Novel zinc-based fixative for high quality DNA, RNA and protein analysis

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ABSTRACT

We have developed a reliable, cost-effective and non-toxic fixative to meet the needs of contemporary molecular pathobiology research, particularly in respect of RNA and DNA integrity. The effects of 25 different fixative recipes on the fixed quality of tissues from C57BL/6 mice were investigated. Results from IHC, PCR, RT–PCR, RNA Agilent Bioanalyser and Real-Time PCR showed that a novel zinc-based fixative (Z7) containing zinc trifluoroacetate, zinc chloride and calcium acetate was significantly better than the standard zinc-based fixative (Z2) and neutral buffered formalin (NBF) for DNA, RNA and protein preservation. DNA sequences up to 2.4 kb in length and RNA fragments up to 361 bp in length were successfully amplified from Z7 fixed tissues, as demonstrated by PCR, RT–PCR and Real-Time PCR. Total protein analysis was achieved using 2-D gel electrophoresis. In addition, nucleic acids and proteins were very stable over a 6–14-month period. This improved, non-toxic and economical tissue fixative could be applied for routine use in pathology laboratories to permit subsequent genomic/proteomic studies.

INTRODUCTION

Fixation is a series of complex chemical modifications of macromolecules present in tissues and cells, to preserve structural and functional components as closely as possible to the living state while inhibiting autolysis, bacterial and fungal decay (1). Short DNA and RNA sequences can be retrieved from conventionally fixed pathology material, but good, long-term preservation of intact nucleic acids and of protein integrity is necessary to meet the increasing number of molecular diagnostic and research techniques which are becoming available. The type and length of fixation determine the degree of preservation of intact nucleic acids in tissue (2–4). Cross-linking fixatives such as formalin and glutaraldehyde bind amino groups and create methylene bridges (5). Precipitant fixatives, including methanol, ethanol, acetone and acetic acid, denature proteins by breaking the hydrophobic bonds that make up the tertiary structure of protein molecules yet preserve secondary structure for immunohistochemistry (IHC). Other compounds include the commercially available HOPE (HEPES-Glutamic acid buffer mediated Organic solvent Protection Effect) which preserves DNA and RNA suitable for polymerase chain reaction (PCR) and reverse-transcription (RT)–PCR (6,7) and the reversible cross-linker dithio-bis[succinimidyl propionate] (DSP) for immunostaining, microdissection and expression profiling (8). The potential value of a new universal molecular fixative (UMFIX) for preservation of macromolecules in paraffin-embedded tissue has been tested which can preserve morphology and macromolecules in paraffin-embedded tissue (9). Despite the number of fixatives available, however, problems still remain for many of them including toxicity, expense, the need for rapid fixation systems and the need to employ denaturants and low melting point wax for embedding. Recently, a zinc-based fixative (zinc acetate, zinc chloride and calcium chloride in Tris buffer) originally described in 1994 (10) was reported to be superior for DNA and protein expression analysis in a broad spectrum of tissues which do not then require heat pre-treatment for antigen retrieval (11). In other studies, zinc-fixed, paraffin-embedded tissues provided superior morphology and improved immunostaining (10). Zinc compounds are non-toxic and inexpensive, non-carcinogenic and are not temperature sensitive.

We evaluated a series of novel fixative recipes for immersion fixation and processing to paraffin in order to

improve DNA, RNA and protein yield whilst maintaining optimal tissue morphology. A range of zinc-based salt solutions, as well as other metal-based salt solutions, was tested for potential fixation properties in comparison with standard fixation procedures. All fixatives were evaluated for morphology using haematoxylin and eosin (H&E) and IHC for actin, a widely distributed antigen not requiring antigen retrieval in formalin fixed material, and for cytokeratin, an epithelial marker and CD3, a T-lymphocyte marker, both of which require pre-treatment when in formalin fixed tissue. Preservation of nucleic acids was tested by PCR and RT–PCR. Additional chemicals were tested with one of the zinc-based fixatives, Z2: dimethyl sulphoxide (DMSO), diethyl pyrocarbonate (DEPC) and ethylenediaminetetraacetic acid (EDTA) at various concentrations.

We describe a reliable, cost-effective and non-toxic fixative, Z7, which demonstrates excellent protein preservation, and which is particularly effective at preserving DNA and RNA integrity in comparison with standard fixation procedures, and allows for detailed molecular analysis procedures on fixed paraffin-embedded samples even after at least a year in storage.

MATERIALS AND METHODS

Fixatives

In all experiments, tissue samples were fixed on a shaking rotor at room temperature (RT) for 24 h.

(i) Standard tissue fixatives:

  (a) NBF (10% formalin, pH 6.7–7.0).
  (b) Zinc-based fixative (Z2) (0.5% zinc chloride, 0.5% zinc acetate, 0.05% calcium acetate in 0.1 M Tris–HCl pH 6.4–6.7).
  (c) HOPE (commercially available),
  (d) Fresh-freezing (FF) in liquid N_2 and storage at $-80°C$.

(ii) Modifications to the zinc-based fixative recipe

  (a) Zinc acetate in the Z2 fixative recipe was replaced by:

    (1) zinc trifluoroacetate (17.16 mM) (Z7)
    (2) zinc citrate (8.10 mM) (Z8)
    (3) zinc trifluoroacetate 17.16 mM + 5% (v/v) DMSO (Z16)
    (4) zinc tartrate (20.05 mM) (Z17)
    (5) zinc tartrate (20.05 mM) + 5% DMSO (Z18)
    (6) zinc isovalerate (18.69 mM) (Z19)

  (b) Replacement of zinc solutions with manganese, magnesium, gallium or vanadium solutions as novel fixative candidates

To investigate whether any other metallic ions could be better fixative candidates than zinc, zinc solutions were replaced by manganese, magnesium, gallium and vanadium salt solutions. These were chosen because they: A) belong to the same family as zinc (same row in the periodic table), B) are divalent cations, C) are non-toxic, D) have a similar atomic mass and E) are low cost. Fixation recipes comprised:

  (1) Manganese chloride (0.5%), manganese acetate (0.5%) and calcium chloride (0.05%) in Tris–HCl pH 6.5 (solution termed Mn2).
  (2) Magnesium chloride (0.5%), magnesium acetate (0.5%) and calcium chloride (0.05%) in Tris–HCl pH 6.5 (solution termed Mg2).
  (3) Gallium chloride (1.0%), and calcium chloride (0.05%) in Tris–HCl pH 6.5 (solution termed Ga2).
  (4) Vanadium chloride (1.0%), and calcium chloride (0.05%) in Tris–HCl pH 6.5 (solution termed V2).

  (c) Addition of chemicals to the standard zinc-based (Z2) fixation recipe

A range of chemicals was examined for their effect on morphology and nucleic acid preservation when used together with the zinc-based fixative Z2. These were:

  (1) DMSO: 2.5% (Z3), 5.0% (Z4), 10.0% (Z5) and 20.0% (Z6)
  (2) EDTA: 1.25% (Z9), 2.5% (Z10), 5.0% (Z11) and 7.5% (Z12)
  (3) DEPC: 0.5% (Z13) and 1.0% (Z14)
  (4) DEPC + DMSO: 0.5% and 5.0%, respectively (Z15)

Tissues

Three types of tissue from C57BL/6 male/female mice 8–12 weeks of age were used in this study. Colon was used for its variety of tissue types including epithelial, endothelial, nervous and muscular tissues and its susceptibility to autolytic degradation. Spleen was used as a solid reticuloendothelial organ and liver as a solid parenchymal organ. This selection provided a range of tissue density and structure to assess physical properties such as penetration of fixatives in different tissue types. Colon, liver and spleen tissues were dissected into pieces up to 5–6-mm thick and immediately immersed in the fixing solutions for 24 h at room temperature (RT). Tissues were also fresh-frozen in liquid N_2 as control samples for DNA and RNA quality. For every experiment, three mice were used and experiments from each mouse were repeated once.

Tissue processing and embedding

Fixation in each of the solutions described above was followed by processing of tissues. Tissue processing was performed overnight using a vacuum infiltrating processor. Briefly, this comprised:

(i) Seventy percent, 80%, 95% ethanol for 1 h 30 min each, followed by 100% ethanol for 1 h × 4, 40°C
(ii) Fifty percent ethanol + 50% xylene, 1 h 30 min, 45°C

(iii) Xylene, 1 h × 2, 45°C
(iv) Wax, 1 h × 2, then again wax for 1 h 30 min × 2, 60°C

When the processing programme was finished, tissue samples were embedded in paraffin standard melting point (52°C–54°C) wax.

Tissue sectioning, H&E staining and imaging

Tissue sections were cut using a rotary microtome, and floated on distilled H_2O at 37°C for 5 min. Sections were picked up on charged slides and dried at 39°C overnight. The blade was wiped with 70% ethanol between blocks to avoid cross-contamination. All sections were stained with H&E and evaluated independently by three observers. All images of IHC and H&E were taken using a digital camera.

Immunocytochemistry

Tissue sections were immunostained with a variety of primary antibodies. No antigen retrieval was performed in any of the experiments and a reagent only (no antigen) negative control was included for every experiment. Antibodies and other reagents are shown in Table 1. The two protocols for immunostaining are summarized below:

(a) Standard avidin-biotin complex (ABC) immunostaining method. One hundred micro litres of the primary antibody in PBS diluent (PBS with 0.1% BSA and 0.1% NaN_3) was applied at 4°C overnight. Sections were incubated for 30 min with 100 µl peroxidase-labelled ABC reagent, counterstained with haematoxylin and mounted in DPX medium.

(b) Immunostaining using the Dako Cytomation Animal Research Kit (ARK)-biotinylated antibody method. The ARK method was used for mouse-on-mouse IHC. One hundred micro litres of ARK-biotinylated antibody was added and sections were incubated overnight at 4°C. Sections were incubated in 100 µl of peroxidase-labelled ABC reagent for 30 min, counterstained with haematoxylin and mounted in DPX medium.

DNA extraction from fixed and fresh-frozen tissues

DNA was extracted from 25-mg fixed paraffin-embedded tissues using a commercially available kit (Qiagen DNA Tissue Kit, Qiagen Ltd, West Sussex, UK). Three to four 20-µm sections were cut using a rotary microtome and placed in a 1.5-ml microfuge tube. One millilitre of xylene was added and the sample centrifuged for 1 min at 12 000 rpm. DNA was extracted according to manufacturer's protocol and eluted with 100 µl Tris/EDTA pH 8.0 buffer. Fresh-frozen tissue was used as a control for DNA integrity. For every experiment, a tissue sample was removed and immediately placed in liquid N_2 followed by storage at −80°C. DNA was extracted from fresh-frozen tissue according to the manufacturer's instructions and eluted with 100 µl Tris/EDTA pH 8.0 buffer.

RNA extraction from fixed and fresh-frozen tissues

RNA from fresh-frozen tissue was extracted using the RNeasy Tissue kit (Qiagen) according to manufacturer's protocol and the RNA was eluted in 50-µl RNAse-free water. RNA from fixed tissue was extracted using the RecoverAll Total Nucleic Acid Isolation extraction kit (Ambion Ltd, Cambridgeshire, UK). Fifteen to twenty milligrams of tissue was used as starting material. Two to three 20-µm sections (according to the size of the tissue block) were taken and placed in a 1.5-ml microfuge tube. Wax was removed with xylene and then the tissue was cleared with 100% alcohol twice. RNA was extracted according to manufacturer's protocol and eluted in 60-µl RNAse-free water.

PCR and RT–PCR

PCR and RT–PCR were performed on genomic DNA to amplify two universally expressed genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and Trefoil factor 2 gene (large fragment), (Table 2 has the primer sequences). In all PCR experiments, extracted DNA was diluted in RNAse-free water and 100 ng of DNA was used from each sample. Experiments were duplicated in order to verify the results. For RNA amplification, cDNA was prepared from 1 µg total RNA using a RT system (Promega Corp., Southampton, UK), and a commercial cDNA mouse β-actin primer was used (R&D Systems, GenBank Accession Number: X03672) which contains a positive control. Another primer also used for RNA amplification was S15 (small ribosomal unit). Mouse β-actin transcripts were amplified and the conditions used are summarized in Table 2. RT–PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide and visualized using an ultra violet gel imager.

Table 1. Reagents for immunocytochemistry

Antibody	Source	Manufacturer	Dilution Factor
Primary antibodies			
Muscle specific actin (MSA)	Mouse/Monoclonal	Novocastra	1:100
Broad range cytokeratin (AE1/AE3)	Mouse/Monoclonal	Dako	1:50
T-cell marker (CD3)	Rabbit/Polyclonal	Merck Biosciences	1:100
Secondary antibodies			
Biotinylated Goat anti-rabbit IgG	Goat polyclonal	Dako	1:400
FITC-labelled Donkey anti-mouse IgG	Donkey polyclonal	Novocastra	1:300

RNA measurement using the Agilent 2100 Bioanalyser

RNA samples were kept on ice and their concentrations measured using a Nanodrop spectrophotometer. RNA samples were prepared according to the Agilent 2100 Bioanalyser protocol and were loaded into the NanoChip or PicoChip and processed for 30 min. An equal amount of RNA was used for each experiment. The 18S and 28S ribosomal peaks were used to quantify RNA.

Real-Time PCR and Real-Time RT–PCR

Real-Time PCR was performed using the SYBR Green Ready Taq Mix (Sigma-Aldrich Ltd, Dorset, UK), using the same primers as for PCR and RT–PCR. A series of seven 2-fold dilutions was prepared in each run starting from 100 ng of DNA and gradually reducing to 1.5 ng of DNA. A reagent-only (no DNA) negative control sample was included in each run. DNA extracted from fresh-frozen samples was used as positive control for each experiment. Complementary DNA was prepared from 1 µg of RNA using the RT system (Promega) described above and Real-Time PCR was performed with S15 primers. A sample that contained no RT was used as negative control in each experiment. Experiments were carried out in triplicate using three sets of tissue (nine tests per tissue in total) to ensure reliability.

Two-dimensional (2-D) polyacrylamide gel electrophoresis

Liver samples fixed by different methods were subjected to 2-D polyacrylamide gel electrophoresis (PAGE) analysis. For fresh-frozen samples, 2–3 sections of 10 µm with an area size of 1 cm^2 were placed in a 1.5 ml microfuge tube with 100 µl Extraction Buffer II (Bio-Rad Laboratories, Hercules, CA). Samples were vortexed for 30 s, transferred in dry ice for 5 min and then thawed at RT. The freeze-thaw-vortex step was repeated three times and samples were centrifuged at 14 000 rpm at 4°C for 8 min. Supernatant (50 µl) from each sample was transferred to a fresh tube. For paraffin embedded samples, 30 10 µm sections were dissolved into 250 µl Extraction Buffer II (Bio-Rad Laboratories, Hercules, CA) and vigorously vortexed at RT. Samples were heated at 55°C for 30 min, 1 ml of xylene was added and samples were centrifuged at

12 000 g for 10 min. The supernatant was transferred to a fresh microfuge tube and centrifuged for 8 min at 12 000 g. The final supernatant of both fresh-frozen and paraffin embedded tissue was combined with a re-hydration buffer mixture containing re-hydration buffer (Bio-Rad Laboratories), Immobilized pH gradient (IPG) buffer (Amersham Biosciences, Piscataway, NJ), and bromophenol blue and subsequently re-hydrated overnight with Immobiline Drystrips (pH 4/7, 11 cm; Amersham Biosciences, Amersham UK). The isometric focusing for the first dimensional electrophoresis was performed with a Multiphore II Electrophoresis System (Amersham Biosciences). The strips were subjected to high voltage at 3500 V. IPG strips were equilibrated with Equilibration Buffer I and Buffer II (Bio-Rad Laboratories) for 15 min each. Precast ExcelGel SDS gels (Amersham Biosciences) were used for the second dimension of protein separation by a Multiphore II Flated System (Amersham Biosciences) under a constant voltage of 700 V for 3 h. A silver staining kit (Amersham Biosciences) was used according to the manufacturer's instructions to detect protein spots. All samples were run in duplicate to guarantee over 90% identity.

Assessment of stability of DNA in fixed tissues over time

Paraffin blocks prepared as described above were stored for 14 months at RT. DNA was extracted from these and compared with DNA extracted from paraffin blocks stored at RT for less than a week. PCR of the GAPDH and the β-actin genes, and Real-Time PCR on the GAPDH gene were performed to assess DNA stability over time.

Assessment of data

Tissues used during this study were assessed for their morphological structure and the integrity of their proteins and nucleic acids. Three independent and experienced histopathologists graded H&E and IHC results. Grades ranged from 1 (lowest) to 10. For the H&E, assessment was based on criteria such as shrinkage, cell morphology, nuclear structure and fixative penetration. For the IHC, criteria for assessment were the intensity and localization

Table 2. Primer names, sequences and PCR conditions used for PCR, RT–PCR, Real-Time PCR and Real-Time RT–PCR

Name	Primer sequence	Size (bp)	PCR conditions
β-actin[a,b]	5′–TGTTACCAACTGGGACGACA–3′ 3′–TCTCAGCTGTGGTGGTGAAG–5′	392	94°C, 55°C, 72°C, 25 cycles 30 s, 30 s, 30 s
β-actin[a]	5′–GACGGCCAGGTCATCACTAT–3′ 3′–CCTGGGCCATTCAGAAATTA–5′	936	94°C, 60°C, 72°C, 25 cycles 45 s, 60 s, 45 s
GAPDH[a,b]	5′–AACTTTGGCATTGTGGAAGG–3′ 3′–TGTGAGGGAGATGCTCAGTG–5′	599	94°C, 55°C, 72°C, 25 cycles 30 s, 30 s, 30 s
Trefoil Factor 2[a]	5′–GTCCCTTGGTGTTTCCACCC– 3′ 3′–ACTGTGATCAGCAGTCATGCG–5′	2400	94°C, 59°C, 72°C, 30 cycles 45 s, 1 min 3 min
β-actin[c]	Commercial Primer (R&D Systems)	302	94°C, 55°C, 72°C, 30 cycles 45 s, 45 s, 45 s
S15[c,d]	5′–TTCCGCAAGTTCACCTACC– 3′ 3′–CGGGCCGGCCATGCTTTACG–5′	361	95°C, 55°C, 72°C, 30 cycles 30 s, 30 s, 30 s

[a]PCR.
[b]Real-Time PCR.
[c]RT–PCR.
[d]Real-Time RT–PCR.

of immunostaining. The PCR and RT–PCR products were scored for their intensity in the agarose gel as a means of quantifying amount of product. A score was also given for the RNA Agilent 2100 Bioanalyser graphs compared to the fresh-frozen control samples. For all the data produced, scores were given in the following manner: 1–3 = poor, 4–6 = adequate, 7–9 = good and 10 = excellent. Statistical analysis of results comprised the Student's *t*-test which was carried out between pairs of fixatives for each of the three organs (liver, spleen and colon). The 2-tailed paired Student's *t*-test (assuming equal and unequal variances) was used for comparison of the mean CT values between fixatives for each tissue. For each of the tests a level of $P = 0.05$ was taken to show significant difference and $P = 0.01$ to show highly significant difference.

RESULTS

Preliminary selection of optimum fixative candidate

Mouse colon, spleen and liver sections were each fixed using the four standard fixation techniques described above, i.e. NBF, Z2, HOPE and fresh-freezing in liquid N_2. PCR and RT-PCR carried out to assess DNA and RNA integrity following the fixation processes were assessed by three independent observers (data not shown). The intensity of the PCR product bands in agarose gels indicated that DNA was poorly preserved by NBF, while the best results were obtained from fixation in Z2 and HOPE. In addition, assessment of morphology following staining with H&E and immunostaining for actin, cytokeratin and CD3 (data not shown) suggested that Z2 was better for structural and protein preservation. Therefore, the zinc-based compound Z2 was chosen for continuing investigation over HOPE, in view of the high cost of HOPE.

Optimization of fixation recipes

(i) Replacement of zinc acetate from the Z2 fixative recipe

Mouse spleen, liver and colon sections were each fixed in each of the zinc acetate replacement solutions described above, i.e. Z7, Z8, Z16, Z17, Z18 and Z19. Examination of morphology and DNA and RNA quality (data not shown) indicated that Z7 gave the best results, and all other combinations were abandoned.

(ii) Replacement of zinc solutions with manganese, magnesium, gallium or vanadium solutions as novel fixative candidates

Mouse spleen, liver and colon tissues each were fixed with each of the zinc replacement solutions, Mn2, Mg2, Ga2 and V2, and compared with fixation with NBF and Z7 as described in Materials and Methods above. Morphological assessment by three independent observers indicated that morphological structure was poorly conserved with all zinc replacement solutions Mn2, Mg2, Ga2 and V2 (data not shown). RNA was extracted from liver, spleen and colon after fixation with Mn2 and Mg2,

while for Ga2 and V2 fixed tissues, RNA was extracted only from mouse liver tissues. RNA concentrations from each fixed tissue was measured using a Nanodrop 1000 spectrophotometer and these together with the results from the Agilent 2100 Bioanalyser showed that the quality of extracted RNA was inferior to that extracted from the zinc-based fixative Z7 (data not shown). For this reason, none of the zinc replacement-based fixatives were pursued further.

(iii) Optimization of zinc-based fixative recipes

Mouse spleen, liver and colon sections were each fixed in each of the zinc-based fixative recipes described above, i.e. Z3, Z4, Z5, Z6, Z9, Z10, Z11, Z12, Z13, Z14 and Z15 and compared with NBF and Z7. Assessment of morphology and DNA and RNA quality (data not shown) suggested that Z4 (Z2 + 5% DMSO) contained the optimal combination and concentrations of chemicals, except for Z7, and all other combinations were abandoned.

Comparison of optimized fixatives with standard fixation

Table 3 shows the scores for morphology and DNA and RNA preservation in the comparison of the zinc-based fixative Z2, the optimum zinc-based fixation recipe Z4, and the zinc acetate replacement fixative Z7 with NBF and fresh-frozen controls. The fresh-frozen samples were only assessed for DNA, RNA and morphological quality and therefore achieved an enhanced total score, since frozen tissue may contain ice crystal artefacts and is avoided for and IHC. From the scores on Table 3, NBF was inferior to the rest of the fixatives tested for DNA and RNA preservation, although it had the best score for morphology. Fresh-frozen samples showed high quality of both DNA and RNA preservation in all tests performed. Z7 was better than NBF and the remaining zinc-based fixatives for protein preservation, morphological structure and RNA preservation and equivalent to Z2 and Z4 fixatives for DNA preservation. Overall, Z7 was given the highest score from the fixatives tested. Immunocytochemistry showed that Z7 was overall the best fixative for antigen preservation without the need for antigen retrieval for all of the antibodies tested.

Assessment of DNA, RNA and protein quality following tissue fixation with Z7

RNA was extracted from mouse liver sections fixed in Z2, Z7 and NBF and from fresh-frozen samples and was

Table 3. Scores from liver, spleen and colon tissue fixed in NBF, Z2, Z4, Z7 and fresh-frozen tissue

Test	Fixative				
	NBF	Z2	Z4	Z7	Frozen (Control)
H&E	9	6	7	8	1
IHC	4	7	7	8	-
DNA (PCR)	4	8	8	8	9
(1) RNA (RT–PCR)	0	0	3	4	9
(2) RNA Bioanalyser	1	2	3	4	10
Overall Score (max. score = 50)	18	23	28	31	29/40

assessed for integrity using the Agilent 2100 Bioanalyser (Figure 1). Typical traces that can be obtained from RNA in various degrees of degradation are shown for purposes of comparison. It can be seen that Z7 has two peaks corresponding to 12S and 18S RNA and that it is significantly better than both Z2 and NBF at preserving RNA structure and integrity.

DNA was extracted from Z7-fixed, Z2-fixed, NBF-fixed and fresh-frozen mouse tissues and was assessed for the maximum size of fragment that could be amplified by Real-Time PCR using GAPDH primers which yield a product of 599 bp. Figure 2 shows results obtained from comparing the mean crossing thresholds of liver, spleen and colon tissues depending on fixation process and gene sequence amplified. Fragments up to 2.4 kb in length could be amplified from the Trefoil Factor 2 gene following Z7 fixation but could not be obtained from amplification of DNA fixed in Z2 or NBF. Results are shown in Figure 6.

Figure 3a shows the results from comparing the mean crossing thresholds (CTs) from real-time RT–PCR using S15 primers, which yield a product of 361 bp, on mouse liver, spleen and colon tissue fixed in NBF and Z7 with fresh-frozen tissue as a control for RNA quality. After RT, the samples were amplified by conventional PCR using commercial β-actin primers (Figure 3b). Results showed there was no contamination with genomic DNA. The smaller band visible (302 bp) in most samples does not correspond to the size expected from genomic DNA contamination (680 bp) but the provenance of this band is not known.

Proteins were extracted from liver fixed with Z7 and NBF and subjected to 2-D PAGE. Fresh-frozen liver tissue was used as positive control. Results are shown in Figure 4. These results show that Z7 is better than NBF and comparable to the fresh-frozen data.

For DNA and RNA quality evaluation, a 2-tailed paired Student's *t*-test (assuming equal and unequal variances) was carried out for comparison of the mean CT values between fixatives, after real-time PCR of mouse liver, spleen, colon and the results are shown in Table 4.

Comparison of quality of archival DNA extracted from Z7- and NBF- fixed paraffin blocks

DNA was extracted from paraffin blocks fixed with NBF and Z7 stored at ambient temperature for 14 months and compared with DNA samples from paraffin blocks stored for less than a week. Conventional PCR results using two housekeeping genes, GAPDH and β-actin, showed that DNA quality from Z7-fixed archival and newly stored tissues was significantly better than from all NBF-fixed tissue (Figure 5a and b). No significant difference was observed between archival stored Z7-fixed and newly stored Z7-fixed samples.

Table 5 shows results from statistical analysis of mean CTs from real-time PCR of mouse liver, spleen and colon tissue fixed in NBF and Z7 when paraffin blocks were stored for 14 months and for less than a week, with fresh-frozen tissue as a control sample for DNA quality. There was no significant difference between the quality of DNA in Z7-fixed material between archived stored paraffin blocks and blocks stored for less than a week ($P = 0.497$). No significant difference in DNA quality was shown between the Z7-fixed and fresh-frozen (positive control) samples ($P = 0.1608$).
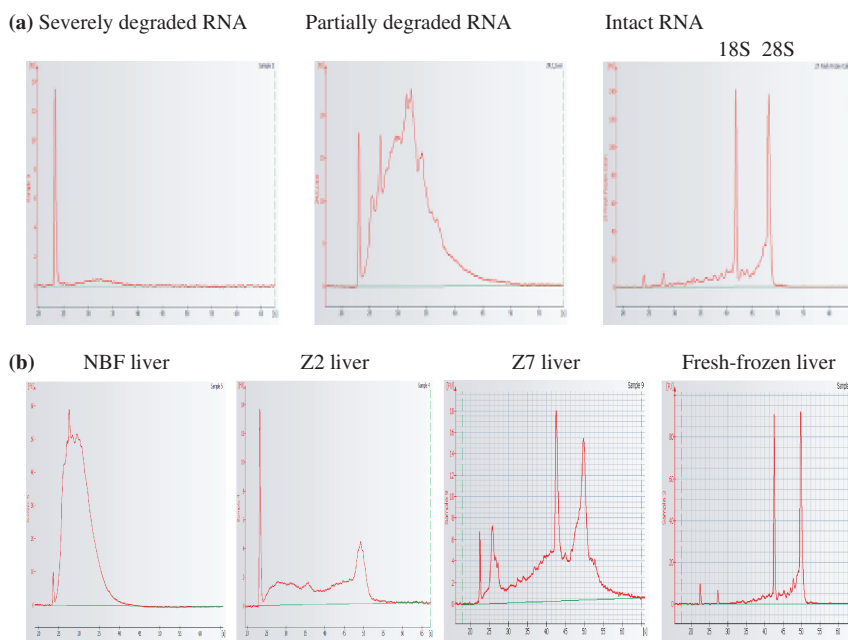

(a) Severely degraded RNA   Partially degraded RNA   Intact RNA

(b)   NBF liver   Z2 liver   Z7 liver   Fresh-frozen liver

Figure 1. Assessment of mouse liver RNA quality using the Agilent 2100 Bioanalyser. (**a**) Typical graphs showing severely degraded, partially degraded and intact RNA. (**b**) Graphs showing RNA quality from tissues fixed in: NBF, Z2, Z7 and also fresh-frozen mouse liver.

DISCUSSION

All our modifications to the zinc-based fixative recipe showed improved quality in terms of morphology and greater quality and quantity of DNA and RNA preservation compared to the standard zinc-based fixative (Z2). The addition of 5% DMSO to the Z2 fixative (our Z4 fixative) markedly improved the morphological structure and nucleic acid and protein preservation. Modifications to the zinc-based recipes included replacing zinc acetate with zinc trifluoroacetate (our Z7 fixative), zinc citrate or zinc tartrate at the same concentration. The rationale for these modifications was the fact that the different counter-ions were expected to lead to different degrees of binding to the zinc and different levels of hydrophobicity, effectively allowing zinc to act faster and with better penetration into the tissue. When Z4 and Z7 were compared, Z7 was found to be a better overall fixative in terms of improved nucleic acid, protein integrity and morphology, although the quality of RNA obtained from
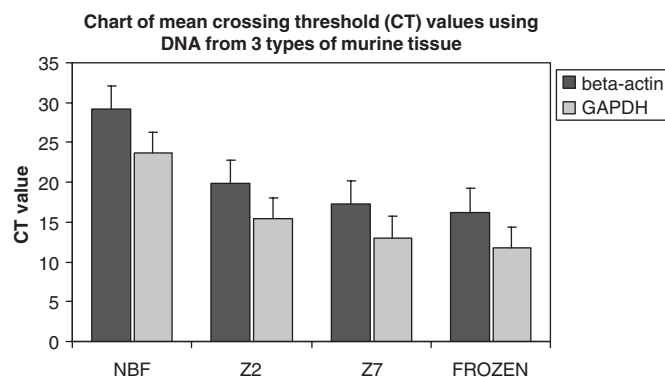
Table 4. Statistical comparison of mean crossing thresholds from real-time RT–PCR of three different tissues (for both DNA and RNA quality)

DNA	*P*-value
Z2 versus NBF	$P<0.01$
Z7 versus NBF	$P<0.01$
FF versus NBF	$P<0.01$
Z7 versus Z2	$P<0.05$
FF versus Z7	$P<0.05$
FF versus Z2	$P<0.05$
RNA	
Z7 versus NBF	$P<0.01$
FF versus NBF	$P<0.01$
FF versus Z7	$P<0.05$

Figure 2. Real-Time PCR results from mouse liver, spleen and colon tissue fixed in NBF, Z2, Z7 and fresh-frozen tissue as a control sample for DNA quality. Bars indicate the standard error mean (±SEM).
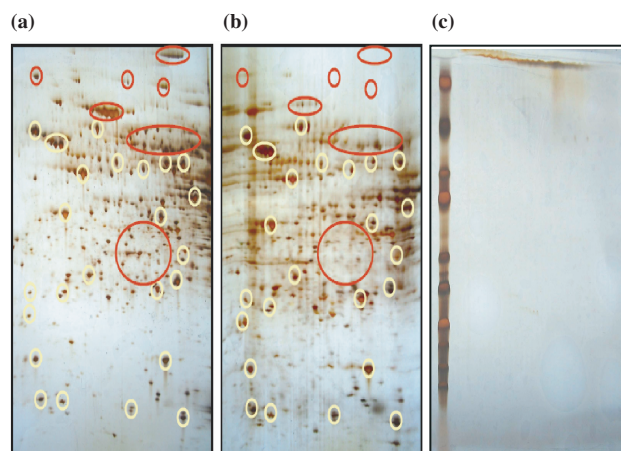
Figure 4. Proteomic comparison between fresh-frozen and zinc-fixed liver tissue by 2-D PAGE. (**a**) Fresh frozen control, (**b**) Z7 fixed sample and (**c**) NBF fixed sample. Representative 2-D PAGE image from one specimen. Yellow and red circles were assigned to indicate similarities and differences respectively in stained protein spots between the two samples.
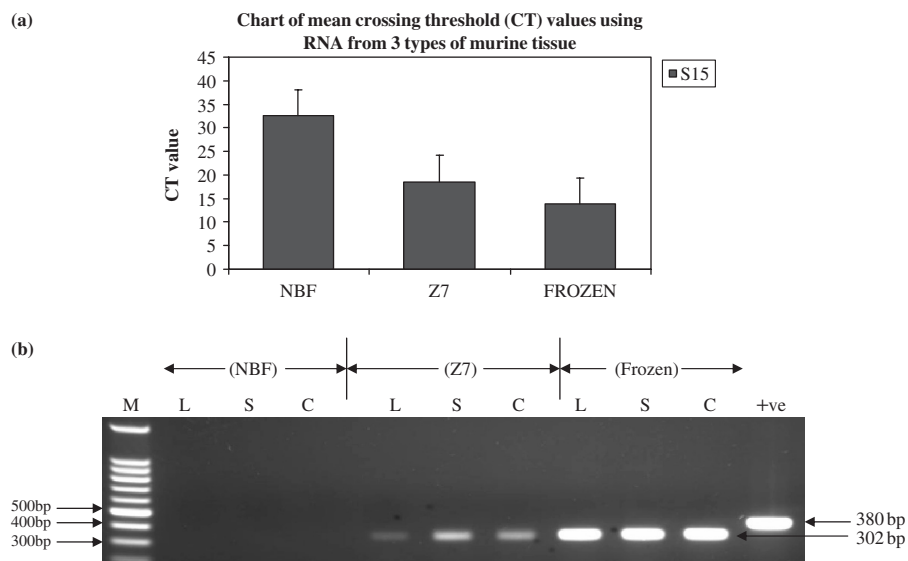
Figure 3. (**a**) Real-time RT–PCR results from mouse liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 with fresh-frozen tissue as positive control and a no-RT negative control sample. Bars indicate the ±SEM. (**b**) Genomic DNA contamination control using RT–PCR with commercial β-actin primers.
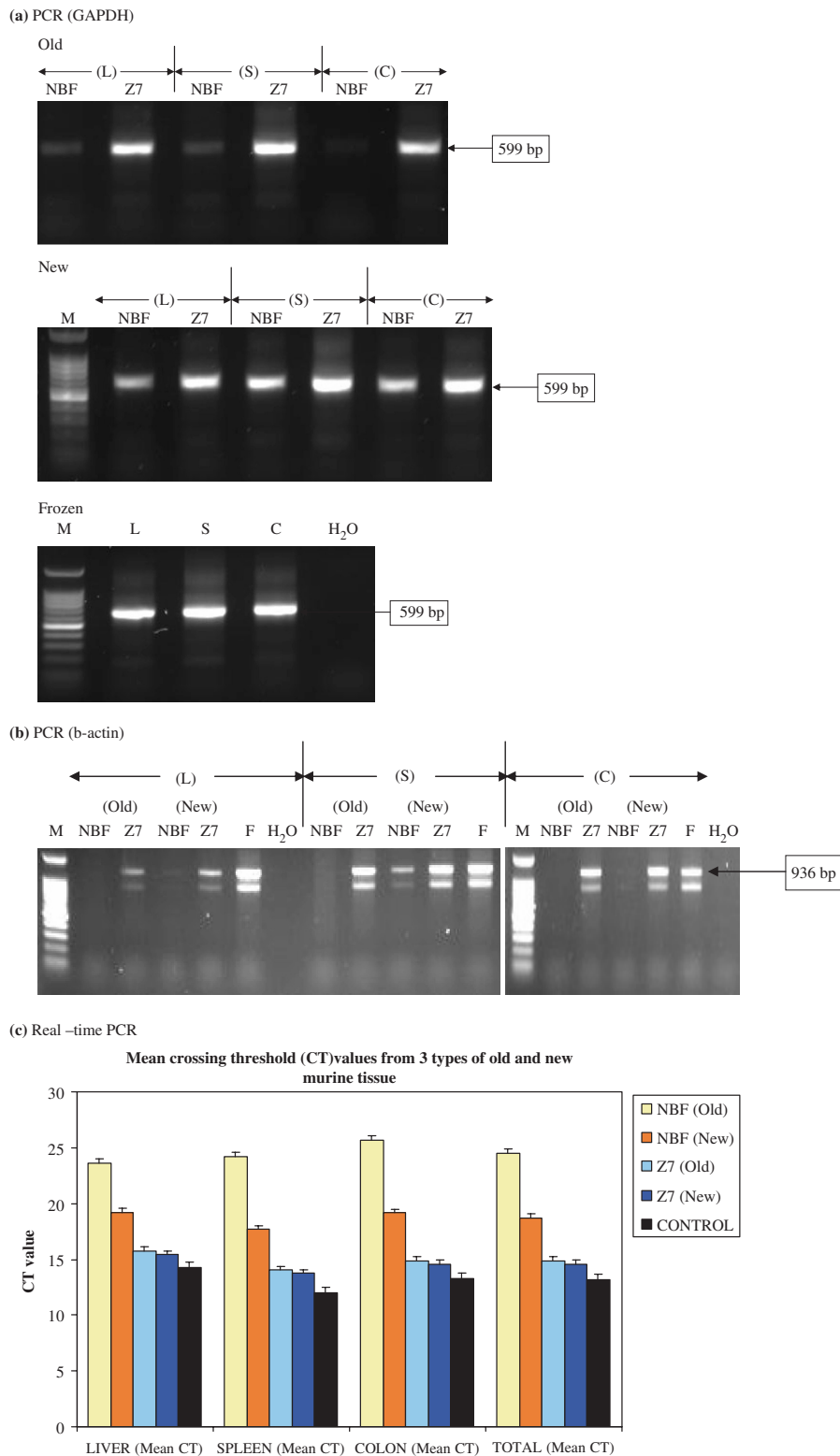
Figure 5. (**a**) PCR of the GAPDH gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for 14 months (Old), compared with DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H_2O) as negative control. (**b**) PCR amplification of the β-actin gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and paraffin blocks stored at RT for 14 months (Old), compared with DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H_2O) as negative control. (**c**) Real-Time PCR showing amplification of the GAPDH gene using DNA extracted from liver, spleen and colon tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for 14 months (Old), compared with DNA extracted from liver, spleen and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H_2O) as negative control. Bars indicate the ±SEM.
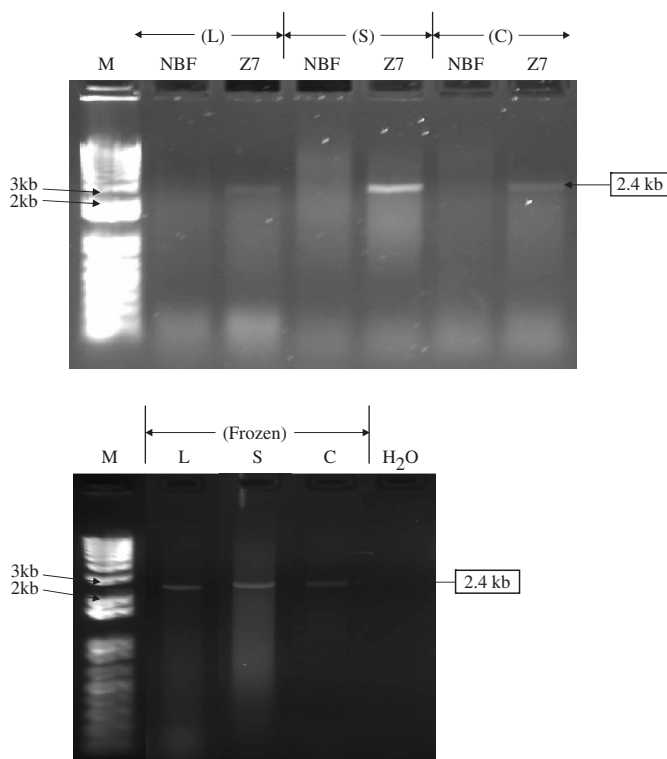
Figure 6. PCR amplification of a 2.4-kb Trefoil Factor 2 gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF and Z7. Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H_2O) as negative control.

Table 5. Statistical comparison of mean CTs from real-time PCR of tissues fixed with three different fixatives and stored for 14 months (Old) or less than a week (New)

	Z7 versus NBF	FF versus NBF	FF versus Z7
Old versus Old	$P<0.01$	$P<0.01$	$P>0.05$
New versus New	$P<0.01$	$P<0.01$	$P>0.05$
	NBF versus NBF	Z7 versus Z7	
Old versus New	$P<0.01$	$P>0.05$	

the Agilent 2100 Bioanalyser was not as good as that obtained from fresh-frozen samples. This is likely to be due to the fact that RNA is degraded throughout the processing stage. Wester *et al.* (2003) indicated that it is the processing and/or storage in paraffin that is responsible for the degradation of RNA. When zinc was replaced with the metallic ions magnesium, manganese, gallium and vanadium to investigate potential fixative properties, poor results were obtained for morphology and RNA quality and these recipes were abandoned.

In the current study, the quality of RNA was assessed using the Agilent 2100 Bioanalyser and agarose gel electrophoresis. Our initial RT–PCR and Real-Time RT–PCR analyses demonstrated that amplicons up to 361 bp in size could be consistently obtained using our methodology, in direct contrast to the results obtained using NBF-fixed tissue, and comparable to fresh-frozen material. Future studies will determine the maximum size of intact RNA and DNA that can be reliably recovered

using our methodology. It was shown that our Z7 fixative was overall the best fixative for DNA, RNA and proteins. DNA fragments up to 2.4 kb were successfully amplified from Z7- fixed samples, compared to 0.6 kb from Z2- and 0.6 kb from NBF-fixed samples. Total protein was analysed successfully by 2-D gel electrophoresis following Z7 fixation, and results were better than those obtained from NBF fixation. The DNA quality of Z7-fixed archival stored samples was similar to freshly fixed and processed samples, and significantly better than that found with NBF fixative.

Although zinc fixation caused shrinkage in all organs tested so far, none of the zinc-based solutions penetrated the tissue as fully as NBF. This resulted in well-fixed edges but apparently not equally well-fixed interiors. In view of the fact that tissues fixed in Z7 and taken through to paraffin blocks are significantly more stable for archiving than other fixatives (except NBF), we believe that this reduction in observed penetration of the fixative does not result in any harm to the nucleic acids.

The search for an 'ideal fixative' suitable for preserving nucleic acid, protein integrity and tissue morphology is an essential requirement for molecular biological analysis of tissues and cells. Our results show that Z7 is a cheap, easily prepared and highly effective fixative that provides significantly improved preservation of DNA, RNA and proteins and allows improved PCR, Real-Time PCR and protein analysis, which may provide an excellent alternative to NBF for contemporary molecular pathobiology research.

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