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Morphological quality and nucleic acid preservation in cytopathology

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TITLE: Morphological quality and nucleic acid preservation in cytopathology.

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KEYWORDS
Cytopathology; DNA/RNA extraction; HRM Analysis; formalin-free fixation; immunocytochemistry.

ABSTRACT

Aim: Fixation is a chemical or physical procedure to prevent the degradation of protein and tissue morphology. To optimize molecular analyses on archival tissues, it is essential that fixation preserves morphology along with protein epitopes and DNA/RNA integrity.
Methods: We employed a new formalin-free alcoholic-based fixative, named FineFix®, in 15 cases of serous effusions and 38 cases of Fine-Needle Aspirates, in order to evaluate the cellular morphology, and the quality of nucleic acids.
Results: The cyto-morphology of the cellular component of effusions and Fine-Needle Aspirates obtained with FineFix® fixation was similar to that obtained with the matched formalin-fixed counterpart. Immunocytochemistry showed comparable results with the traditional fixative, but the formalin-free fixation preserved higher molecular weight DNA and RNA as demonstrated by successful PCR of large amplification products of more 2000 bp.
Conclusions: The formalin-free fixative produced not only satisfactory results for immunocytochemistry on cytological smears and cell blocks, but also excellent preservation of DNA/RNA that can be efficiently employed also for sophisticated molecular techniques.

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INTRODUCTION

Gene and protein expression profiles are increasingly employed in pathology to better understand the molecular events leading to disease and identify new prognostic and therapeutic markers.[1-5] Morphological evaluation of bioptic and cytological material remain the gold standards of diagnosis, from which any new molecular insight into disease takes origin. Pathologists are therefore in the need of tissues with a satisfactory morphological quality along with the preservation of sub-cellular components for molecular investigations.[6-8]

In pathology, tissue fixation is a most critical procedure for the preservation of specimens-Tissues are routinely fixed in formalin, with the advantages of its low cost, rapid diffusion, solution stability and availability, but with disadvantages such as toxicity, carcinogenicity and nucleic acid fragmentation.[7, 9] The extraction of good quality DNA and RNA from formalin-fixed paraffin-embedded tissues (PET) is compromised due to incomplete removal of the protein-nucleic acid crosslinks.[7-11] Non-crosslinking alcoholic fixatives provide superior better results than aldehydes in the preservation of nucleic acids, but they properly fix only small tissue fragments with poor morphology, due to shrinkage of cells.[7] Recently, an alcohol-based fixative showed good results not only for tissue morphology, but also for DNA/RNA quality and protein preservation.[12]

In this paper, we present the morphological, immunocytochemical and molecular results of an alcohol-based fixative in cytology material from effusions, and lung, liver, breast, lymph nodes, thyroid and subcutaneous nodules.

MATERIALS AND METHODS

Sample collection

We examined 15 effusions and 38 Fine Needle Aspirates (FNAs) at the Department of Pathology, Padova University.

The effusions arrived unfixed at our laboratory and were centrifuged immediately at 2000 rpm for 20'. A part of the pellet was smeared onto two slides for cytology; one-third of the fresh cells were used immediately for DNA/RNA extraction. The remaining material was fixed in formalin, and in FineFIX© (FF , Milestone, Italy) FF is a mixture of ethanol, distilled water, glycerol, polyvinyl alcohol, and monomeric carbohydrates. The fixation time varied from 24 hours to one week.

The FNAs arrived at our laboratory in formalin or FF within one hour from the sampling. Cells were centrifuged at 2000 rpm for 20' for cell blocks.

Cell blocks

Cells were resuspended in 1% low melting point agarose equal to the volume of the pellet, and solidified in microfuge tubes at 4°C, placed in cassettes, processed and embedded in paraffin.

Immunocytochemistry (ICC)

Five-micron-thick sections from the cell blocks of FF/formalin-fixed FNAs and effusions were deparaffinized before hematoxylin and eosin (H&E) staining and ICC.

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Table 1. Panel of antibodies and ICC reaction results for samples fixed by FF and formalin.

Antigen/Antibody	Dilution	Source
Vimentin	1:100	Biogenex
Pan-Cytokeratin (MNF116)	1:250	DakoCytomation
Cluster of Differentiation 45 (CD45)	1:400	DakoCytomation
Placental-Like Alkaline Phosphatase (PLAP)	1:100	Biogenex
Cluster of Differentiation 10 (CD10)	1:50	Novocastra
Cytocheratin 7	1:100	Novocastra

Table 1 reports the antibodies and the dilutions; the staining was performed using an indirect immunoperoxidase technique with a automated system (Bond Polymer Refine Detection; Bond-maX; Vision BioSystems, UK). Antigen retrieval was performed by heating sections in Bond Epitope Retrieval Solution 1. Antigen was detected by incubation with labelled polymer and diaminobenzidine.

DNA extraction and amplification

The fresh cells were resuspended in extraction buffer (NaCl 5M, Tris-HCl 1M, pH 8, EDTA 0,5 M, pH8, SDS 10%) with Proteinase K (20 mg/ml). Three 10 µm sections of formalin/FF-fixed PET from effusions were cut into small pieces, deparaffinized, rehydrated, and air-dried at room temperature. Finally, the sample sections were resuspended in extraction buffer containing Proteinase K and incubated at 56°C for 60'.

To reverse cross-linking, sections were deparaffinized adding 100 µl 0.5% Tween-20 and heating to 90°C for 10' on a thermal cycler, cooled to 56°C and digested in extraction buffer with Proteinase K.

DNA extraction was performed by phenol:chloroform protocol. Samples were amplified with specific primers for the human *beta-catenin 1* gene *(CTNNB1,* Gene ID: 1499*)* and PCR was performed in 25 µl final volume using the following conditions: denaturation at 95°C for 5', 35 cycles of 95°C for 30'', annealing temperature for 30''and 72°C for 30''; elongation step at 72°C for 5'. PCR primers and amplicon lengths are reported in Table 2.

Table 2. Primers for DNA amplification.

GENE	Primer sequence 5'-3'	Amplicon	T° annealing	DMSO
CTNNB1	F GGACTTCACCTGACAGATCCA 4R GAGTCCCAAGGAGACCTTCC	199	56	0
CTNNB1	F GGACTTCACCTGACAGATCCA R CACAAATTGCTGCTGTGTCC	941	50	10%
CTNNB1	2F CAGGGAGAACCCCTTGGATA 1R GGTACTGACTTTGCTTGCTTT	1900	56	10%
CTNNB1	2F CAGGGAGAACCCCTTGGATA 2R ACCGCATTTTCTCTTGAAGC	2361	56	10%

RNA extraction, reverse transcription (RT) and RT PCR

Three 10 µm sections formalin/FF-fixed paraffin embedded from effusions were cut into small pieces. RNA was extracted using the PureLink FFPE Total RNA Isolation kit (Invitrogen, Carlsbad, CA, USA). DNase I digestion was performed to eliminate genomic DNA contamination.

RNA integrity was controlled using the RNA 6000 Pico assay kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

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Reverse transcription was performed using 100 to 500 ng of total RNA, M-MLV Reverse Transcriptase and 250 µM random primers (Invitrogen, Carlsbad, CA, USA). Every cDNA sample was amplified using specific primers for human *beta-actin* gene (*ACTB***,** Gene ID: 60). PCR was performed in 25 µl final volume using standard conditions. PCR primers and amplicon lengths are reported in Table 3.

Table 3. Primers for RNA amplification.

GENE	Primer sequence 5'-3'	Amplicon	T° annealing
beta actin	FOR3 TGAGGCACTCTTCCAGCCT REV5 GACAGCACTGTGTTGG	126	65
beta actin	FOR3 TGAGGCACTCTTCCAGCCT REV2 GCTGGAAGGTGGACAGCGA	287	65
beta actin	FOR2 GGCATCGTGATGGACTCCG REV2 GCTGGAAGGTGGACAGCGA	617	68
beta actin	FOR3 TGAGGCACTCTTCCAGCCT REV4 GAGACCAAAAGCCTTCATAC	1199	65

High Resolution Melting Analysis (HRMA)

HRMA is a screening method for mutations and deletions based on PCR amplification and melting curve analysis. DNA from the 11 lung cancer aspirates was amplified using primers for the human *Epidermal Growth Factor Receptor* (*EGFR,* Gene ID: 1956) gene. The sequences for amplification of DEL region in exon 19 were: Fw Ex19 5'-CGTCTTCCTTCTCTCTCTGTC-3'; and Rev Ex19 5'-GACATGAGAAAAGGTGGGC-3'. The sequences for amplification of the region of L858R mutation in exon 21 were: Fw Ex21 5'- GCATGAACTACTTGGAGGAC-3' and Rev Ex21 5'- GGCTGACCTAAAGCCAC-3'. DNA was amplified via real-time PCR in the presence of a proprietary saturating DNA dye contained in the LightCycler 480 High Resolution Melting Master. A melting curve was produced using high data acquisition rates, and data were analyzed with the LightCycler 480 Gene Scanning Software Module.

RESULTS

Effects of FF and formalin on cellular morphology and ICC

Cellular morphology and ICC were analyzed on cell blocks obtained from 15 effusions samples and 38 FNA after fixation and paraffin-embedding using either formalin or FF as fixative (Tables 4 and 5).

Table 4. Collection and type of fixation for effusions.

Body Site	n° effusions		
	Fresh	FF	Formalin
Lung	11	11	11
Peritoneal	2	2	2
Ascitis	2	2	2
Total	15	15	15

Table 5. Collection and type of fixation for FNAs.

Body Site	n° FNA		Tot
	FF	Formalin	
Lung	9	6	15

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Breast	1	2	3
Liver	1	2	3
Lymph node	2	0	2
Thyroid	5	4	9
Subcutaneous nodules	3	3	6
Total	21	17	38

FF clearly preserved cell integrity, giving morphological information similar to formalin: no differences were identified in cell architecture, cytoplasmatic and nuclear morphology, or tinctorial reactions (Figure 1A and 1B). The only exception was a moderate swelling of erythrocytes in FF samples fixed for more than 48 hours, but this did not impair the cellular details.

Immunoreactivity was similar in FF and formalin-fixed samples, except for an higher sensitivity for vimentin antibody in FF-fixed samples.. No cell shrinkage was observed, and nuclear details were satisfactory (Figure 1C-F). In addition, in FF-fixed samples nuclear antigenicity was investigated using MIB-1, Wilms Tumor (WT) and Vimentin, with satisfactory results.

Quality of DNA extracted from FF- and formalin-fixed samples

The DNA was extracted from the cellular component of fresh effusions and from FF/formalin-fixed cell blocks counterparts. The DNA extracted from formalin-fixed samples was evidently degraded, as demonstrated by the smear observed on the agarose gel (Figure 2) and failed to amplify large PCR products (Table 6).

Table 6. Genomic DNA amplification. Comparison between formalin and FF, numbers of positive results after PCR amplification.

Length (bases)	**Formalin fixed**	**FF fixed**	**Fresh**
112	15/15 (100%)	15/15 (100%)	15/15 (100%)
199	15/15 (100%)	15/15 (100%)	15/15 (100%)
941	0/15 (0%)	15/15 (100%)	15/15 (100%)
1900	0/15 (0%)	15/15 (100%)	15/15 (100%)
2361	0/15 (0%)	15/15 (100%)	15/15 (100%)

For formalin fixed samples, cross links reversion obtained less than 300 bp DNA length.

High quality genomic DNA was obtained from FF-fixed samples, comparable to fresh samples (Figure 2). In addition, the integrity of DNA from FF-fixed samples permitted the amplification of a 2361 bp amplicon for *CTNNB1*, exactly as from the fresh counterpart (Table 6, Figure 3) and allowed more sophisticated analyses, as HRMA. This technique can detect missense mutations, deletions and insertions in tumor DNA isolated.[13, 14]

In HRMA, mutations/deletion detection takes place only if the amplification curve reaches the plateau between 20 and 40 amplification cycles. In the analysis of exon 19 of *EGFR*, we obtained two comparable amplification curves using DNA from fresh and FF-fixed material at the 23rd and 25th cycles, respectively. In fact, the plateau is reached by fresh and FF-fixed samples, but not by formalin-fixed samples. The amplification of DNA extracted from formalin-fixed samples was delayed beyond the 40th cycle, inadequate for detection of deletion/mutations (Figure 4).The same results were obtained for the analysis of exon 21 of *EGFR*. Mutation in

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exon21 was detected only in FF fixed samples, while it could not possible for formalin fixed specimen due to the DNA degradation (figure 4 B and C).

FF and RNA quality

The quality of RNA extracted from the fresh and FF samples was comparable, whereas RNA extracted from formalin-fixed cells were significantly degraded, as indicated by the absence of 28S and 18S ribosomal RNA bands (Figure 5). To estimate the lengths of preserved transcripts in samples prepared in different fixatives and embedded in paraffin, we amplified various sizes of cytoplasmic *beta-actin* mRNA fragments by RT PCR from total RNA. In 6 out of 15 (40%) formalin-fixed samples, all successfully amplified amplicons were at maximum 287 bps; no amplification products were detected for longer fragments (Table 7, Figure 6).

Table 7. RNA amplification. Comparison between formalin and FF, numbers of positive results after RT PCR amplification.

Length (bases)	Formalin fixed	FF fixed	Fresh
126	15/15 (100%)	15/15 (100%)	15/15 (100%)
287	6/15 (40%)	15/15 (100%)	15/15 (100%)
617	0/15 (0%)	9/15 (60%)	15/15 (100%)
1199	0/15 (0%)	3/15 (20%)	15/15 (100%)

FF-fixed samples showed different results. In Table 7 we report the results of cDNA amplification from fresh, formalin and FF cytological samples. The cDNA amplification efficiency for FF-fixed samples was comparable to the fresh counterpart for fragments no longer than 300 bp, while a decreased efficiency with FF-fixed samples was noticed in the amplification of PCR fragments longer than 617 bps. In particular, we obtained an RT PCR product of 1199 bps in only 3 FF-fixed effusions out of 15 (20%) .

DISCUSSION

Archived tissues are a resource for DNA and RNA for molecular biological studies in cancer research and for screening for genetic-based diseases, requiring the optimal preservation of proteins and nucleic acids. To date, the common technique of fixation with formalin prevents the extraction of intact nucleic acids, and the molecular information conserved in the pathology archives cannot be exploited. Furthermore, formalin is toxic, an allergen, and a carcinogen, and its disposal is becoming increasingly costly for laboratories.[15-18] The carcinogenicity of formaldehyde has been evaluated by the International Agency for Research on Cancer (IARC) as "carcinogenic to humans" (group 1) on the basis of induction of nasopharyngeal cancer, although there is uncertainty ("strong but not sufficient evidence") of a causal association with myeloid leukemia and a limited association with nasopharyngeal carcinoma.[19]

It is not surprising, therefore, that, in recent years, many fixatives have been introduced as formalin replacements. Alcohol-based fixatives exert their effect by protein precipitation, and are superior in preserving a number of cellular antigens.[20, 21] Advantages also include the elimination of carcinogenic vapors, greater staining avidity, no enzyme predigestion in ICC, and simple and rapid disposal. Disadvantages include slightly increased viscosity, variability of tissue staining and nuclear shrinkage, artifactual pigment deposition in bloody specimens, and increased flammability.[22]

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Recently, a new ethanol-based fixative was developed, named FF, which overcomes these problems and allows for improved molecular analyses. FF fixation has been described for the study of PET by Stanta, with good tissue histomorphology, and low denaturation of proteins and nucleic acids preservation.[11] In histopathology, FF is best used with a microwave-processing apparatus for a better fixation of the deeper layers of the specimen.[12, 23] In this cytopathology study, we considered the use of microwaves unnecessary due to the monolayer of cytological preparation.. FF was validated for DNA integrity in fixed and paraffin-embedded cytological material by showing high yields of extracted DNA and the amplification of a fragment that was longer than 2300 bp in all the FF fixed samples. The quality of the DNA from FF-fixed material is excellent, allowing the application of fine and sophisticated methodologies, such as HRMA, with better results than formalin.

FF is a non-crosslinking fixative that preserves RNA, by precipitation and inactivation of endogenous RNAses. We obtained high-quality RNA from FF-fixed, in contrast to what obtained from formalin-fixed and paraffin-embedded samples. To test the quality of RNA, we evaluated RT PCR amplification from extracted RNA samples. From the FF-treated specimens, we were able to amplify products that, in some cases, were longer than 1000 bases.

FF is an efficient fixative not only in protecting nucleic acids from degradation, but also for the preservation of cell morphology. The ICC experiments demonstrated excellent preservation of cellular morphology, maintaining antigen properties and giving morphological information similar to formalin fixed tissues.

In conclusion, FF fixation proved to preserve cytomorphology, immunoreactivity, DNA and RNA of effusions and aspirates with a quality comparable to fresh material, without modifying the routine laboratory procedures, and being safe and non-toxic. As a satisfactory replace of formalin the pathology archives will yield even more fascinating information for the next generations.

Take-home messages

- FineFix is an ethanol-based fixative;
- It can be successfully employed in cytological smears with good definition of cytoplasmic and nuclear details, and on cell blocs for immunocytochemistry;
- FineFix efficiently protects nucleic acids from degradation, and DNA and RNA can be extracted and amplified up to 2300 bp;
- FineFix is non toxic and will possibly replace formalin in pathology laboratories.

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Conflict of interest statement

The Authors have no conflict of interest to disclose.

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REFERENCES

1 Schena M, Shalon D, Davis R, et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467-470.

2 Velculescu V, Zhang L, Volgelstein B, et al. Serial analysis of gene expression. Science 1995;270:484-487.

3 Emmert-Buck M, Gillespie J, Paweletz C, et al. An approach to proteomic analysis of human tumors. Mol Carcinog 2000;27:158-165.

4 Lim M, Elenitoba-Johnson K. Proteomics in pathology research. Lab Invest 2004;84:1227-1244.

5 Bustin S. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169-193.

6 Goldsworthy S, Stockton P, Trempus C, et al. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissues. Mol Carcinog 1999;25:83-91.

7 Srinivasan M, Sedmark D, Jewel S. Effects of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Path 2002;61:1961-1971.

8 Leong ASY, James CL, Thomas AC. Handbook of surgical pathology. New York: Churchill Livingstone 1996.

9 Binetti R, Costamagna FM, Marcello I. Development of carcinogenicity classifications and evaluations: the case of formaldehyde. Ann Ist Super Sanita 2006;42(2):132-43.

10 Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acid Res 1999;27:4436-4443.

11 Finke J, Fritzen R, Ternes P, et al. An improved strategy and a useful housekeeping gene for RNA analysis from formalin-fixed paraffin-embedded tissues by PCR. Biotechniques 1993;14:448-453.

12 Stanta G, Pozzi Macelli S, Petrera F, et al. A novel fixative improves opportunity of nucleic acids and proteomic analysis in human archive's tissues. Diagn Mol Pathol 2006;15:115-123.

13 Zhang X, Chang A. Somatic mutations of the epidermal growth factor receptor and non-small-cell lung cancer. J Med Genet 2007;44:166-172.

14 Nomoto K, Tsuta K, Takano T, et al. Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. Am J Clin Pathol 2006;126(4):608-15.

15 Bramwell NH, Burns BF. The effects of fixative type and fixation time on the quantity and quality of extractable DNA for hybridization studies on lymphoid tissue. Exp Hematol 1988;16:730-732.

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16 Serth J, Kuczyk MA, Paeslack U, et al. Quantization of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. Am J Pathol 2000;156:1189-1196.

17 Yagi N, Satonaka K, Horio M, et al. The role of DNase and EDTA on DNA degradation in formaldehyde fixed tissues. Biotech Histochem 1996;71:123-129.

18 Diaz-Cano SJ, Brady SP. DNA extraction from formalin-fixed, paraffin-embedded tissues: protein digestion as a limiting step for retrieval of high-quality DNA. Diagn Mol Pathol 1997;6:342-346.

19 International Agency for Research on Cancer. Formaldehyde, 2-butoxyethanol, and 1-tert-butoxy-2-propanol. Lyon, France: IARC; 2006 (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 88).

20 Mitchell D, Ibrahim S, Gusterson BA. Improved immunohistochemical localization of tissue antigens using methacarn fixation. J Histochem Cytochem 1985;33:491–495.

21 Nadji M, Nassiri M, Vincek V, et al. Immunohistochemistry of Tissue Prepared by a Molecular-Friendly Fixation and Processing System. Appl Immunohistochem Mol Morphol 2005;13:277–282.

22 Bostwick DG, al Annouf N, Choi C. Establishment of the formalin-free surgical pathology laboratory. Utility of an alcohol-based fixative. Arch Pathol Lab Med 1994;118(3):298-302.

23 Iesurum A, Balbi T, Vasapollo D, et al. Microwave processing and ethanol-based fixation in forensic pathology. Am J Forensic Med Pathol 2006;27(2):178-82.

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TITLES AND LEGENDS TO FIGURES

Figure 1: ICC staining. 5 μm thick sections from formalin-fixed (A, C and E) and FF-fixed (B, D and F) fixed cell blocks (mesothelioma, pleural effusion) (40x). H&E staining (A and B) and ICC with anti-human-Cytocheratin 7 (C and D) and anti-human-vimentin (E and F)**.** The insert in F is a particular of the section under 63x magnification.

Figure 2: Quality of genomic DNA. DNA from fresh (lane 1), FF- (lane 2), formalin- (lane 3) fixed samples and, at lane 4, 1Kb Plus DNA Ladder (Invitrogen, with bands ranging in size from 100 to 12000 bp). High molecular weight DNA was extracted by FF-fixed and fresh samples, while the DNA obtained from the formalin-fixed sample is completely fragmented.

Figure 3: Genomic DNA and PCR. PCR amplification for DNA extracted from a fresh (lane 1), FF- (lane 2) and formalin- (lane 3) fixed samples. Lane 4, negative control; lane 5, DNA Molecular Weight Marker XIII (ROCHE, with bands ranging in size from 50 to 2462 bp). *CTNNB1* amplicons of: (A) 199 bp; (B) 941 bp; (C) 1900 bp and (D) 2361 bp.

Figure 4: HRMA. A, amplification curves of DNA extracted from fresh sample, FF-, and formalin-fixed (Form) counterparts in the analysis of EGFR exon 19. The curves of the fresh and FF-fixed samples are comparable, and the PCR amplification occurred at the 23rd and 25th cycles, respectively. The amplification of DNA extracted from formalin-fixed counterparts was delayed with respect to the previous specimens and took place after the 40th cycle. B, a sample fixed in FF and formalin shows different amplification curves. The plateau was reached only with FF-fixed sample. C, the FF-fixed sample was analyzed with 10 different FF-fixed specimens. The plot shows 2 different melting profiles, corresponding to the wild type exon 21 in the 10 samples and a positive sample to the mutation L858R in exon 21 (arrow).

Figure 5: RNA quality. Electropherogram profiles of RNA extracted from FF (A) and formalin-fixed (B) cell blocks of a pleural effusion. Peaks of 18S and 28S of ribosomal RNA are evident in the FF-fixed sample, whereas they are absent in the formalin-fixed sample. Total RNA extracted from fresh had a RIN of 9.6, from FF-fixed of 6.3, and from formalin-fixed of 2.1.

Figure 6: cDNA and PCR. PCR amplification for cDNA obtained after retro-transcription of total RNA extracted from a fresh sample (lane 1) and FF- (lane 2) and formalin- (lane 3) fixed samples. Lane 4, negative control; lane 5, molecular weight marker, 1Kb Plus DNA Ladder (Invitrogen, with bands ranging in size from 100 to 12000 bp). *Beta actin* amplicons of: (A) 126 bp; (B) 287 bp; (C) 617 bp and (D) 1199 bp.

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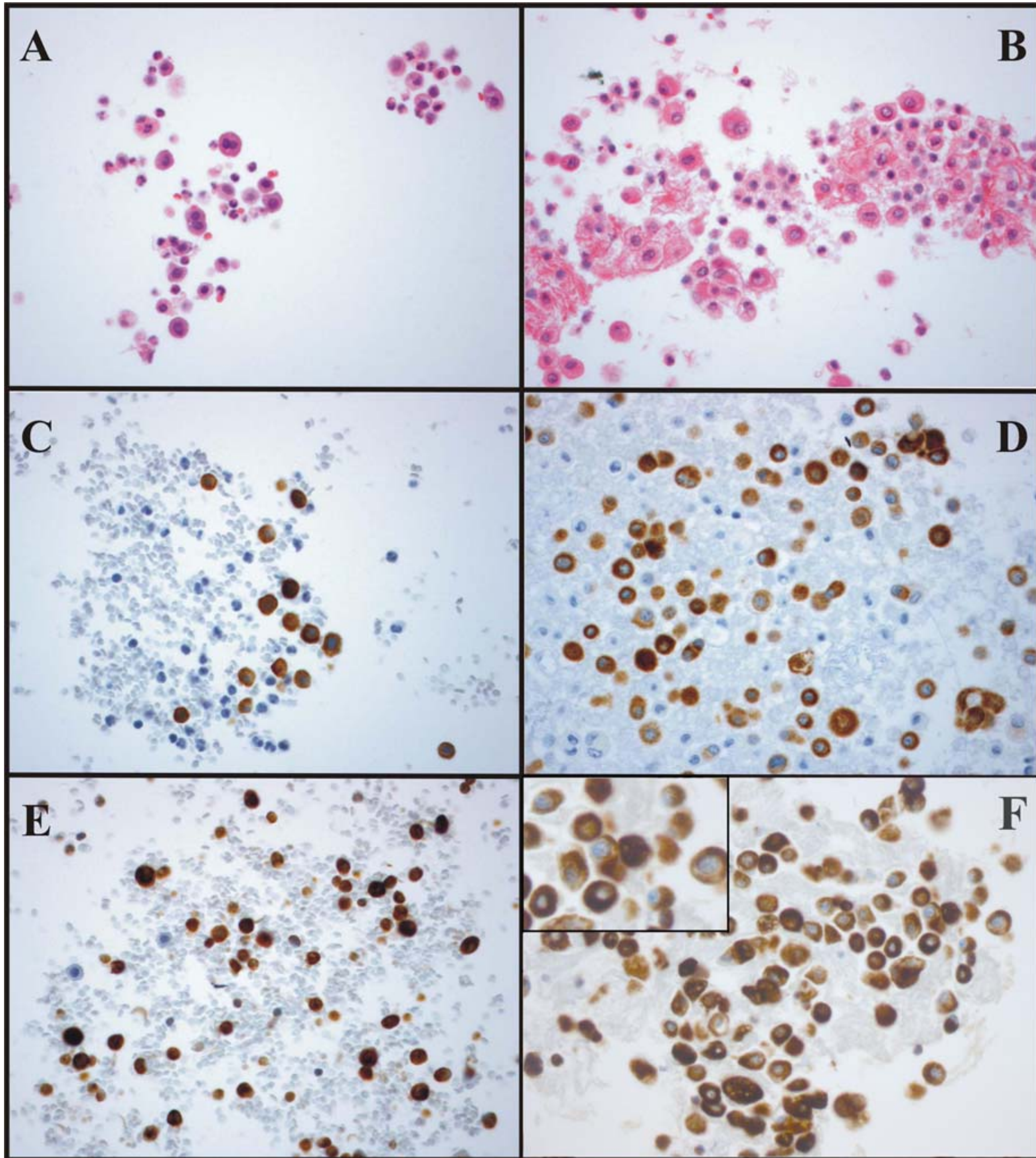

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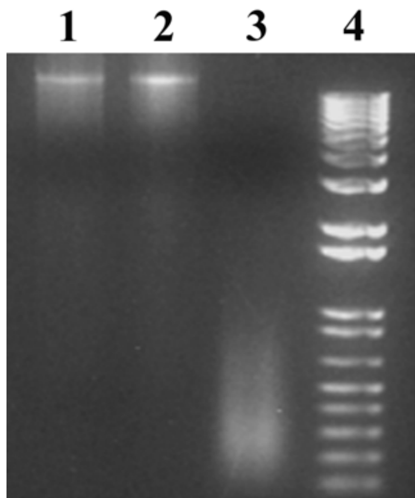

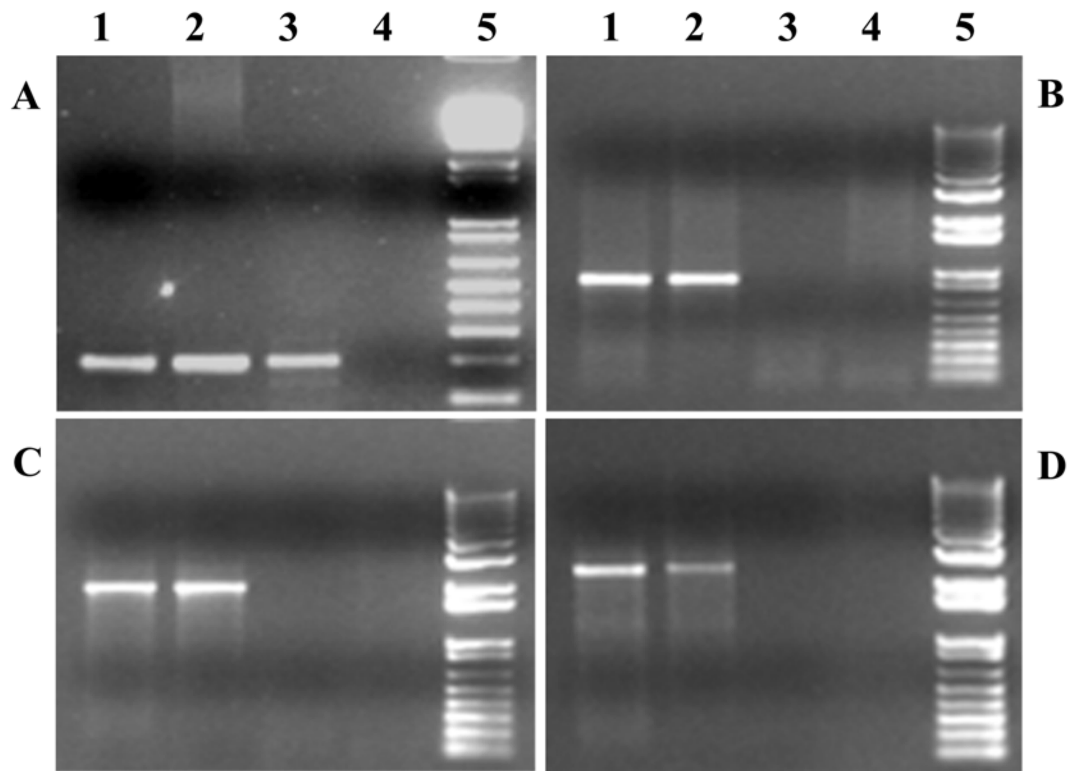

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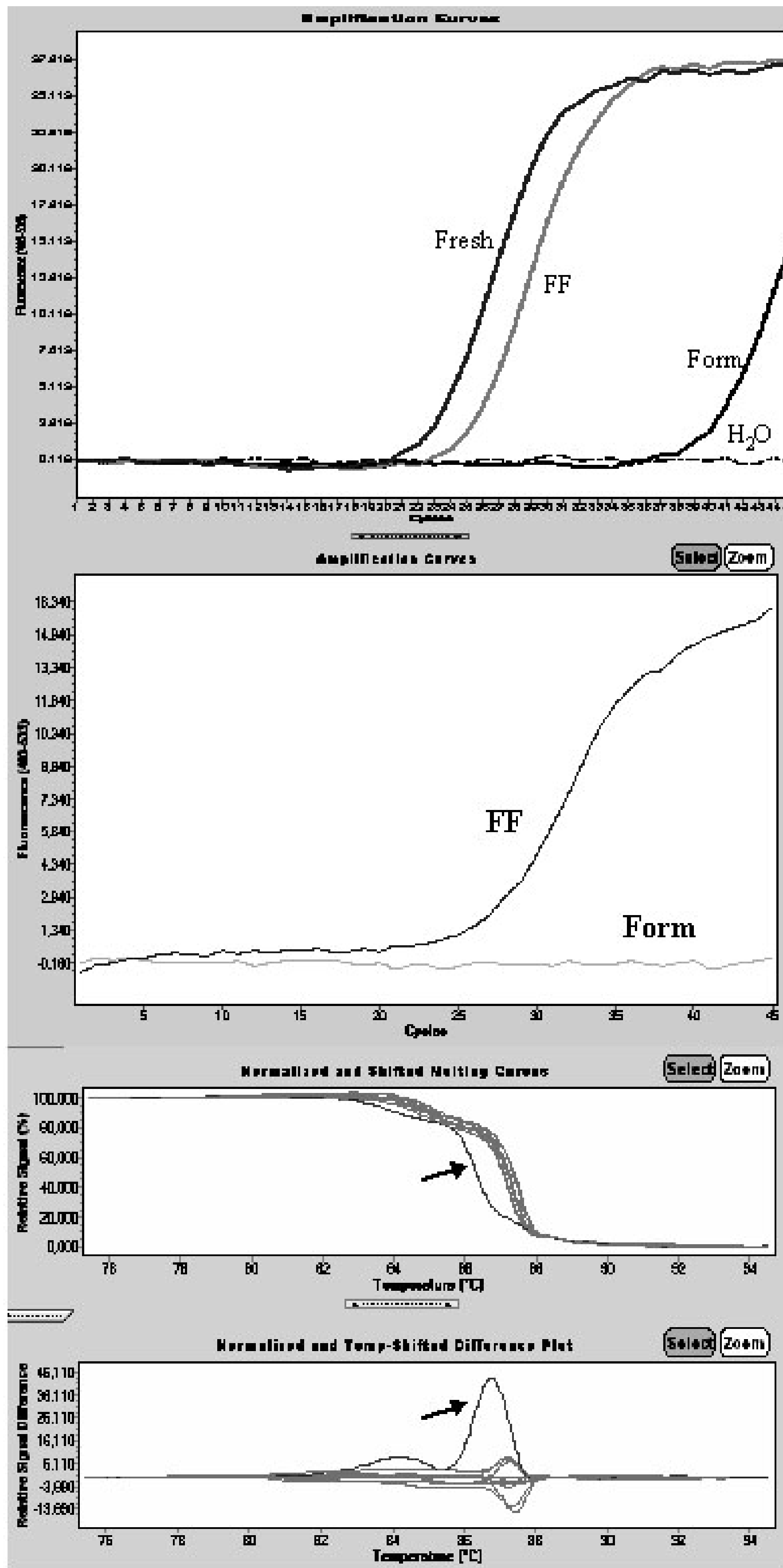

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