The Korean Journal of Pathology 2011; 45: 549-556
http://dx.doi.org/10.4132/KoreanJPathol.2011.45.6.549

Comparison of the DNA Preservation in Neutral-Buffered Formalin Fixed Paraffin-Embedded Tissue and in Non-Buffered Formalin Fixed Paraffin-Embedded Tissue

An Na Seo · Jae-Hoon Kim[1]
Dakeun Lee · Ji Yun Jeong
Ji-Young Park

Department of Pathology, Kyungpook National University School of Medicine, [1]Kyungpook National University Hospital, Kyungpook National University School of Medicine, Daegu, Korea

Received: July 2, 2011
Accepted: October 6, 2011

Corresponding Author
Ji-Young Park, M.D.
Department of Pathology, Kyungpook National University Hospital, Kyungpook National University School of Medicine, 50 Samduk-dong 2-ga, Jung-gu, Daegu 700-721, Korea
Tel: +82-53-420-5247
Fax: +82-53-426-1525
E-mail: jyparkmd@knu.ac.kr

*This research was supported by Kyungpook National University Research Fund, 2011.

Background: The preservation of optimized DNA and its extraction from formalin-fixed, paraffin-embedded (FFPE) tissues are important issues. There has been some doubt over whether 10% neutral-buffered formalin is an ideal fixation solution for DNA preservation over non-buffered formalin, as conventionally recommended. In this study, the correlation between the efficiency of DNA extraction from FFPE tissues and buffered formalin was evaluated. **Methods:** Several tissues with same conditions except fixatives were fixed in four different formalin solution groups and were routinely processed as paraffin-embedding protocols. DNAs were extracted from four different FFPE tissues that were stored for over 3 months and over 9 months. The quantity and quality of the DNAs were assessed with a NanoDrop ND-1000 spectrophotometer, and the polymerase chain reaction (PCR) amplification and degradation were analyzed via microchip electrophoresis. *KRAS* mutation analysis and microsatellite instability (BAT25) PCR were performed with each sample. **Results:** The results showed no remarkable difference in the four groups. **Conclusions:** The study findings demonstrate that DNA preservation is fairly unaffected by a neutral buffer where there is short formalin manufacture period and an adequate formalin fixation time before embedding in paraffin.

Key Words: Tissue fixation; Formaldehyde; Tissue preservation; Pathology, molecular; DNA degradation

Formalin, a traditional fixative, has been used for several decades to preserve tissues in pathology.[1,2] Most pathological specimens are routinely formalin-fixed, paraffin-embedded (FFPE), and stained with hematoxylin and eosin for diagnosis.[1,3] These methods provide excellent morphological preservation and high consistency under various conditions, and offer simple and economical processing and handling.[2]

The recent dynamic changes in molecular biology have had great utility in medical practice[2,4] and have revolutionized pathological diagnosis.[2] Initially, molecular studies were limited to the immediate freezing of fresh tissues,[2,5] but various studies for optimizing nucleic-acid extraction from FFPE tissues have accelerated technological advancements.[1,5-11]

The conventional tissue fixation method was in 10% neutral-buffered formalin (NBF) for 16-24 hours at room temperature.[2] As a progressive acidification of formalin into formic acid, the use of buffered formalin stabilizes the solution for a longer time compared to the use of non-buffered formalin. However, because commercial 10% NBF is expensive, the large pathology laboratories sustain significant daily costs in fixing surgical tissue samples in 10% NBF. Moreover, in the pathology laboratory of the authors of this study, new NBF is prepared daily, and most surgical specimens are fixed in this formalin. Then, these tissues are processed and embedded in a paraffin block within 24 hours from the time of formalin fixation. Therefore, the aforementioned issue regarding the high price of formalin is an important issue in this laboratory because the expensive NBF, which has recently been made daily and has not yet been degraded in the laboratory, contributes significantly more to the good quality of the DNA preserves obtained from FFPE tissues compared to the inexpensive non-buffered formalin. This study was designed to determine the correlation between the efficiency of DNA extraction from FFPE tissue sections and neutral-buffered formalin. It was demonstrated that the DNA yield in the same

samples, which were fixed at the same time before paraffin embedding, does not clearly show a difference between the neutral-buffered and non-buffered formalin where there is a short manufacture period of formalin and an adequate formalin fixation time before embedding in paraffin.

MATERIALS AND METHODS

Tissue preparation

A total of 19 surgically removed human samples were obtained within 2-5 hour excision. These samples included the following: uterus, pancreas, breast, liver, soft tissue, colon, stomach, and lung. Fresh tissue samples were sectioned into four slices, each 10×5×5 mm in length×width×thickness. Four different types of formalin solution were newly prepared before fixation, as follows: group 1, 10% NBF (3.7% formaldehyde [OCI Co. Ltd., Seoul, Korea] in distilled water, with the following buffers added: $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4 [Sigma-Aldrich Co., St. Louis, MO, USA]); group 2, formalin mixed with tap water instead of distilled water (the other ingredients that were used were the same as in the first type of formalin solution); group 3, preserved commercial 10% NBF (Sigma-Aldrich Co., St. Louis, MO, USA) (for comparison with the other solutions); and group 4, 10% formalin prepared by mixing 3.7% formaldehyde with tap water, without adding buffers. Each tissue slice was fixed in 1 L of one of the four formalin solution groups at room temperature for 16-24 hours, but the other conditions, including size, temperature, fixation time and other process were the same. The formalin fixture time was 16-24 hours, and the fixed tissues were temporarily stored in 70% ethanol until processing. The fixed slices were processed routinely through dehydration in graded ethanol, were cleared in xylene, and were then made to infiltrate paraffin with an automatic tissue processor (Tissue-Tek VIP, Sakura Finetek, Torrance, CA, USA) for 15 hours overnight. Then, the tissues were embedded in paraffin blocks using embedding equipment (Tissue-Tek TEK, Sakura Finetek, Torrance, CA, USA).

A total of 76 paraffin blocks were stored in a 4°C refrigerator until they were ready for use. Then, the paraffin blocks were used for DNA extraction at five-day average storage periods. Three and nine months after the initial studies, DNA was extracted from the refrigerated paraffin blocks for comparison of the results with the initial results.

DNA extraction

For DNA extraction, five 10-μm sections were cut from each paraffin block and were collected in an autoclaved plastic microtube (1.5 mL), then a total of 76 paraffin block sections were treated according to the manufacturer's recommendations for the QIAamp DNA minikit (Qiagen Ltd., Crawley, UK). The eluted DNA was stored for later analysis.

Estimation of absorbance ratios

To quantify the DNA extract, all the samples were analyzed using a Thermo Scientific NanoDrop ND-1000 spectrophotometer (Labtech International Ltd., East Sussex, UK) with 1-μL samples. To evaluate the purity of the extracted DNA, the absorbance ratios were measured at 260/280 nm (DNA/protein) and 260/230 nm (DNA/humic acids). The ratios of the fixed FFPE tissues in the four different types of formalin solution were compared.

Detection of amplification products via microchip electrophoresis

For a precise comparison, targeted polymerase chain reaction (PCR) reactions of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were carried out, and the amplified products were analyzed via automated chip-based micro-capillary electrophoresis on an Agilent 2100 bioanalyzer DNA 1000 kit (Agilent Technologies, Santa Clara, CA, USA). This instrument performs a highly sensitive assay over agarose gel electrophoresis with ethidium bromide staining. Using the Agilent 2001 bioanalyzer, nucleic acid samples were automatically separated and analyzed via capillary electrophoresis with laser-induced fluorescence detection. All the experiments were performed according to the standard protocol recommended by the manufacturer, and amplicon sizing and relative quantification was performed with Agilent biosizing software (ver. B.02.02). Forward: 5´-GACACCCACTCCTCCACCTTTG-3´ and reverse: 5´-CACCACCCTGTTGCTGTAGCCA-3´ were the primers used.

Detection of DNA degradation via microchip electrophoresis

To compare the quality and size of the DNA degradation over time, the chip was prepared and 1 μL of DNA samples that represented each of the groups at 3 and 9 months storage periods were loaded as recommended by the manufacturer.

KRAS sequencing

To compare the utility and quality of the DNA sequencing on four different groups, DNA was extracted from the FFPE tissues in a lung cancer patient that had been stored for 3 and 9 months. The *KRAS* exon-2 fragment of the lung cancer tissues was amplified and sequenced according to the standard protocol. The primer sequences were: forward 5´-GTCCTGGTGGAGTATTTGAT-3´ and reverse 5´-AGAATGGTCCTGCACCAGTA-3´. The reaction was analyzed with an Applied Biosystems 3130Xl genetic analyzer (Applied Biosystems, Foster City, CA, USA), with the fluorescent BigDye terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Microsatellite instability (MSI) (BAT25) typing via fluorescent PCR-based capillary electrophoresis

The FFPE tissue blocks, which were stored for over 9 months, of normal and tumor tissues from a colorectal cancer patient were examined for MSI (BAT25), representatively. To amplify the mononucleotide marker panels, high performance liquid chromatography-purified IRD700 (for BAT25) labeled forward and unlabeled reverse primers that were obtained from Metabions were used. The primer sequences were forward 5´-TCGCCTCCAAGAATGTAAGT-3´ and reverse 5´-TCTGCATTTTAACTATGGCTC-3´. PCR was carried out according to the standard protocol, and the fluorescent PCR products were analyzed via capillary electrophoresis using an Applied Biosystems 3130Xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The electropherogram peak with the largest area was defined as the "main product."

RESULTS

Nucleic acid purity

The average purity (based on the absorbance ratio) of the DNA was estimated for each formalin solution group in the three experiments (Tables 1-3). The absorbance 260/280 ratio of over 1.8 was generally accepted as pure for DNA, and the ratios of 1.8 and 2.0 were considered "rules of thumb." Also, the expected 260/230 ratio was commonly in the range of 2.0-2.2. In the experiments, the average 260/280 values of all the groups were higher than 1.8, and the 260/230 values were in the range of 1.6-2.3, although a slight difference between the four types of formalin solutions was revealed in the three experiments and

Table 1. Results of the DNA purity assessment with a NanoDrop ND-1000 spectrophotometer in the initial studies

Case No.	Group 1		Group 2		Group 3		Group 4	
	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio
1	1.50	1.94	1.53	1.80	1.60	0.92	1.64	2.36
2	2.04	2.07	2.07	2.21	2.06	2.16	2.03	2.22
3	0.95	1.27	1.92	2.15	1.58	2.17	1.60	3.33
4	1.80	2.38	1.72	2.32	1.90	2.55	1.82	1.86
5	2.22	2.13	1.98	2.39	2.05	2.32	1.90	2.17
6	2.31	1.20	2.18	1.39	2.18	0.55	2.03	1.54
7	2.07	1.85	2.00	1.87	2.06	1.79	1.97	2.16
8	1.95	1.85	1.96	2.14	1.98	1.88	1.95	2.70
9	1.86	1.07	1.67	0.83	1.98	2.00	2.01	2.17
10	2.45	0.50	2.38	0.22	2.00	0.64	1.64	0.94
11	1.96	2.19	1.91	2.09	1.71	1.44	1.77	1.25
12	1.84	1.97	1.92	2.18	1.92	2.24	1.94	2.25
13	1.82	2.59	2.07	1.50	1.98	2.83	1.89	1.85
14	2.06	1.40	2.12	1.05	2.21	1.78	1.92	0.98
15	1.94	2.02	2.21	2.03	2.04	1.33	1.95	1.66
16	1.94	1.40	0.77	1.05	1.52	1.78	0.99	1.71
17	1.95	1.60	2.07	1.21	2.12	1.08	2.18	1.83
18	1.83	1.93	1.85	1.14	1.90	2.19	1.90	1.68
19	1.88	2.18	1.98	2.25	1.95	2.29	1.96	1.44
Average	1.914210526	1.765263158	1.911052632	1.674736842	1.933684211	1.786315789	1.90894737	1.90

The DNA purity of all the samples is assessed by a Thermo Scientific NanoDrop ND-1000 spectrophotometer. The results are expressed for the measurement using a 260/280 nm absorbance ratio and 260/230 nm absorbance ratio.
Group 1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; Group 2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; Group 3, commercial 10% neutral-buffered formalin; Group 4, manufacture of 3.7% formaldehyde with tap water.

Table 2. Results of the DNA purity assessment with a NanoDrop ND-1000 spectrophotometer after 3 mo

Case No.	Group 1		Group 2		Group 3		Group 4	
	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio
1	1.88	1.94	1.87	1.34	1.94	2.44	1.94	2.53
2	2.06	2.24	2.06	2.23	2.05	2.31	1.97	2.35
3	2.06	1.89	2.03	2.13	2.06	1.81	2.04	2.74
4	2.17	2.42	2.08	1.99	2.08	2.35	2.01	2.44
5	2.04	2.35	2.10	2.60	2.04	1.85	1.96	2.41
6	2.15	1.60	2.09	1.91	1.98	1.62	2.03	2.11
7	2.11	1.66	2.08	1.64	2.11	1.85	2.02	2.00
8	2.05	2.18	1.99	2.34	1.96	2.20	1.98	2.42
9	2.05	1.66	2.05	2.25	2.07	1.99	2.06	2.09
10	2.91	4.58	2.76	1.77	2.45	1.25	2.16	3.18
11	2.02	2.20	1.97	2.18	2.35	5.25	2.02	2.17
12	2.03	2.04	2.03	2.22	2.09	1.46	1.99	2.28
13	2.08	2.44	2.06	1.86	2.08	1.91	1.96	2.12
14	2.07	2.05	1.86	1.30	1.96	1.80	1.93	2.08
15	2.09	2.22	2.07	1.95	2.04	2.07	1.98	2.16
16	2.14	2.12	2.04	2.11	1.86	1.71	2.11	2.31
17	2.04	2.11	2.10	2.10	2.11	2.11	2.07	2.07
18	2.14	2.20	2.06	2.20	1.94	2.26	1.92	2.30
19	1.99	2.20	2.02	2.25	1.98	2.08	1.92	2.26
Average	2.109473684	2.215789474	2.069473684	2.019473684	2.060526316	2.122105263	2.003684211	2.316842105

The DNA purity of all the samples is assessed by a Thermo Scientific NanoDrop ND-1000 spectrophotometer. The results are expressed for the measurement using a 260/280 nm absorbance ratio and 260/230 nm absorbance ratio.
Group 1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; Group 2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; Group 3, commercial 10% neutral-buffered formalin; Group 4, manufacture of 3.7% formaldehyde with tap water.

Table 3. Results of the DNA purity assessment with a NanoDrop ND-1000 spectrophotometer after 9 mo

Case No.	Group 1		Group 2		Group 3		Group 4	
	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio	260/280 ratio	260/230 ratio
1	1.99	1.67	1.98	1.77	1.92	1.97	1.90	2.01
2	2.03	2.19	2.04	2.10	2.03	2.07	1.97	2.23
3	1.99	2.11	2.06	1.10	2.01	1.61	1.93	1.53
4	2.07	2.24	1.99	1.61	2.06	1.98	1.97	2.23
5	1.97	2.00	1.98	2.05	2.00	2.03	1.93	2.19
6	2.01	2.20	1.98	1.86	2.00	2.20	2.00	1.85
7	1.95	2.11	1.93	2.05	1.93	2.17	1.93	2.17
8	1.95	1.76	1.91	2.07	1.94	2.23	1.88	2.11
9	2.06	2.17	2.01	2.21	2.05	2.05	2.02	2.24
10	2.18	1.02	1.83	1.02	1.98	2.14	2.03	1.14
11	1.99	2.24	1.95	2.09	1.97	2.05	1.92	2.24
12	1.88	1.99	2.00	2.10	1.94	2.00	1.87	1.62
13	2.04	1.04	1.95	0.95	1.98	1.97	1.95	2.24
14	1.90	1.73	1.92	2.37	2.00	1.42	1.91	2.05
15	1.95	2.07	1.98	2.10	1.98	2.21	1.98	2.21
16	1.99	2.17	1.99	2.16	2.05	2.10	1.96	2.38
17	2.03	2.18	2.01	1.76	2.02	2.19	1.99	2.23
18	1.72	1.54	1.80	2.27	1.93	2.30	1.83	2.23
19	1.95	2.22	1.97	2.25	2.01	2.23	1.90	2.28
Average	1.981578947	1.928947368	1.962105263	1.888947368	1.989473684	2.048421053	1.940526316	2.062105263

The DNA purity of all the samples is assessed by a Thermo Scientific NanoDrop ND-1000 spectrophotometer. The results are expressed for the measurement using a 260/280 nm absorbance ratio and 260/230 nm absorbance ratio.
Group 1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; Group 2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; Group 3, commercial 10% neutral-buffered formalin; Group 4, manufacture of 3.7% formaldehyde with tap water.

a difference was shown that was unrelated to the buffer.

Comparison of the DNA yields of the different groups

The *GAPDH* PCR products from the DNA that was extracted from the four different groups of FFPE tissues are shown in Figs. 1-3. The results of case 10 were excluded from these results because its DNA amplification was the worst among the 19 cases in the initial studies. It is apparent that any type of formalin solution will have completely unaffected DNA yields over time.

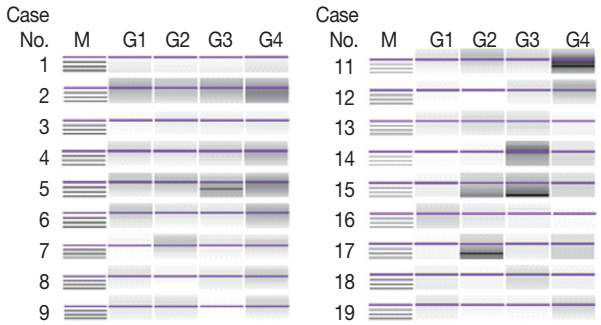

Fig. 1. Microchip electrophoresis of polymerase chain reaction products with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primer pair of DNA extracted from formalin-fixed, paraffin-embedded tissues in the initial studies.
M, size marker (10,380 bp DNA ladder); G, group; G1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; G2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; G3, commercial 10% neutral-buffered formalin; G4, manufacture of 3.7% formaldehyde with tap water.

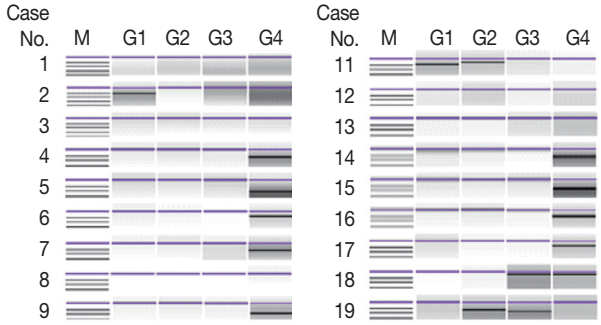

Fig. 2. Microchip electrophoresis of polymerase chain reaction products with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primer pair of DNA extracted from formalin-fixed, paraffin-embedded tissues after 3 mo.
M, size marker (10,380 bp DNA ladder); G, group; G1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; G2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; G3, commercial 10% neutral-buffered formalin; G4, manufacture of 3.7% formaldehyde with tap water.

DNA degradation yields over time

Since the buffer might have been affected by the DNA degradation of the FFPE tissue blocks during their storage, blocks from a representative case were used, the average storage period of which was over 3 months and 9 months, to test the DNA degradation yields for the storage time of the tissue block. Fig. 4 shows the microchip electrophoretic pattern of the DNA degradation from the FFPE tissue section fixing in four different formalin solutions. The figure shows that all the solutions yielded satisfactory results that had no significant difference. Three months and nine months are short-term periods, however, and as such, the authors' next attempt should be to test the degradation of the DNA from tissue blocks that have been stored for a long time.

Quality of DNA sequencing

Fig. 5 shows the *KRAS* sequence for the lung cancer samples using a BigDye terminator. The homogeneity of the peak heights and areas was of better quality for group 1 than for the three other groups. The difference between group 1 and group 2 is only slight, however, and there was no ambiguity in the interpretation of the electropherograms for the different groups.

MSI (BAT25) patterns

The patterns of the representative electropherograms in the MSI (BAT25) region are shown in Fig. 6 for the patient samples

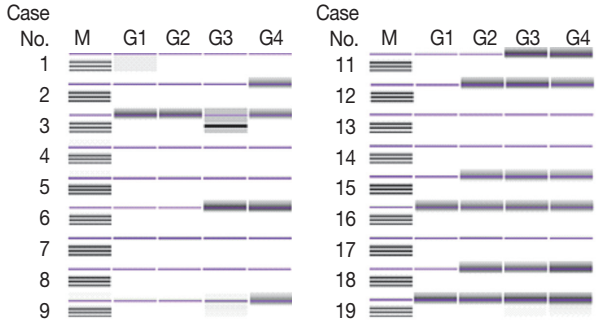

Fig. 3. Microchip electrophoresis of polymerase chain reaction products with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primer pair of DNA extracted from formalin-fixed, paraffin-embedded tissues after 9 mo.
M, size marker (10,380 bp DNA ladder); G, group; G1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; G2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; G3, commercial 10% neutral-buffered formalin; G4, manufacture of 3.7% formaldehyde with tap water.

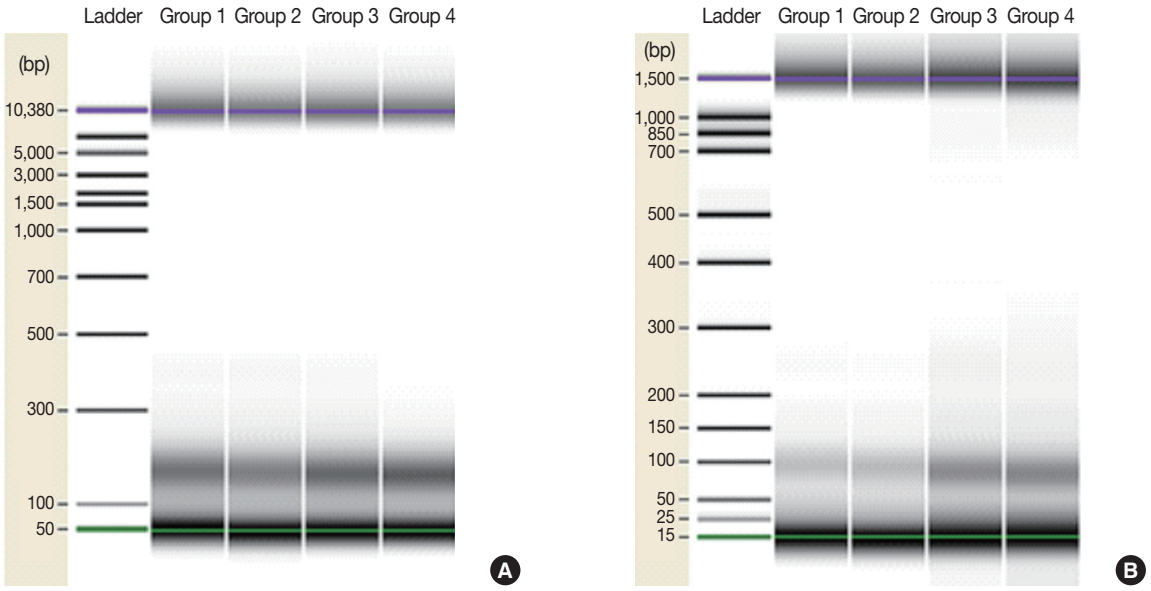

Fig. 4. Microchip electrophoresis of degradation of DNA extracted from representative formalin-fixed, paraffin-embedded tissues over 3 mo (A) and 9 mo (B).
Group 1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; Group 2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; Group 3, commercial 10% neutral-buffered formalin; Group 4, manufacture of 3.7% formaldehyde with tap water.

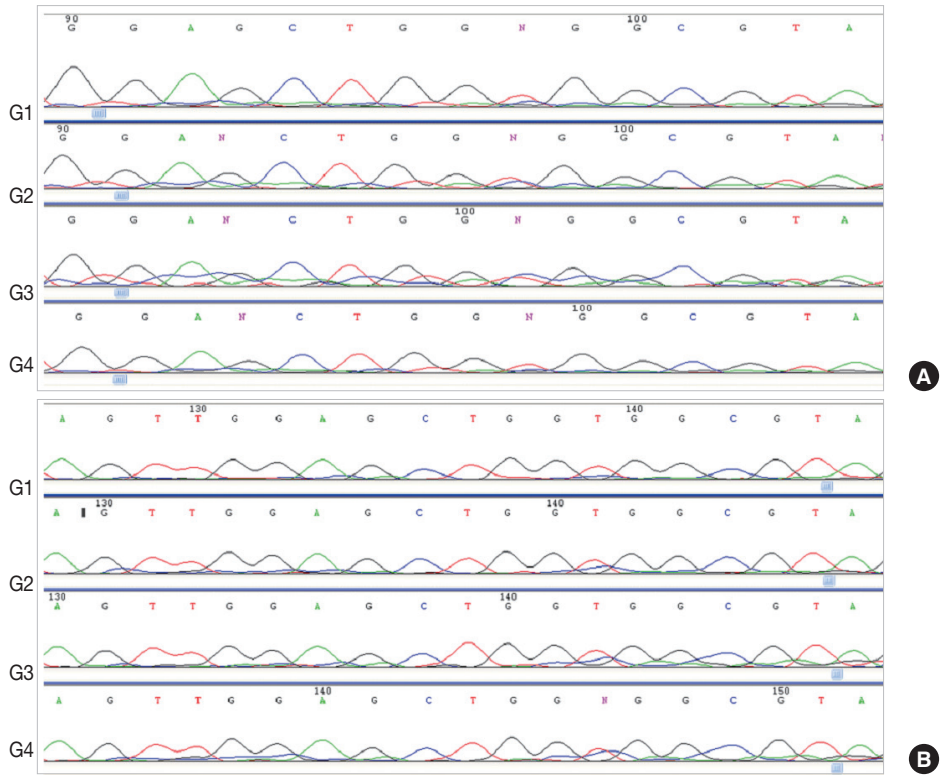

Fig. 5. The representative electropherograms of *KRAS* exon-2 fragment obtained by each group. Amplification and sequence are performed by DNA extracted from formalin-fixed, paraffin-embedded tissue that had been stored for 3 mo (A) and for 9 mo (B).
G, group; G1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; G2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; G3, commercial 10% neutral-buffered formalin; G4, manufacture of 3.7% formaldehyde with tap water.

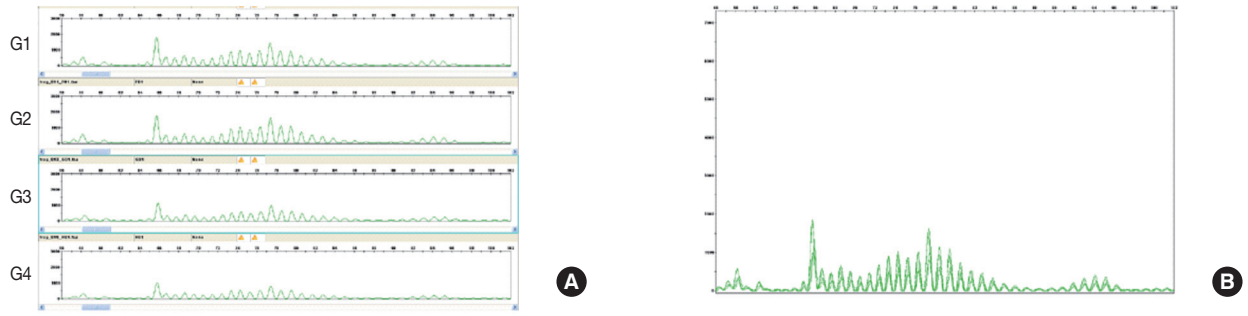

Fig. 6. The representative microsatellite instability (MSI) pattern of BAT 25 marker in formalin-fixed, paraffin-embedded tissues that have been stored for over 9 mo. The four graphs that are placed in order from top to bottom show each group's representative samples in order (A). All the representative results of MSI (BAT25) from the four groups are added together and the differences from each result are compared (B).
G, group; G1, manufacture of 3.7% formaldehyde in distilled water with neutral buffers added; G2, manufacture of 3.7% formaldehyde in tap water with neutral buffers added; G3, commercial 10% neutral-buffered formalin; G4, manufacture of 3.7% formaldehyde with tap water.

of colorectal cancer. There was no significant difference in the "main products" of the four groups.

DISCUSSION

10% NBF is commonly known to be an ideal fixation solution for soft-tissue preservation[1,2] and is the most widely used fixation solution in pathology laboratories.[1,3] In the present study, four different types of formalin solution were prepared, the same tissue was fixed in each of the four formalin solutions, paraffin blocks were produced, and the DNA preservation in each of the four FFPE tissue groups was compared with the other groups. As the fixation time may influence the DNA preservation,[12-16] all the samples were fixed at room temperature for 16-24 hours. In the case where the formalin fixture time was more than 24 hours, the fixed tissues were temporarily stored in 70% ethanol until processing. Next, as the storage conditions and periods of the paraffin-embedded blocks may affect the DNA preservation,[4,11-15,17] all the blocks were stored in a 4˚C refrigerator.

As shown in Tables 1-3, the purity of DNA between each group revealed no significant difference. Furthermore, no formalin solution was dominant over the others, as shown in the DNA yield assessed using a DNA 1000 kit (Figs. 1-3). The patterns of the microchip electrophoresis in the DNA degradation were also shown to have no noteworthy difference. The representative electropherogram of the DNA template in *KRAS*, however, revealed that group 1 showed a better quality than the other groups. The difference between group 1 and group 2 was slight, however, and group 2 showed a better quality than group 3 (10% commercial NBF). Also, the interpretation of the electropherograms for the four different groups was not ambiguous, and there was no problem with the mutation analysis. Furthermore, other factors might have affected the sequencing results such as excess or scarce templates, salt, protein, residual detergent, etc. The representative electropherograms of MSI were shown to agree with the main products. Therefore, with regard to DNA preservation in FFPE tissues, the results of this study revealed that the use of 10% NBF is not a crucial factor when there is a suitable formalin fixation time for embedding in paraffin and a short formalin storage period before degradation. Other factors may be more important than the neutral buffer in these circumstances.

However, non-buffered formalin is definitely degraded over time, and the degraded formalin is believed to contribute to the poor quality of the nucleic acids that are obtained from FFPE tissues.[3,4] As such, to find the optimal non-buffered formalin fixation time for adequate DNA preservation before degradation, further study is required. In addition, as the DNA quality of FFPE tissues may change over time, the future studies require long-term follow-up in terms of the changes in the respective groups' DNA preservation after years of storage of paraffin-embedded tissue. In almost all laboratories, however, including these authors' laboratory, formalin is newly manufactured and is expended before degradation, and the maximum formalin fixation time of the tissues before embedding in paraffin is 48 hours. In this case, the buffer will not have a significant influence on the DNA preservation of the FFPE tissues. In most pathology laboratories, molecular diagnoses have been conducted using DNA from FFPE tissues rather than RNA, within 30 days. Hence, the FFPE tissues that had been fixed in a mixture of 3.7% formaldehyde with tap water without degradation are sufficient for use in molecular diagnoses. Further, it seems that

the purity average and yield of DNA for the four formalin solution groups slightly differ, although the storage period was over nine months. Thus, it appears that the storage environment of FFPE tissues is more important than the buffer in DNA preservation. To optimize the storage environment, the used FFPE tissues were covered in paraffin, and were promptly stored in a 4˚C refrigerator.

In summary, the results of this study showed that formalin newly manufactured by directly mixing 3.7% formaldehyde with distilled water and adding buffers is the best fixation solution for DNA purity analyzed with the use of a spectrophotometer, and DNA quality analyzed with the use of a microchip electrophoresis of PCR products with *GAPDH* primer pair. The amplification and sequence of the DNA extracted from the representative sample showed similar patterns for each group in repeated tests. Although the distinction was fine, however, it is negligible considering cost-effectiveness. In addition, as this study differs from those of the general public,[9,18] the results of this study will be considerably disputed. Therefore, more detailed studies must be conducted with a large number of samples and with long intervals, and several pathology laboratories must conduct studies to ascertain the merits of the use of a buffer.

REFERENCES

1. Shi SR, Cote RJ, Wu L, *et al.* DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. J Histochem Cytochem 2002; 50: 1005-11.
2. Chu WS, Furusato B, Wong K, *et al.* Ultrasound-accelerated formalin fixation of tissue improves morphology, antigen and mRNA preservation. Mod Pathol 2005; 18: 850-63.
3. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Res 1999; 27: e12.
4. Hewitt SM, Lewis FA, Cao Y, *et al.* Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med 2008; 132: 1929-35.
5. Ribeiro-Silva A, Zhang H, Jeffrey SS. RNA extraction from ten year old formalin-fixed paraffin-embedded breast cancer samples: a comparison of column purification and magnetic bead-based technologies. BMC Mol Biol 2007; 8: 118.
6. Coura R, Prolla JC, Meurer L, Ashton-Prolla P. An alternative protocol for DNA extraction from formalin fixed and paraffin wax embedded tissue. J Clin Pathol 2005; 58: 894-5.
7. Shi SR, Datar R, Liu C, *et al.* DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem Cell Biol 2004; 122: 211-8.
8. Körbler T, Grsković M, Dominis M, Antica M. A simple method for RNA isolation from formalin-fixed and paraffin-embedded lymphatic tissues. Exp Mol Pathol 2003; 74: 336-40.
9. Hamatani K, Eguchi H, Takahashi K, *et al.* Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. J Histochem Cytochem 2006; 54: 773-80.
10. Bonin S, Hlubek F, Benhattar J, *et al.* Multicentre validation study of nucleic acids extraction from FFPE tissues. Virchows Arch 2010; 457: 309-17.
11. Penland SK, Keku TO, Torrice C, *et al.* RNA expression analysis of formalin-fixed paraffin-embedded tumors. Lab Invest 2007; 87: 383-91.
12. Volkenandt M, Dicker AP, Fanin R, Banerjee D, Albino A, Bertino JR. Polymerase chain reaction analysis of DNA from paraffin-embedded tissue. Methods Mol Biol 1993; 15: 81-8.
13. Xie R, Chung JY, Ylaya K, *et al.* Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. J Histochem Cytochem 2011; 59: 356-65.
14. Wolff C, Schott C, Porschewski P, Reischauer B, Becker KF. Successful protein extraction from over-fixed and long-term stored formalin-fixed tissues. PLoS One 2011; 6: e16353.
15. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002; 161: 1961-71.
16. Bresters D, Schipper ME, Reesink HW, Boeser-Nunnink BD, Cuypers HT. The duration of fixation influences the yield of HCV cDNA-PCR products from formalin-fixed, paraffin-embedded liver tissue. J Virol Methods 1994; 48: 267-72.
17. Ferrer I, Armstrong J, Capellari S, *et al.* Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: a BrainNet Europe study. Brain Pathol 2007; 17: 297-303.
18. Zsikla V, Baumann M, Cathomas G. Effect of buffered formalin on amplification of DNA from paraffin wax embedded small biopsies using real-time PCR. J Clin Pathol 2004; 57: 654-6.