Nucleic Acid Quality Preservation by an Alcohol-based Fixative

Comparison With Frozen Tumors in a Routine Pathology Setting

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Abstract: Pathologic diagnosis requires tissue fixation for histologic and immunohistologic analysis, and formalin is routinely used for this. The disadvantage of this fixative is its inability to preserve nucleic acids. Pathologic tumor diagnosis requires extensive molecular analyses, for which formalin fixation may be not adequate. Recently, an alcohol-based fixative (molecular fixative, MF) was described that allows nucleic acid preservation as well as histologic and immuno-histologic studies. Moreover, the MF fixation processing system (Xpress) is fast and is well adapted to a routine process. We evaluated RNA and DNA quality within 1 month and after 1 year for 10 breast carcinomas and 20 sarcomas fixed in MF in comparison with the corresponding frozen tumors. The quality of DNA extracted from the MF-fixed tissue was similar to that extracted from the frozen tumors. The quality of RNA extracted from the MF-fixed tissue was lower than that of frozen tumors; nevertheless, a majority of RNA integrity number (RIN) values were greater than 7. Gene expression quantification by real-time polymerase chain reaction gave comparable results between tumors fixed with MF and frozen tumors. Tissue fixation at 4°C with the MF improved the RNA quality measured by the RIN value. However, after storage for 1 year at room temperature, although DNA quality was preserved, RNA extracted from tissues fixed with the MF was degraded. Tissue fixation with the MF is an important improvement for molecular pathologic diagnosis, enabling a combination of routine pathologic diagnoses and current molecular diagnoses if they are carried out near the processing time.

Key Words: tissue fixation, alcohol-based fixative, nucleic acid preservation, paraffin-embedded tissue

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Formalin is a fixative used for routine diagnosis in pathology. It allows good tissue preservation for histologic diagnosis, and is also adapted for immunohistochemistry and fluorescence in-situ hybridization. However, this fixative modifies the chemical structure of nucleic acids inducing degraded RNA and DNA.[1,2] Formalin fixation and classic tissue processing may no longer be adapted to modern pathology that requires nucleic acid preservation for molecular studies and rapid diagnosis. Although several publications have reported optimized protocols for recovering higher DNA and RNA quality,[3–6] nucleic acids are still degraded and less suitable than when extracted from frozen tissues for the more recent molecular biology techniques.[7,8] Molecular pathologists are searching for a fixative suitable to preserve histologic tissue structure and to allow immunohistologic and molecular analyses. Such a fixative may be an improvement for molecular pathologic diagnosis if nucleic acid preservation is equivalent to frozen tissues.

For 3 years, we have been testing a high throughput formalin-free tissue processing system (Xpress from Sakura Finetek SAS, Villeneuve d'Ascq, France) and an alcohol-based molecular-friendly fixative (MF).[9–13] This system, originally developed by Morales et al, greatly accelerates the turn-around time for tissue processing, while yielding histologic material of comparable morphologic and immunohistochemical quality to formalin-fixed tumors.[9]

Our aim in this study was to assess the quality of nucleic acids extracted, within 1 month and after 1 year, from tumor specimens received in our laboratory on a routine basis, fixed in MF, and processed in the Xpress machine, to evaluate whether they were equivalent to paired frozen tumor samples.

MATERIALS AND METHODS

Tissue Samples

Fresh tumor samples from 30 consecutive sarcomas (1 pleomorphic liposarcoma, 1 myxoid chondrosarcoma, 8 dedifferentiated liposarcomas, 2 dermatofibrosarcoma protuberans, 4 leiomyosarcomas, 4 myxofibrosarcomas, 3 unclassified sarcomas, 3 gastrointestinal stromal tumors,

1 synovialsarcoma, 1 osteosarcoma, 1 rhabdomyosarcoma, and 1 spindle cell liposarcoma) and 10 consecutive breast tumors (9 invasive ductal carcinomas and 1 invasive lobular carcinoma) received between January 2007 and July 2008 by the Institut Bergonié Department of Pathology were prospectively included in this study. To be eligible for inclusion, tumors had to be of sufficient size to perform routine pathologic analysis, systematic frozen tumor banking, and fixation in MF. Tumor fragments designated to tumor banking were frozen in liquid nitrogen, weighed, and stored in a $-140^{\circ}C$ freezer. Tumor fragments processed with the Xpress machine were cut beforehand in 2-mm-thick slices and stored for 1 to 68 hours in MF at room temperature for all cases and at $4^{\circ}C$ for 6 cases.

The tumor fragments were then placed in a preprocessing solution containing acetone and isopropylic alcohol, according to the manufacturer's instructions for a standard time of 20 minutes before being processed in the Xpress machine with the 120-minute program. Processing in the Xpress machine was performed in 4 stages: a fixation/dehydration step in an acetone and isopropylic alcohol solution, a dehydration/clearing step in an isopropylic alcohol and mineral oil solution, a clearing/paraffin impregnation step in a mineral oil solution, and an impregnation step in paraffin wax. The first 2 steps were carried out under the combined action of microwave heating and agitation.

Subsequently, the tumor samples were embedded in paraffin and stored at room temperature ($\approx 20^{\circ}C$).

Nucleic acids were extracted from MF-fixed tumors [molecular-fixed paraffin-embedded (MFPE)] between less than 1 week and 28 weeks after the fixation process (median value = 2 wk).

The use of human tumors in this study has been approved by the institution's review committee.

DNA Extraction

For MFPE tissues, fifteen 10 μm sections were deparaffinized twice with toluene, rinsed twice with absolute ethanol, and then dried in a speed vacuum. Sections were digested with 500 μL of guanidine-HCl 6 M and proteinase K (final concentration: 10 μg in 100 μL of tris acetate ethylene diamino tetra acetic acid, Promega, Madison, WI) at $55^{\circ}C$ under agitation. Subsequently, DNA was extracted with phenol-choroform according to a standard procedure. In the paraffin blocks, the remaining tissue was protected from the air with a paraffin layer.

For frozen tissues, DNA was extracted from 50 mg of tumor with phenol-choroform according to standard procedure.

DNA quantity was evaluated by spectrophotometry using a nanodrop analyzer (Nanodrop technologies, Agilent technologies, Waidbrom, Germany).

DNA integrity was evaluated by electrophoresis on a 1% agarose gel.

RNA Extraction
Optimization of the RNA Extraction Protocol

For MFPE tissues, ten 10 μm sections were deparaffinized twice with toluene, rinsed twice with absolute ethanol, and then dried with a vacuum. Three protocols of RNA extraction from fixed tumors were tested. Tissues were disrupted in 3 different buffers according to protocols 1 to 3.

Protocol 1: Sections were disrupted in 700 μL of the solution (guanidine thiocyanate 4 M, sodium citrate 0.75 M, pH 7, 0.5% sarcosyl, pH = 7) containing 1% β-mercaptoethanol. Once dissociated, tissues were incubated for 1 hour under agitation at room temperature. Next, 70 μL of sodium acetate 2 M, pH 4, 140 μL of chloroform-isoamylic alcohol (49:1), and 700 μL of phenol saturated with sodium acetate 0.02 M, pH 5 were added to the lysate, shaken, and incubated on ice for 15 minutes.

Protocol 2: Sections were disrupted in 700 μL of the Trizol LS reagents (Invitrogen, Carlsbad, CA). Next, 300 μL of a solution of chloroform-isoamylic alcohol (49:1) was added to the solution.

For protocols 1 and 2, after centrifugation for 20 minutes at 10,000*g*, the aqueous phase was retrieved and mixed with an equal volume of 70% ethanol. RNA was then purified on an RNeasy column (Qiagen Inc, Valencia, CA).

Protocol 3: RNA was extracted with the commercially available RNeasy kit according to the manufacturer's instructions (Qiagen Inc, Valencia, CA).

RNA from frozen tissues was extracted according to the manufacturer's instructions (Quiagen Inc, Valencia, CA).

RNA quantification was performed with the nanodrop analyzer. RNA integrity[14] was evaluated on an RNA 6000 Nano chip (Agilent technologies, Waidbrom, Germany). The profile was scored from 0 to 3 according to the RNA profile on gel electrophoresis, with 3 corresponding to the visualization of RNA 28S and 18S with RNA 28S intensity staining >RNA 18S; 2 corresponding to the visualization of the 2 RNA with RNA 18S intensity staining > RNA 28S or visualization of 1 of the 2 RNA; and 1 corresponding to the absence of visualization of the 2 RNA.

Qualitative DNA Analysis
Multiplex PCR

Polymerase chain reaction (PCR) was performed with a multiplex PCR kit (Quiagen Inc, Valencia, CA) to amplify 5 PCR products from 100 to 600 bp (Table 1),[15] with 250 ng of DNA and 2.5 pmole for each primer, except the *AFA* primers (5 pmoles).

EGFR Exon 20 PCR Product Analysis by DHPLC

PCR amplifications were performed on exon 20 of the *EGFR* gene (Table 1). Four hundred nanograms of DNA were amplified with the thermal cycling profile: $95^{\circ}C$ for 5 minutes, 40 cycles of $95^{\circ}C$ for 30 seconds, $60^{\circ}C$

TABLE 1. Primers Sequences

Multiplex PCR gene		PCR Product Size
AFA exon 3		
Forward	5′-GGA GCA GCA TTC CAT CCA GC-3′	
Reverse	5′-CAT CCA TGG GCC GGA CAT AA-3′	600 bp
AFA exon 11		
Forward	5′-CCG CAG CAA GCA ACG AAC C-3′	
Reverse	5′-GCT TTC CTC TGG CGG CTC C-3′	400 bp
PLZF		
Forward	5′-TGC GAT GTG GTC ATC ATG GT G-3′	
Reverse	5′-CGT GTC ATT GTC GTC TGA GG C-3′	300 bp
RAGI		
Forward	5′-TGT TGA CTC GAT CCA CCC CA-3′	
Reverse	5′-TGA GCT GCA AGT TTG CTG GA A-3′	200 bp
TBXAS		
Forward	5′-GCC CGA CAT TCT GCA AGT CC-3′	
Reverse	3′-GGT GTT GCC GGG AAG GGT T-3′	100 bp
Quantitative PCR		
ALB		
Forward	5′-GCT GCT ATC TCT TGT GGG CTG T -3′	
Reverse	5′-ACT CAT GGG AGC TGC TGG TT C-3′	
Quantitative RT-PCR		
TBP		
Forward	5′-TTT TCT TGC TGC CAG TCT GGA C -3′	
Reverse	5′-CAC GAA CCA CGG CAC TGA TT -3′	
PGK		
Forward	5′-CAG TTT GGA GCT CCT GGA AG -3′	
Reverse	5′-TGC AAA TCC AGG GTG CAG TG -3′	
EGFR sequencing		
Forward	5′-GAA GCC ACA CTG ACG TGC C-3′	
Reverse	5′-TCC CCT CCC CGT ATC TCC-3′).	

PCR indicates polymerase chain reaction; RT, reverse transcriptase.

for 45 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. To detect homozygous mutations by denaturing high-performance liquid chromatography (DHPLC), PCR products obtained from the tumoral DNA were mixed with an equal proportion of a PCR product obtained from a wild-type DNA. This process leads to artificial heteroduplexes. A temperature cycle was added for the PCR product run on DHPLC for heteroduplex formation: 98°C for 10 minutes, 60°C for 30 minutes, and then cooling at 4°C.

The WAVE DNA Fragment Analysis System (Transgenomic, Inc, Omaha, NE) was used to detect the heteroduplex PCR products, and the elution temperatures were 59°C and 60°C.

EGFR Exon 20 Sequence Analysis

PCR products were sequenced with automated cycle sequencing for both strands using the Bid-Dye DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were carried out on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Quantitative DNA Analysis

PCR amplification was performed in duplicate using the ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with a 25 μL final reaction mixture containing 200 nM of each primer (Table 1), 2X qPCR master mix containing sybergreen, and *N*-uracyl glycosylase (Eurogentec, Liege, Belgium). The PCR reaction was preheated at 50°C for 2 minutes, at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute, followed by 1 dissociation stage (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). Four DNA quantities of DNA were amplified (100, 50, 25, and 10 ng).

Quantitative cDNA Analysis

Reverse Transcription

One microgram of RNA was reverse transcribed to cDNA with expanded reverse transcriptase (Promega corporation, Madison, WI) and random hexamers.

Quantitative PCR was performed in duplicate to amplify PCR products of 2 sizes (*TBP* 89 bp and *PGK* 247 bp, Table 1). For real-time PCR, different quantities of cDNA (100, 50, 25, and 12.5 ng) were amplified with 200 nM of each primer in a 2X qPCR master mix containing sybergreen and *N*-uracyl glycosylase (Eurogentec, Liege, Belgium). The PCR reaction was preheated at 50°C for 2 minutes, at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute, followed by 1 dissociation stage (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s).

Relative expression of the *PGK* mRNA was calculated using the comparative cycle at the threshold (CT) method described earlier.[16] The quantity of RNA input was controlled by amplification of a portion of the *TBP89* cDNA with a short PCR product size of 89 bp. Finally, results from the fixed tissues were normalized to frozen tissue results for each PCR-amplified cDNA quantity, with 2-delta delta CT (ddCT) mean values equal to the mean value obtained for each cDNA input.

RESULTS

The time between the surgical excision of the specimen and freezing or immersion in MF varied from 10 to 66 minutes (median value = 25 min). Fixation duration for MFPE tumors, depending on the moment in the week, varied from 1 hour to 68 hours (median = 21 h).

DNA Quality Analysis

DNA was successfully extracted from all frozen and fixed tissues. The quantity extracted from 15 MFPE sections varied from 20 to 525 μg (mean value = 125 μg), depending on the size and cellularity of the tumor samples. DNA extracted was of high molecular weight,
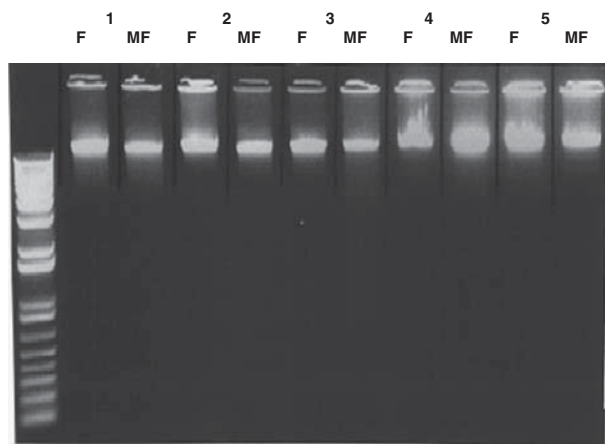
FIGURE 1. Agarose gel electrophoresis of DNA extracted from paired frozen (F) and methanol-fixed paraffin-embedded (MF) breast tumors (1, 2, and 3) and sarcoma tumors (4, 5).

the same as DNA extracted from the paired frozen tissues (Fig. 1). The DO260/DO280 ratio ranged from 1.8 to 2.02 for the frozen tissues and from 1.75 to 2.04 for the MF-fixed tissues. DNA was simultaneously amplified by PCR for 5 targets, with PCR product sizes ranging from 100 to 600 bp. For all the paired tissues (frozen and MFPE), the 5 PCR products were amplified (Fig. 2).

Four paired molecular-fixed and frozen tumors were analyzed for EGFR exon 20 polymorphism detection by DHPLC and sequenced. For 3 cases, an abnormal DHPLC profile was detected (Fig. 3). The same profile was observed for each paired fixed and frozen tumor. After sequencing, the c.2361G >A polymorphism was
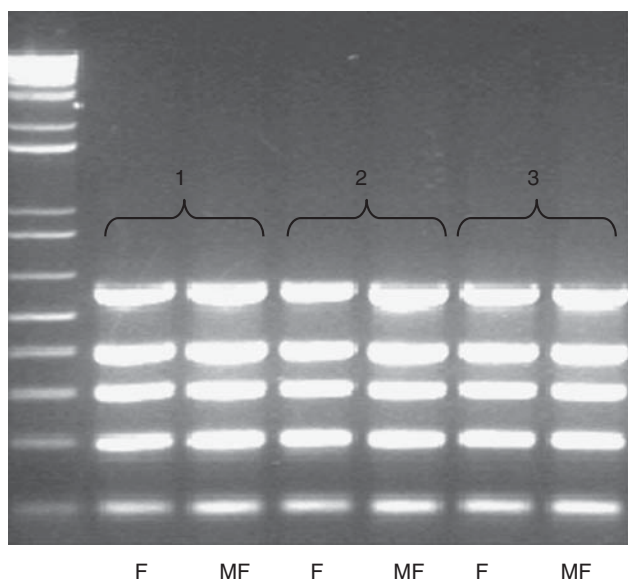

FIGURE 2. Multiplex PCR amplification for 5 different PCR sizes of products ranging from 100 to 600 bp for 3 paired frozen (F) and methanol-fixed paraffin-embedded (MF) tumors. PCR indicates polymerase chain reaction.

detected for the 3 cases with an abnormal DHPLC profile, and the sequence was normal for the case with a normal DHPLC profile. However, all the paired samples were sequenced for the EGFR exon 20 PCR product. In all the cases (40 paired tumors), the same sequence was detected for the paired fixed and frozen tumors, with 39 cases bearing the c.2355C > T polymorphism (22 cases were heterozygous and 17 homozygous). No discordance was observed for EGFR exon 20 PCR product sequences between the frozen and the molecular-fixed tumors (Fig. 4).

The PCR efficiency was compared between the frozen and the paired MFPE tumors for the 40 samples analyzed. Four quantities of DNA (100, 50, 25, and 10 ng) were amplified in duplicate by real-time PCR for the albumin gene. According to the dissociation curves, no primer dimers were observed. Slopes relating to PCR efficiency were compared between each pair of frozen and fixed tumors. In all cases, the correlation coefficient was greater than 95%. The maximum difference between the absolute value of the slope obtained from the frozen tumor and the corresponding fixed tumor was 0.6 (Fig. 5). For MFPE tissues, the slope value ranged from −2.8 to −3.7 (mean = −3.2). For frozen tissues, the slope value ranged from −2.3 to −3.7 (mean = −3.15).

RNA Quality Analysis

Optimization of RNA Extraction Protocol

Protocol 1 was found to be the most suitable for RNA extraction from MFPE tissues. RNA extraction with trizol (protocol 2) impaired the 28S RNA recovery and protocol 3 gave a low yield of RNA recovery. These protocols were also tested on the paired frozen tumors, and protocols 1 and 3 gave the best RNA extraction from frozen tumors. Therefore, RNA was extracted from frozen tumors according to protocol 1. The quantity of RNA extracted from 10 MFPE sections varied from 5 to 35 μg (median value = 14.8 μg).

RNA Electrophoresis

RNA quality was assessed on an Agilent Technologies bioanalyzer for 38 paired MFPE and frozen tumors. The detection of the 28S and 18S RNA was scored as described in Materials and Methods section. For all the frozen tumors, the score was 3. The scores were 3, 2, and 1 for 30, 7, and 1 MFPE tumors, respectively (Fig. 6). For frozen tumors, the RNA integrity number (RIN) varied from 7.8 to 10 (median = 9.3). For molecular-fixed tumors, the RIN varied from 2.1 to 9 (median = 7.3). For MFPE-breast carcinoma tumors, the RIN varied from 4.8 to 8.7 (median = 7.9), and for the MFPE-sarcoma tumors, the RIN varied from 2.1 to 9 (median = 7.3) (Fig. 7).

Quantitative Reverse Transcriptase PCR

For each sample analyzed, the PCR efficiency was greater than 95%. The ddCT value varied from −3.1 to 2.2 (mean = 0.03). When the RIN value was greater than 7 (52% of cases), the ddCT value varied from −2.2 to 1
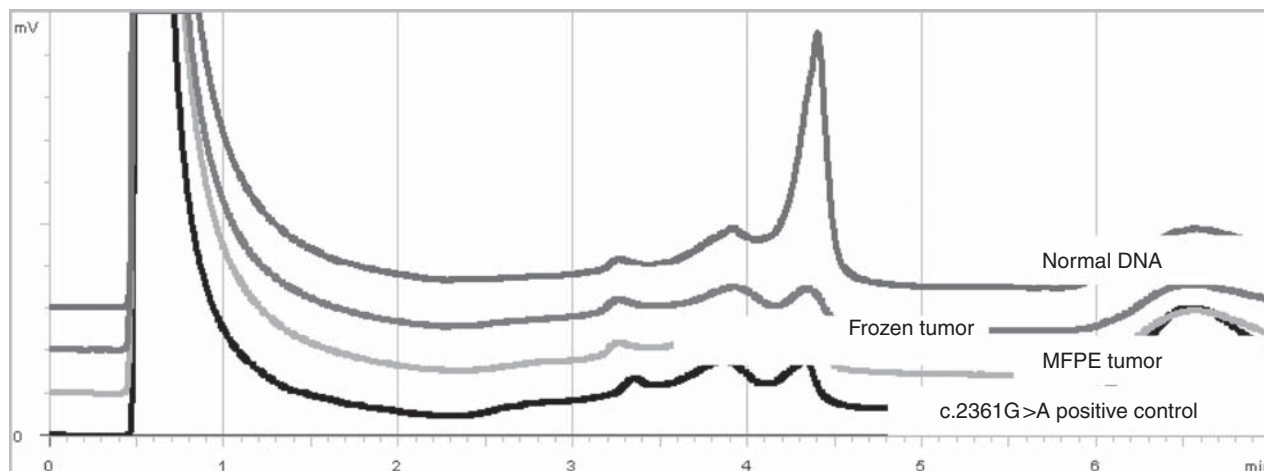
FIGURE 3. Comparison of denaturing high-performance liquid chromatography profile of EGFR exon 20 polymerase chain reaction product between the normal DNA control, c.2361G>A positive control, and frozen and molecular-fixed paraffin-embedded (MFPE) tumor material (breast carcinoma).

(mean = 0.01) (Fig. 8), and when the RIN value was less than 7, it varied from −3.1 (RIN = 2.1) to 2.2 (RIN = 5.1) (mean = 0.1).

The ratio for PGK transcript between the paired frozen tumor and the MFPE tumor varied from 0.2 to 9.8 (mean = 1) (Fig. 9).

Effect of Tissue Fixation at 4°C With MF

RIN values were compared between 6 matched frozen tumors and MF tumors fixed at 20°C and 4°C. There was an increase in the RIN value when the tissue was fixed at 4°C with MF (mean RIN value = 8.9 for frozen tumors and mean RIN value = 5.6 and 7.2 for
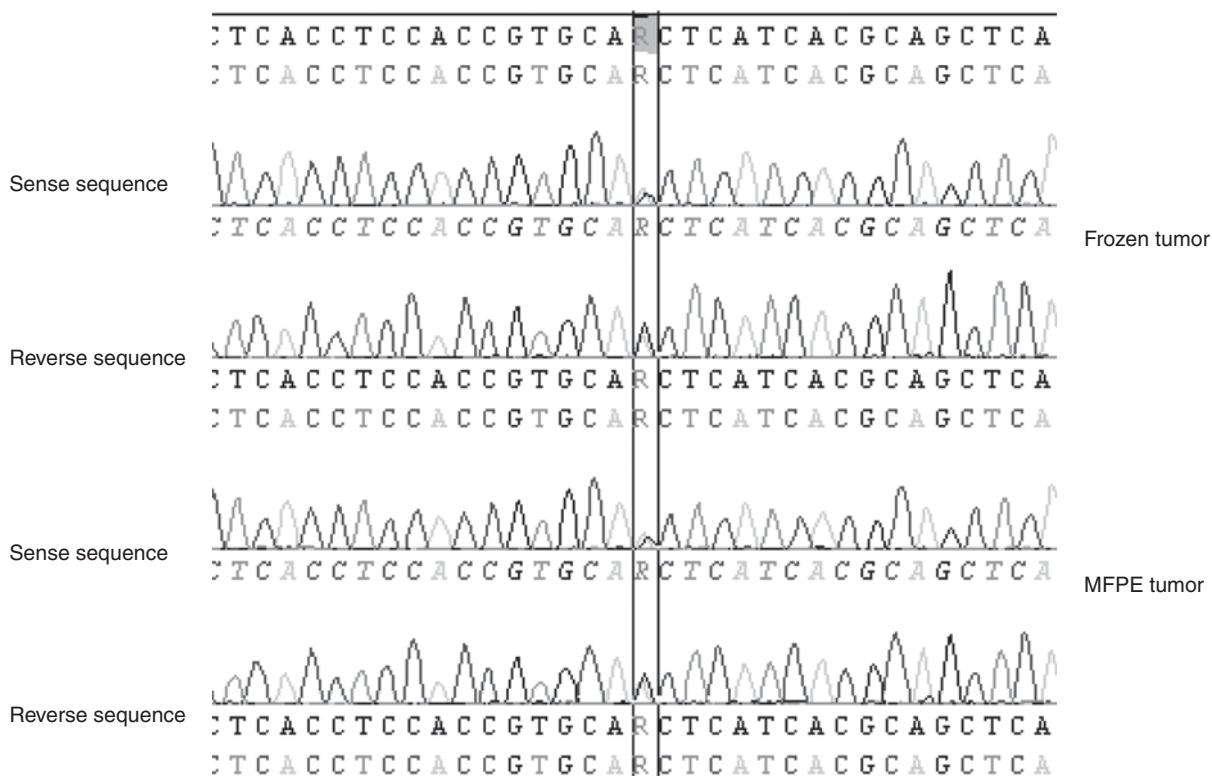

FIGURE 4. Electropherogram profile for EGFR exon 20 polymerase chain reaction product. Detection of the c.2361G>A polymorphism for the frozen and the matched molecular-fixed paraffin-embedded (MFPE) fixed tumors (breast carcinoma).
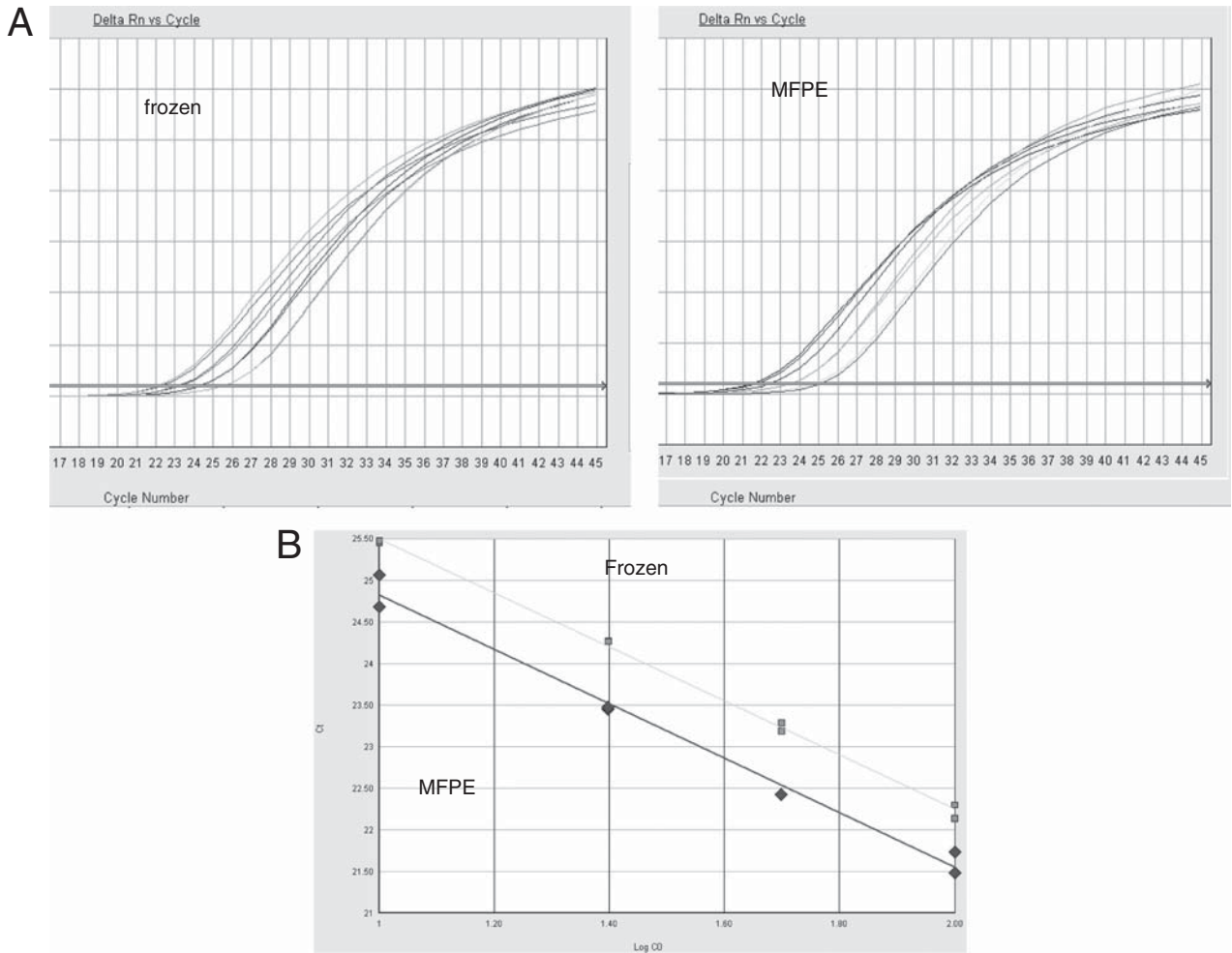
FIGURE 5. Real-time PCR amplification for the albumin gene. Four different quantities of DNA were amplified by PCR (A). A similar slope of the regression line was measured between the frozen tumors and the molecular-fixed paraffin-embedded (MFPE) tumors (B). PCR indicates polymerase chain reaction.
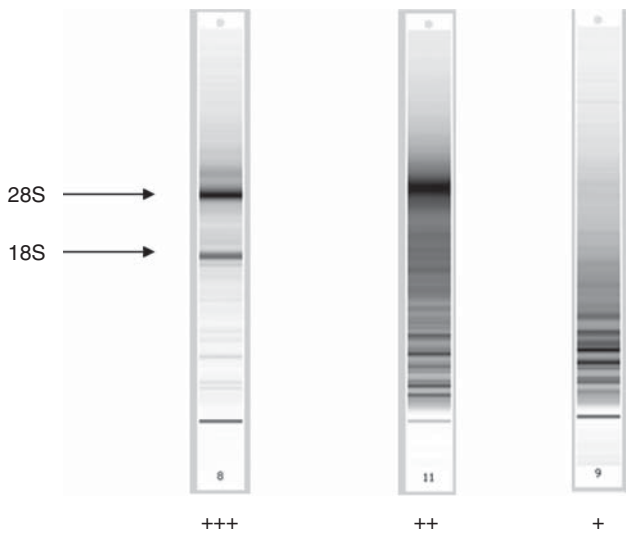

FIGURE 6. RNA quality scoring on a gel electrophoresis. +++ indicates good quality; ++, medium quality; +, poor quality.

MF tumors fixed at room temperature and at 4°C, respectively) (Fig. 10).

Nucleic acid quality after 1 year of storage of MFPE tissues at room temperature.

DNA and RNA were extracted from 25 frozen tumors and the paired molecular-fixed tumors stored for 1 year.

The DNA was of high molecular weight after 1 year of storage of the paraffin block. The DO260/280 ratio ranged from 1.84 to 2.17 and from 1.86 to 2.06 for frozen and fixed tumors, respectively.

However, there was degradation of RNA extracted from molecular-fixed tumors after 1 year of storage at room temperature. For frozen and fixed tumors, the RIN value ranged from 7.9 to 10 and from 1.9 to 6.4, respectively (Table 2). Figure 11 shows the RIN value for each RNA extracted from the frozen tumors and the matched MFPE tumors at the time of extraction and after 1 year of block storage. When the RIN value for frozen tumors remained unchanged,
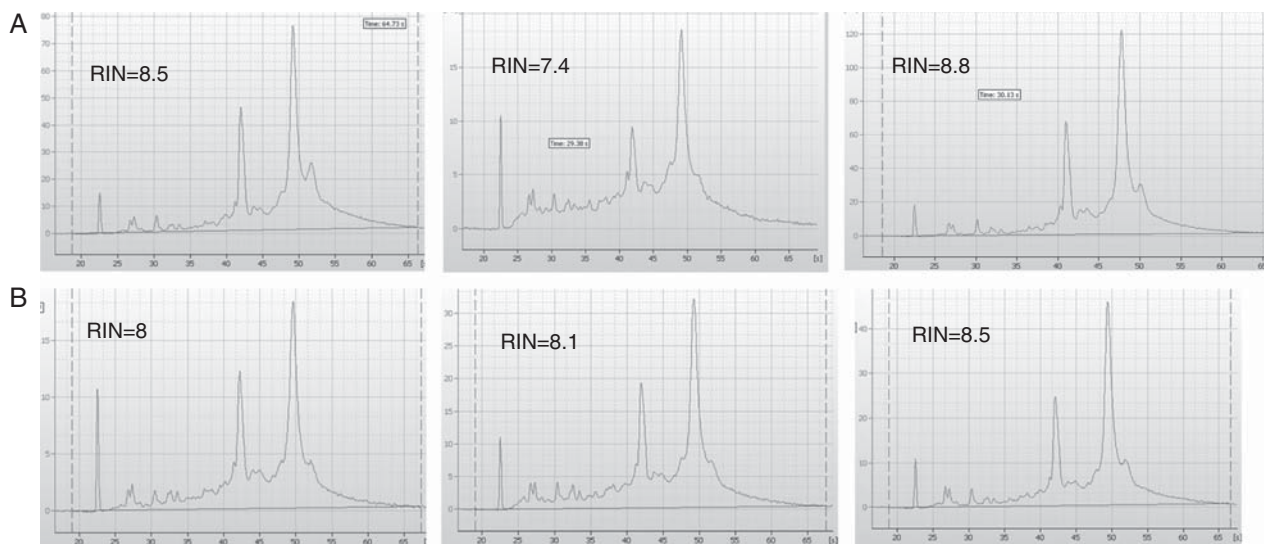
FIGURE 7. Multiple agilent profiles obtained from MFPE sarcomas (A) and MFPE-breast carcinomas (B). MFPE indicates molecular-fixed paraffin-embedded; RIN, RNA integrity number.

there was a strong decrease in RNA quality for MFPE tumors (Fig. 12).

DISCUSSION

For routine pathologic diagnosis, the main material available for histologic and immunohistologic analysis is the FFPE tumor. At present, pathologic diagnosis in oncology is increasingly dependent on molecular diagnosis, helpful not only for tumor diagnosis, but also for predicting the response to targeted treatment. Optimal DNA and RNA qualities are required for relevant results. In fact, nucleic acids extracted from formalin-fixed tumors are of poor quality.[1,2] Formalin-based fixatives induce not only chemical modifications such as the

FIGURE 8. Comparable PCR efficiency for reverse transcription-quantitative PCR amplification for PGK and TBP between frozen (RIN = 8.8) and molecular-fixed paraffin-embedded (MFPE) (RIN = 8.7) breast tumors. The delta delta Ct value is −0.1. PCR indicates polymerase chain reaction; RIN, RNA integrity number.

addition of mono-methylol groups to nucleic acids,[2] but also double and single strand breaks, giving small-sized DNA or RNA fragments. In these conditions, PCR is optimal only for small PCR fragments, usually up to 250 bp.[17] Moreover, during PCR, Taq polymerase can insert a wrong nucleotide residue before jumping to another template, giving an artificial heterozygous mutation as a consequence of such chemical modifications.[18] No fixative has been found that is capable of preserving the histologic structure of the tumor, suitable for immunohistologic analysis, and capable of giving DNA, RNA of optimal quality, comparable with that obtained from frozen material. No formal comparison between MFPE and frozen tumors has been made in a routine setting until now.[13,19] Several fixatives mainly based on alcohol dehydration have been tested for preservation of nucleic acids.[11–13,18–26] The recently described fixative MF was evaluated in the laboratory because it has a rapid turn-around capability adaptable to a routine histologic process with preservation of macromolecules.[20] Histologic examination and immunohistologic analysis show adequate preservation of the tissue.[9,12] In this study, we evaluated the quality of nucleic acids extracted from breast and sarcoma tumors fixed with the MF in comparison with those extracted from frozen tumors.

In this study, we found the quality of DNA extracted from the MFPE tumors to be similar to the DNA extracted from the corresponding frozen tumors. It is of high molecular weight and the DO260/280 is the same for MFPE as for frozen tumors. Moreover, DNA extraction does not require a specific protocol, as it is the same as for frozen tumors, and the extraction was successful for 100% of samples. The quality of DNA was evaluated by different methods. First, although PCR fragment size needs to be less than approximately 250 bp when DNA is extracted from formalin-fixed tissues,
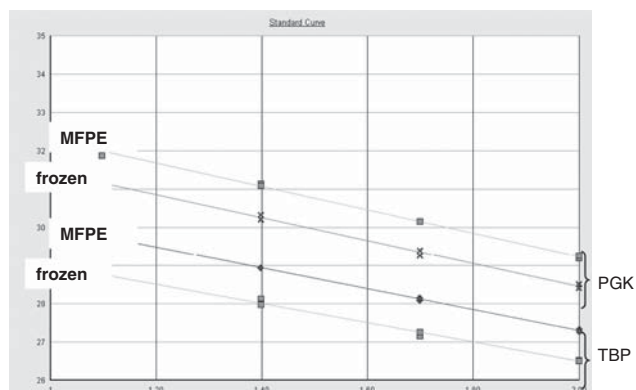
PGK transcript ratio between
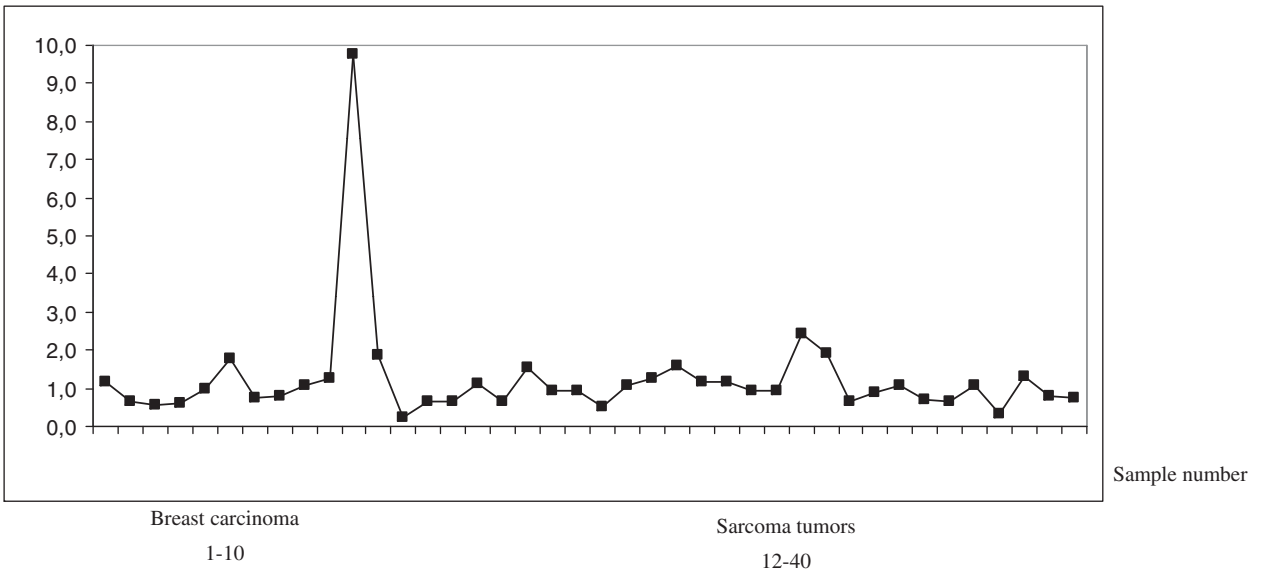
MFPE and frozen tumors

FIGURE 9. PGK transcript level ratio between molecular-fixed paraffin-embedded (MFPE) and frozen tumors for breast carcinoma and sarcoma tumors.
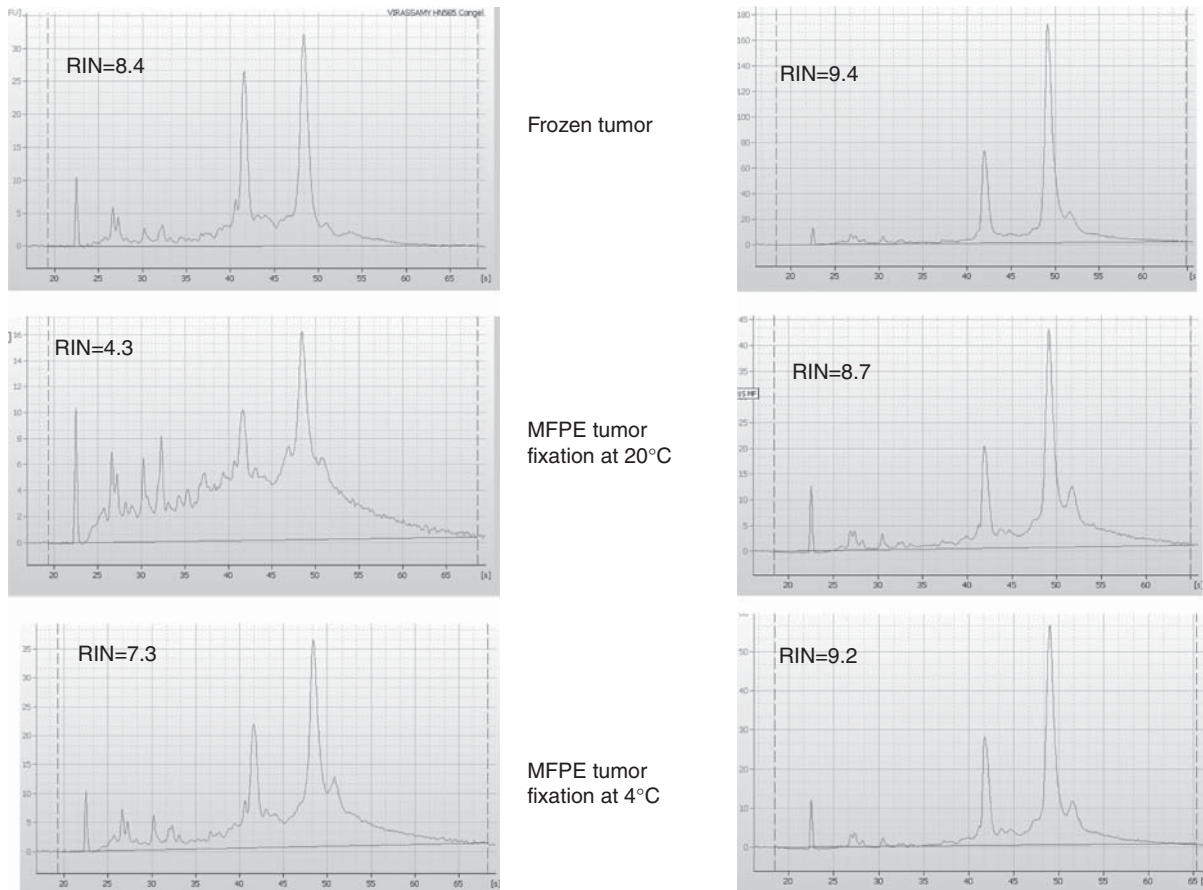

FIGURE 10. Effect on RNA quality after fixation with molecular fixative at 4°C (sarcomas). MFPE indicates molecular-fixed paraffin-embedded; RIN, RNA integrity number.

TABLE 2. Comparison of the Mean RIN Value for Frozen and the Paired Fixed Tumors After 1 Year Storage of the Blocks at Room Temperature (Mean Values, n = 23)

	T = 0	T + 1
Frozen	9.4	9.37
MFPE	6.9	3.4

T = 0 corresponds to the time of fixation.
T + 1 corresponds to 1 year storage of the MFPE block at room temperature. MFPE indicates molecular-fixed paraffin-embedded; RIN, RNA integrity number.

successful amplification up to 600 bp is obtained for the 40 tumors analyzed. Mutation screening may require a prescreening stage before sequencing to reduce the costs and to improve the delay of results. For this purpose, we evaluated the ability to detect mutations by DHPLC screening when DNA was extracted from MFPE tissues. Exon 20 of EGFR was studied because this test is performed routinely in the laboratory and because a highly frequent polymorphism (c.2361G >A) is present. We observed 100% agreement between the 4 frozen tumors and the paired MF fixed tumors analyzed. One hundred percent agreement was also observed when all the paired tumors were sequenced for this exon. No sequence artifact was detected in the fixed tumors, and the quality of the electropherogram was the same as for the frozen tumors. Subsequently, real-time PCR showed a good agreement between the fixed tumor and the paired frozen tumor. Although RNA is fragile, it was successfully extracted from all the tumors fixed with the MF. However, only RNA extraction protocol 1 was suitable for a good RNA quality recovery. The principle of fixation with the MF is based on alcohol dehydration. We

can suppose that in this condition, RNA is in a particular conformation and that RNA recovery needs special chemical conditions in comparison with RNA extracted from frozen tumors. This could explain the low level of RNA recovery in protocol 3, probably corresponding to a low level of RNA retention on the purification column. For the majority of cases, the RIN value was greater than 7 for RNA extracted from MFPE tumors. Yet the RIN value was lower for fixed tumors than for frozen tumors. During the course of this study, some fixation parameters were improved, such as reducing the thickness of the tissue slices to less than 2 mm, putting tissue sections in larger volumes of fixative, and ensuring strict cleanness of the dissecting material. These arrangements improved the quality of the RNA extracted over time (data not shown). The fixative penetration in the tissue is probably dependent on the structure and size of the tissue, which may explain the difference in the median RIN value for the breast carcinoma compared with typically larger sarcoma samples. RNA was also found suitable for quantitative reverse transcription-PCR. Messenger RNA expression level in MFPE tumors is comparable with their expression level in the paired frozen tumors.

MF tissue penetration is 0.5 mm per 15 minutes, which could explain partial RNase activity during the fixation step. RNA quality was greatly enhanced when tumors were fixed at 4°C, which seems to confirm RNase activity in partial RNA degradation. Fixation at 4°C is not convenient for all routine diagnostic processes, but could be considered for pretherapeutic tumor microbiopsies obtained in a clinical trial setting.

RNA stability in the MFPE tumors at room temperature is not very good, and we observed a high decrease in RNA quality after 1 year of storage of the
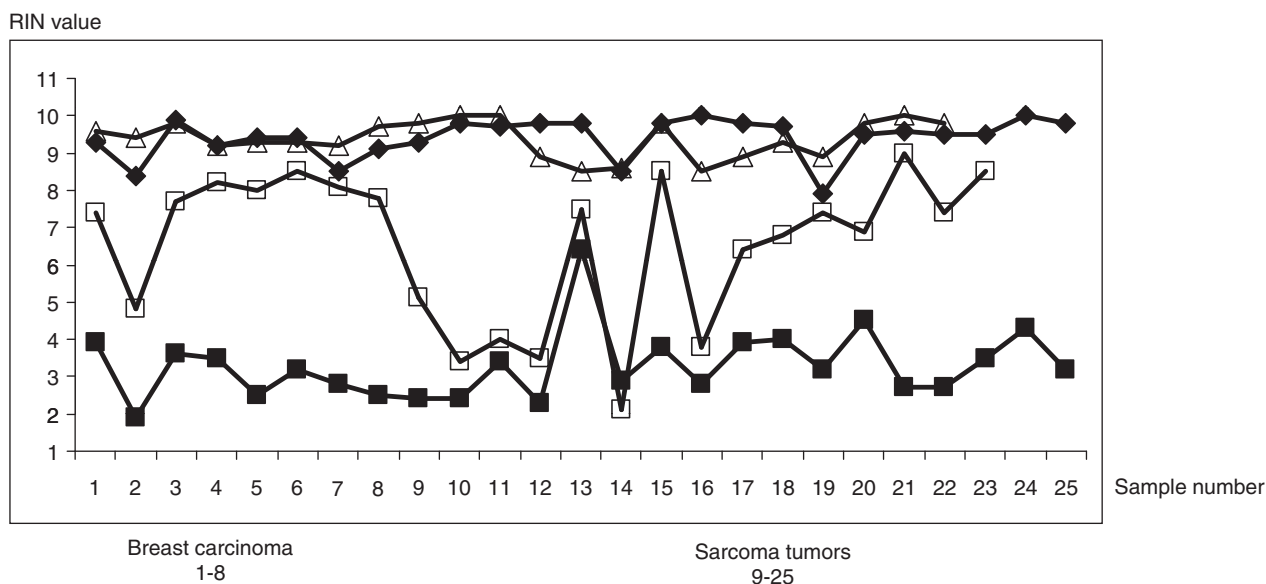

FIGURE 11. RNA integrity number (RIN) value comparison between frozen (△) and molecular-fixed paraffin-embedded tumors (□) at the time of fixation (empty characters) and after 1 year of storage (replete characters).
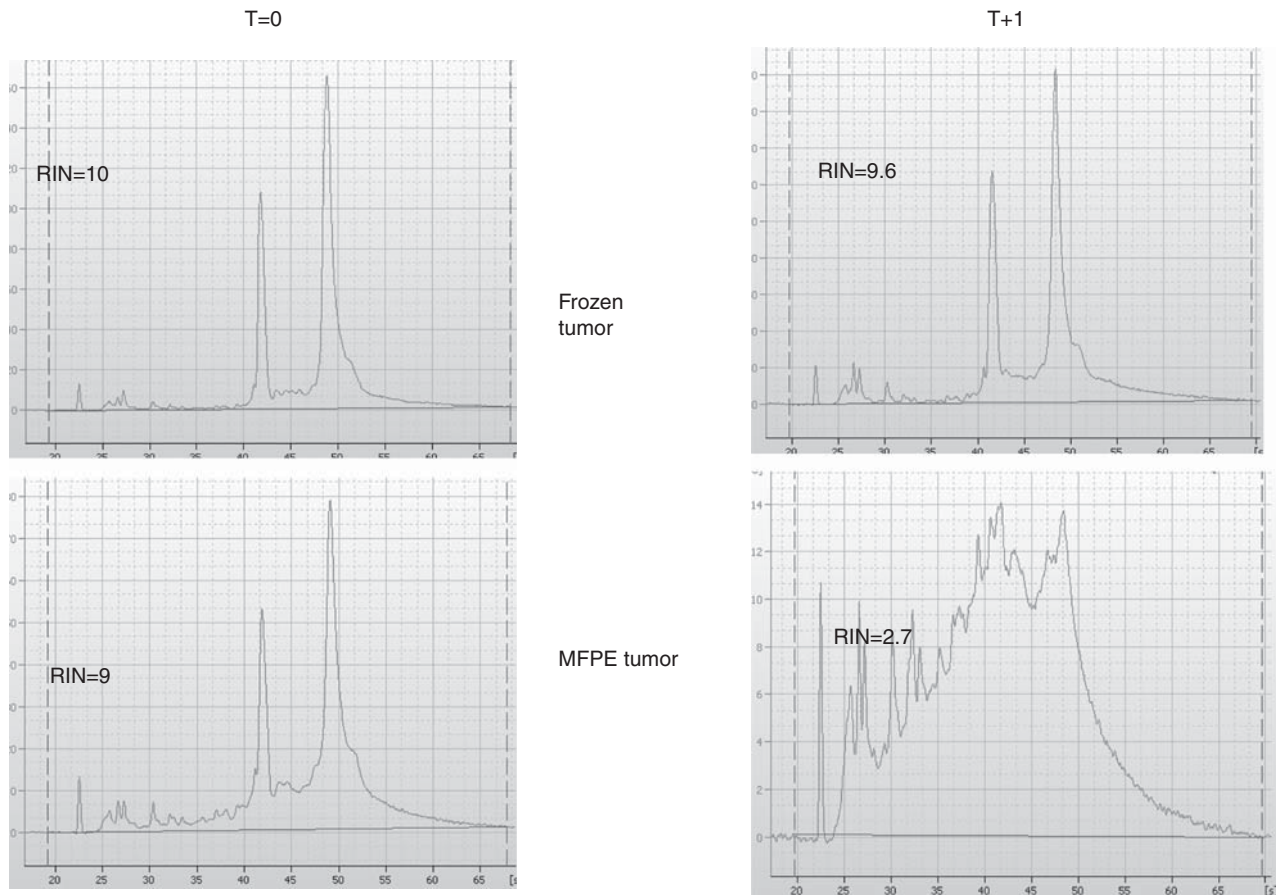
T=0   T+1

Frozen tumor

MFPE tumor

FIGURE 12. RNA electrophoretic profile after 1 year of storage of the MFPE tumor block at room temperature. Comparison between frozen and matched MFPE fragments (sarcoma tumor). MFPE indicates molecular-fixed paraffin-embedded; RIN, RNA integrity number.

paraffin blocks. Although the top of the block is protected from the air by a paraffin layer, oxidation may damage the nucleic acids, particularly RNA that are more sensitive to degradation. The same observation was made in an earlier publication in which tissues were fixed with methacarn.[27] A solution to this problem would be to store blocks of interest at −20°C. In contrast, DNA stability over 1 year in the paraffin block is as good as that observed for some zinc-based fixatives.[21]

In conclusion, we evaluated nucleic acid quality from a large number of molecular-fixed tumors in comparison with frozen tumors. The MF protects DNA and RNA from degradation, and nucleic acids are suitable for molecular analyses comparable with frozen tumors. Although RNA quality in MFPE material is slightly lower than in frozen tumors, it can be enhanced by fixation at 4°C. Unfortunately, RNA quality decreases when MFPE tumors are stored at room temperature over 1 year, and thus retrospective molecular analyses may be compromised. Nevertheless, the MF and Xpress processing system seem promising for combining routine pathologic diagnoses and current molecular diagnoses if they are made near the processing time. The next step in the validation of MF would be to compare the RNA expression and DNA copy number profiles obtained from MFPE and corresponding frozen tissues.

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