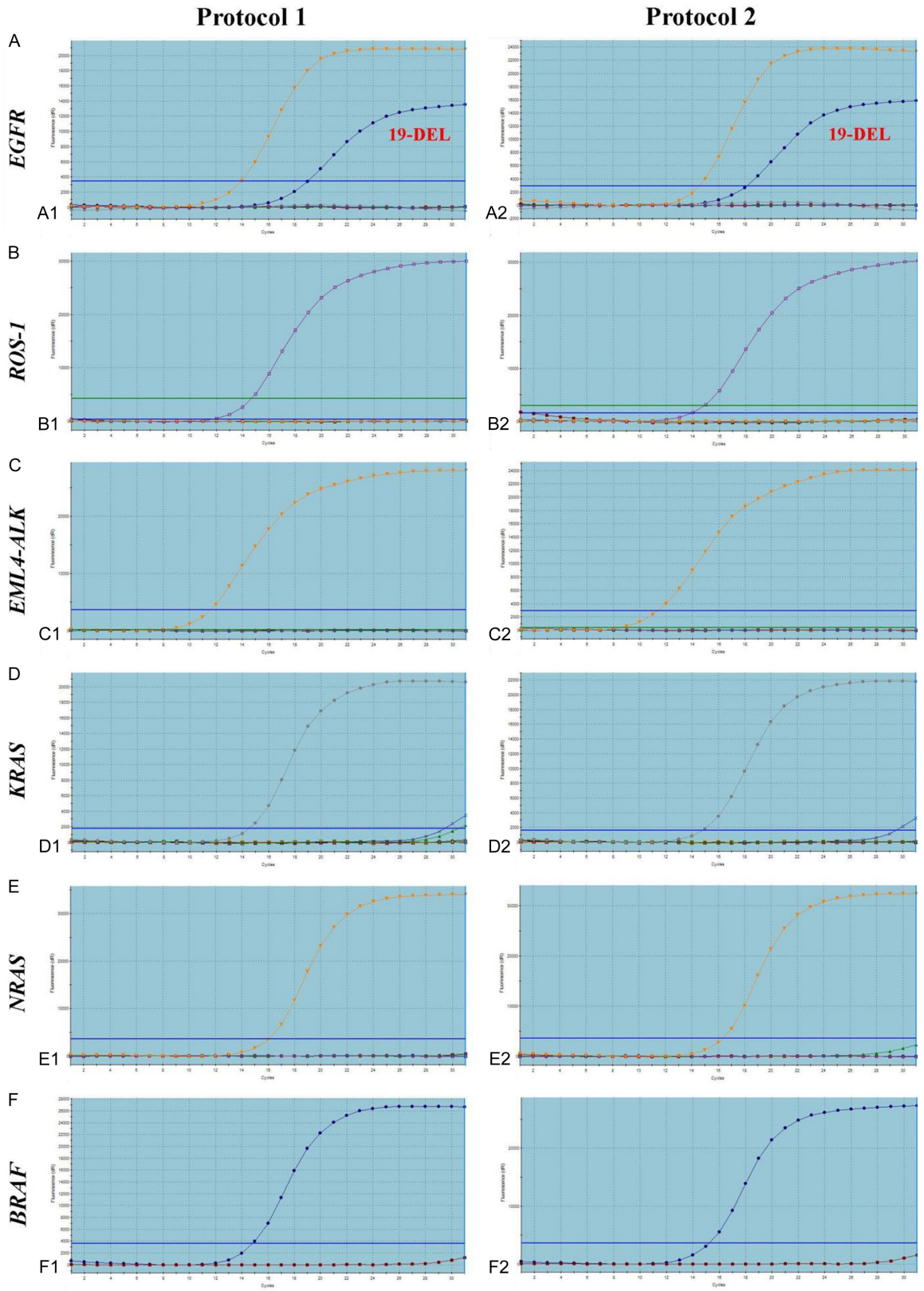

Figure 4. Quantitative PCR results. Genes including EGFR, ROS1, EML4-ALK, KRAS, NRAS and BRAF were tested and compared for samples from protocol 1 and 2. A 19-DEL mutation were detected in both samples (blue-colored curve). The other five genes were all negative, only the signal for house-keeping genes were observed.

Table 3. Summary of Ct values

House-keeping gene/Mutation	Protocol 1 (Ct value)	Protocol 2 (Ct value)
EGFR	14.63/18.20	13.97/19.03
ROS1	14.72	14.93
EML4-ALK	11.40	11.66
KRAS	14.59	14.85
NRAS	16.41	16.07
BRAF	15.30	14.87

(**Figure 4D-F**). T-test shown no significant difference between the CT values. Overall there was no evidence of a difference in PCR success rates between protocol 1 and 2.

DNA length

DNA length was found highly correlated with time in formalin and paraffin. Most surgical samples from protocol 1 only retained fragment size within 300 bp. Moreover, the biopsy samples showed more fragmentation than the big tissue. In comparison, all sample blocks from protocol 2 had fragment sizes longer than 600 bp size (**Figure 5B**). This analysis revealed that paraffin had significantly better effect in preserving DNA from fragmentation.

RNA yields

RNA from total 30 FFPE blocks were ranging from 3.1-524.3 ng/ul. The variation of DNA yields between paired blocks were from 0.7 ng/ul to 137.7 ng/ul. Moreover, the quality of all the blocks were high (260/280 ratio ≥2.0 and 260/230 ration ≥1.8). This rough RNA isolated test showed that protocol 2 also have a similar RNA preservation.

Sequencing results

We selected GIST, colon cancer, lung biopsy and gastroscopic tissue for Sanger sequencing. In both GIST samples, we observed same 502_503InsAY mutation in *KIT* exon 9 (**Figure 6**) and the sequencing signals from both methods did not present obvious differences.

Discussion

Formalin fixation and paraffin embedded tissue samples are the major sources in the pathology department for routine histopathological diagnose and research. Formalin can prevent tissue autolysis, stabilize proteins and limit antigen and nucleic acids degradation thereby nicely preserve the tissue morphology. But formalin introduce protein-protein and proteins-nucleic cross-linking, as well as chemical modifications of nucleic acids [9]. These reactions lead to significant degradation of nucleic acids in FFPE blocks. Further fragmentation of DNA and RNA can be caused by suboptimal fixation, prolonged storage of FFPE blocks, tissue processing, sectioning and staining procedures [10, 11]. With the development of molecular biology and genetics, requirements for nucleic acids preservation in clinical specimens become high. Besides, increased applications of minimal invasive surgery and biopsy produce much smaller pieces of pathology specimens. Therefore, finding better ways for pathological specimen processing and storage are of great necessity. Many groups from worldwide had published various papers aiming at nucleic preservation for molecular diagnosis. But these studies were mainly focus on testing different types of fixatives or DNA isolation methods [12-14]. Whereas different tissue processing protocols are not given enough attention.

In this study, we compared two dehydration protocols for HE staining, IHC staining, FISH, DNA/RNA isolation, quantitative PCR, RT-PCR, Sanger sequencing, fragment analysis. HE slides from dehydration protocol 1 and 2 showed no obvious differences in tissue morphology. One of the pathologists thought the contrast of slides from protocol 2 was better than slides from protocol 1. This slight difference may be caused by better eosin staining of cell cytoplasma and mesenchyme. The effect of long time immersion in paraffin had also been studied on IHC and FISH. Of the tested 28 antibodies, staining intensities for most antigens were generally equal in protocol 2. For FISH, the *CEP17, HER-2* and DAPI showed similar fluorescent intensity. In nucleic acids analysis, concentration and purity of isolated DNA/RNA and following quantitative PCR, RT-PCR and Sanger sequencing were compared between both dehydration protocols. Protocol 2 showed generally equal isolation success, same mutation types and similar Ct values.

Concentration and size distribution of genomic DNA isolated FFPE tissues are important for some downstream assays. It has been suggest-

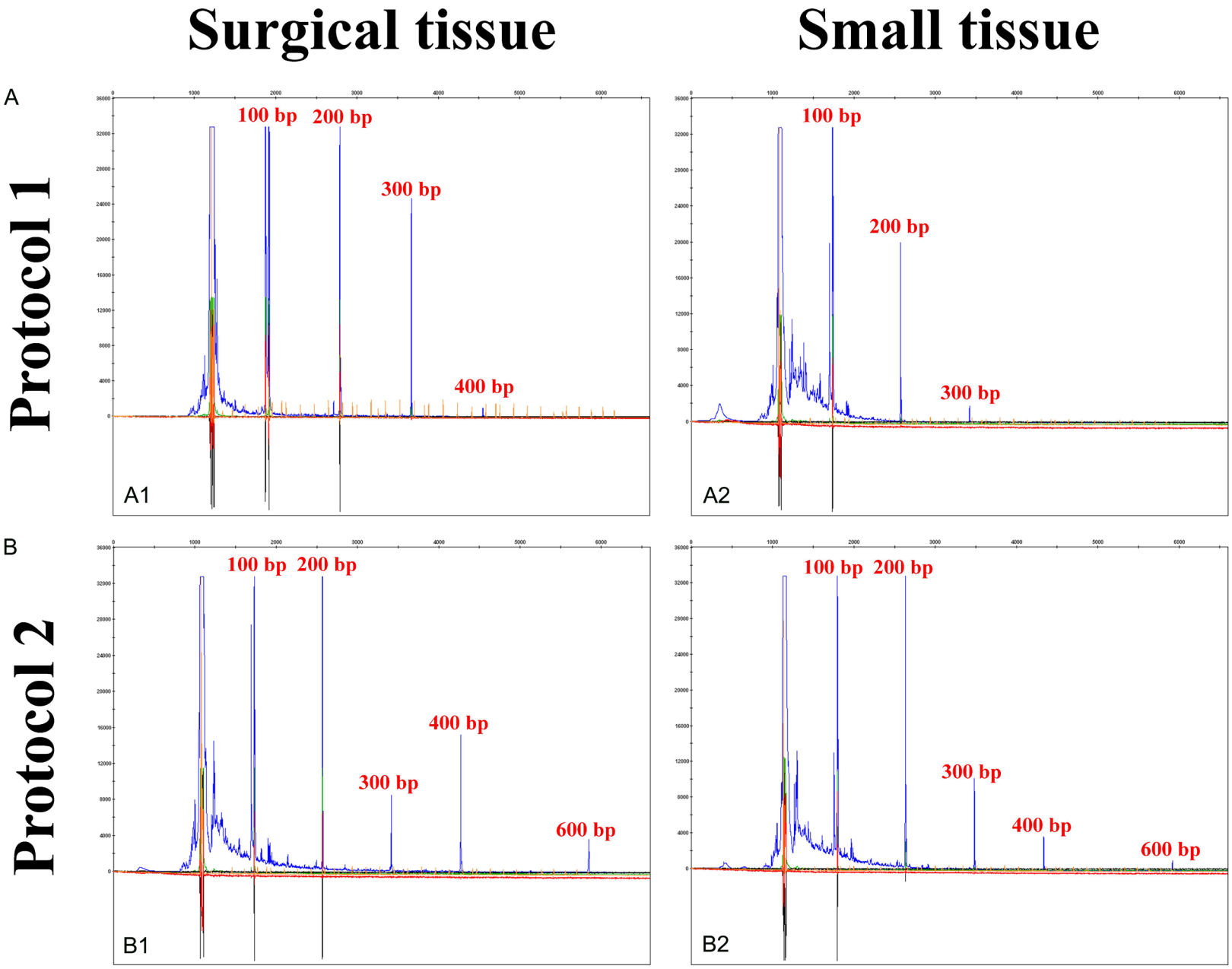
Surgical tissue
Small tissue
A
Protocol 1
100 bp
200 bp
300 bp
400 bp
A1
100 bp
200 bp
300 bp
A2
B
Protocol 2
100 bp
200 bp
400 bp
300 bp
600 bp
B1
100 bp
200 bp
300 bp
400 bp
600 bp
B2

Figure 5. Fragment analysis. Both surgical and biopsy samples were analyzed for fragmentation. Small tissues tended to have severe fragmentation than big samples at same tissue processing conditions. Tissues from protocol 2 showed nice long fragment preservation (>400 bp or 600 bp) whereas tissues from protocol 1 only had fragments smaller than 300 bp.

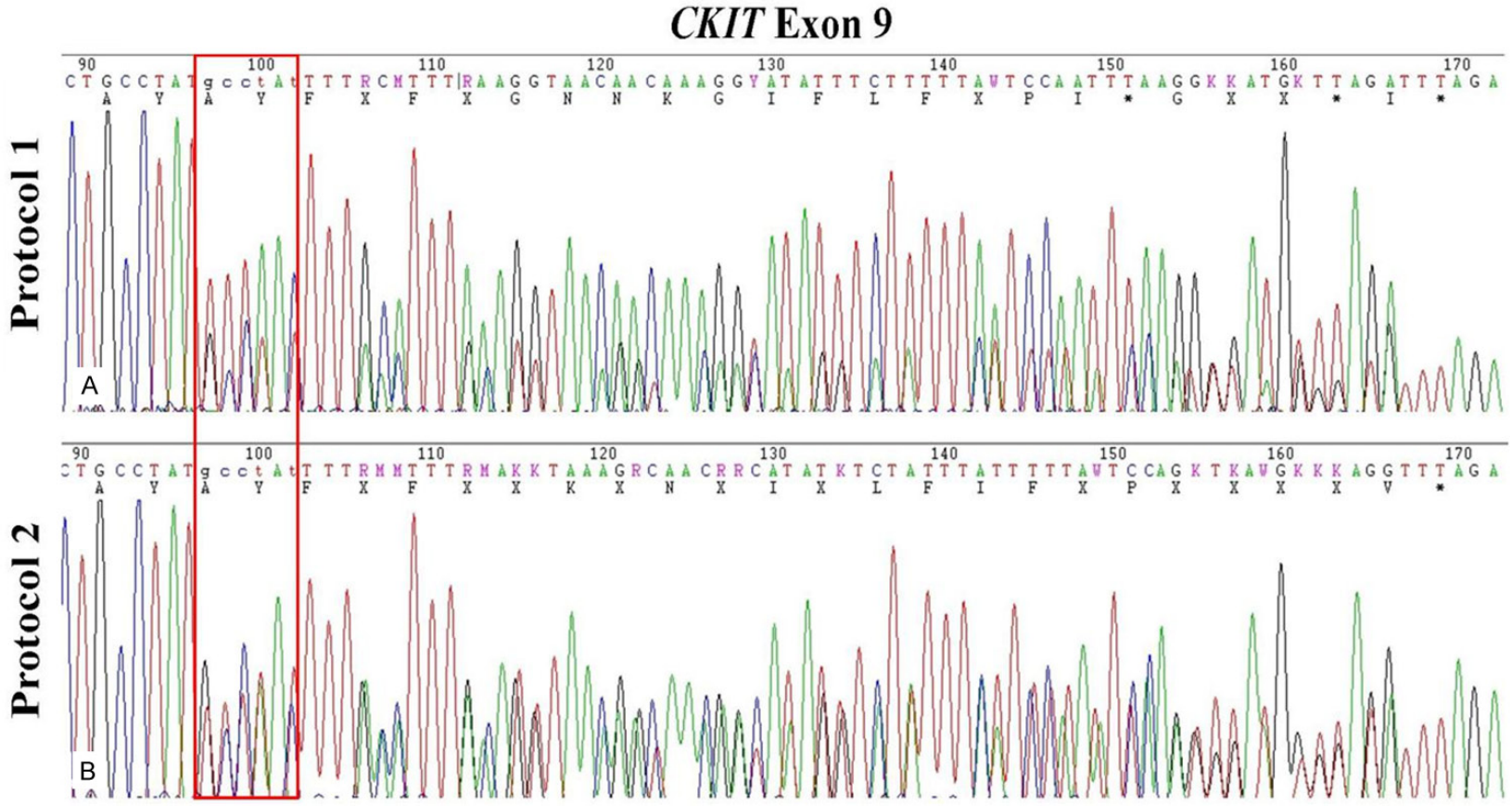

Figure 6. Sanger sequencing. Surgical GIST samples were selected for *KIT* exon 9, 11, 13, 17 and *PDGFRa* exon 12, 18 tests. Same 502_503 InsAY were found in exon 9 (red rectangle). Samples from protocol 1 and 2 had similar signal intensity.

ed lymphoma rearrangement should only be performed if the sample has the 300 bp fragment at the minimum [15]. This indicates that tissue treatment and duration of storage are highly related with assay success rates. Protocol 1 had significantly DNA degradation and contained short amplicons (<300 bp). This outcome will surely influence downstream molecular diagnostic assays. In contrast, protocol 2 appeared to have better nucleic acid preservation and yield long DNA fragment (>400 and 600 bp).

Overall, hydration protocol 1 and 2 showed no differences in H&E, IHC, FISH, DNA/RNA extraction, quantitative PCR, RT-PCR and Sange sequencing but protocol 2 had been demonstrated to be a better method for long-fragment analysis from FFPE tissue specimens. The paraffin used in our department has a low melting point (54-56°C) and stable chemical properties and long-time immersion in paraffin showed no harm to DNA, RNA or proteins. Outcome of this tissue specimen treatment method have not been studied in previous articles.

In conclusion, we designed a new tissue processing protocol which the time of tissues in the paraffin, instead of in the formalin, is extended. All the data show that this method can retain all pathological features and more importantly, preserve large DNA fragment from fragmentation. This protocol can be widely applied in routine pathology departments with molecular diagnostic demands, especially during weekend and holidays.

Disclosure of conflict of interest

None.

Address correspondence to: Wei Ding, Department of Pathology, The First Affiliated Hospital, College of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang, China. Tel: +86-13958082289; Fax: +86-571-87236364; E-mail: 1181010@zju.edu.cn

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