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The Effect of Formaldehyde Fixation on RNA

Optimization of Formaldehyde Adduct Removal

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Formalin-fixed, paraffin-embedded tissues generally provide low yields of extractable RNA that exhibit both covalent modification of nucleic acid bases and strand cleavage. This frustrates efforts to perform retrospective analyses of gene expression using archival tissue specimens. A variety of conditions have been reported to demodify formaldehyde-fixed RNA in different model systems. We studied the reversal of formaldehyde fixation of RNA using a 50 base RNA oligonucleotide and total cellular RNA. Formaldehyde-adducted, native, and hydrolyzed RNA species were identified by their bioanalyzer electrophoretic migration patterns and RT–quantitative PCR. Demodification conditions included temperature, time, buffer, and pH. The reversal of formaldehyde-fixed RNA to native species without apparent RNA hydrolysis was most successfully performed in dilute Tris, phosphate, or similar buffers (pH 8) at 70°C for 30 minutes. Amines were not required for efficient formaldehyde demodification. Formaldehyde-fixed RNA was more labile than native RNA to treatment with heat and buffer, suggesting that antigen retrieval methods for proteins may impede RNA hybridization or RNA extraction. Taken together, the data indicate that reliable conditions may be used to remove formaldehyde adducts from RNA to improve the quality of RNA available for molecular studies. ***(J Mol Diagn 2011, 13:282–288; DOI: 10.1016/j.jmoldx.2011.01.010)***

Formaldehyde fixation followed by dehydration and paraffin embedding (FFPE) is commonly used to preserve tissue specimens for histological studies and provides most archival tissue samples. The isolation of high-quality RNA from FFPE tissues remains a challenge for molecular studies, despite the availability of multiple published and commercial methods.[1] RNA degradation and formaldehyde modification of RNA appear to be the major contributors to this challenge. Degradation of RNA to low molecular weight species may be because of either sample treatment before and during fixation[2] or long-term (1 year or longer) storage in paraffin.[3] RNA extracted from FFPE tissues is usually fragmented to an average of 100 bases in length.[4,5] Reproducible RT-PCR on FFPE-extracted RNA is limited to amplicons of fewer than 300 bases.[6] Most laboratories strive to amplify segments of 150 or fewer bases. Degraded RNA can often be quantified by techniques that use short oligonucleotides, such as microarray and micro-RNA analyses, and by RT–quantitative PCR (qRT-PCR), but the results are almost invariably less sensitive and less reproducible than achieved using RNA extracted from fresh or fresh-frozen sources.[7,8]

Formaldehyde modification of nucleic acid bases reduces or blocks the base pairing necessary for molecular analysis by hybridization techniques. It is also responsible for cross-links to other macromolecules that reduce the yield of extracted RNA.[9] An improved understanding of these modifications may lead to better strategies for their reversal and to the extraction of RNA that is more suitable for molecular analysis. Previous investigations demonstrated that formaldehyde-induced adducts, such as methylol (hydroxymethyl) groups and methylene bridge cross-links on the amine moiety of an adenine base, were reversible in model systems, such as mononucleotides[9–11] and octamer ribonucleotides.[12] Similar to the heat-induced antigen retrieval methods developed for the analysis of proteins,[13] several groups[3,12,14,15] reported that heating the RNA extracted from FFPE tissues in dilute buffer improved the quality of RNA available for

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molecular studies, presumably by reversing formaldehyde modifications.

Although formaldehyde demodification treatment of FFPE-extracted RNA clearly improves molecular analyses, various studies report different results and demodification conditions. RNA extracted from FFPE tissues heated in Tris-EDTA yielded positive RT-PCR results for amplicons up to approximately 1700 bp.[12] However, others[16] were unable to reproduce these findings. Although some[4,12] used Tris buffers (pH 7 to 8) to remove formaldehyde adducts from RNA, others[11,14] used citrate or Tris-acetate buffers (pH 4).

We initiated this study for three reasons. First, based on our knowledge of pH-dependent hydrolysis of RNA, we wanted to investigate whether optimized formaldehyde demodification conditions could be separated from those that produce hydrolysis. Second, we wanted to optimize conditions for the reversal of formaldehyde-induced RNA modifications. Third, we wanted to determine whether the ability of Tris buffer's amine to form Schiff bases and methylols was critical in its success as a demodification reagent. We reasoned that a 50 base RNA (50mer) oligonucleotide and total cellular RNA might produce more meaningful results than mononucleotides[11] or RNA eightmers.[12] The results of these experiments were expected to provide insights into the mechanism(s) of RNA degradation in FFPE tissues that could lead to improvements in the RNA obtained for molecular analyses.

Materials and Methods

Reagents

Diethyl-pyrocarbonate (DEPC)–treated water was purchased from Fisher Scientific, Fair Lawn, NJ; and aqueous 10% (v/v) methanol-free formaldehyde was purchased from Polysciences, Inc., Warrington, PA. A 50× Tris-acetate EDTA (TAE; 1× is 40 mmol/L Tris/acetic acid and 1 mmol/L EDTA; pH 8) buffer was purchased from Invitrogen, Carlsbad, CA. A 50mer RNA oligonucleotide (RNA 50mer), corresponding to a β2-microglobulin amplicon with a 3′ poly A tail, consisted of the following sequence: 5′-UGACUUUGUCACAGCCCAAGAUAGUA-AGUGAAAAAAAAAAAAAAAAAAAA-3′; it was synthesized by Integrated DNA Technologies (Coralville, IA). HeLa cells were grown to confluence in 100-mm tissue culture dishes. Total cellular RNA was harvested according to the manufacturer's instructions with 0.75-mL Trizol LS (Invitrogen) per dish and taken up in DEPC–treated water.

Formaldehyde Fixation and Postfixation Processing

RNA 50mer (4 μg/μL) in 20 mmol/L phosphate buffer (pH 7.4) or total HeLa RNA (1.2 μg/μL) in 2× PBS (2× is 50 mmol/L KH_2PO_4/Na_2HPO_4 and 150 mmol/L NaCl; pH 7.2) was mixed with an equal volume of 10% (v/v) aqueous formaldehyde and left at room temperature for 2 hours. It was critical to avoid buffering these reactions with Tris[17] because adding formaldehyde to RNA in 20 mmol/L Tris solutions buffered to neutral pH resulted in lowered pH of approximately 4 and incubating for 1 hour at room temperature resulted in RNA degradation (data not shown). Excess formaldehyde either remained or was removed by washing the aliquots five times with 0.5-mL DEPC–treated water in a Microcon YM-3 or YM-10 concentrator (Millipore, Billerica, MA), as listed in Table 1. Native RNA samples were placed into two groups: with or without fixation and with or without heat. All native and formaldehyde-fixed RNA samples were added to an equal volume of a 2× buffer, such that the final buffer concentrations were 20 mmol/L to 40 mmol/L with or without 1 mmol/L

Table 1. Optimization of Formaldehyde Fixation Reversal Conditions on a 50mer RNA Oligonucleotide as Measured by Electrophoretic Mobility

Formaldehyde fixation reversal conditions				RNA species recovered (range)		
Buffer	Temperature	Time (minutes)	pH	Low mol. wt.	Native	High mol. wt.
Formaldehyde Fixed, No Reversal						
–	–	–	–	–	–	++
Without Removing Excess Formaldehyde						
T, TE, TAE	70°C	30	4, 7	++	–	–
T, TE, TAE	70°C	30	9	–	–	++
TAE	42°C	Overnight	4, 7	++	–	–
P	70°C	30	4, 7	–	–	++
P	70°C	30	8, 9	+	–	+
PE	70°C	30	4, 7, 9	–	–	++
With Excess Formaldehyde Removed						
TAE	70°C	30	4	++	–	–
TAE	70°C	30	7, 9	–	++	–
P, PE	70°C	30	3	+	+	–
P, PE	70°C	30	7	–	++	–
P, PE	70°C	30	9	–	+	+
AA	70°C	30	7	–	–	++
AB	70°C	30	7, 9	–	–	++

T, Tris; TE, Tris-EDTA; TAE, Tris-acetate EDTA; P, potassium phosphate; PE, potassium phosphate EDTA; AA, ammonium acetate; AB, ammonium bicarbonate; -, none; +, some; ++, nearly all.

EDTA. The native and formaldehyde-fixed RNA samples were then heated at the indicated temperatures for the indicated times before analysis.

Electrophoresis

The compositions of the total HeLa RNA and RNA 50mer preparations were characterized by capillary electrophoresis using a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Each RNA solution, 1 μL (0.5 to 1 μg per well), was loaded into a nano total RNA chip (Agilent 6000) and run according to the manufacturer's instructions. RNA size and concentration were calculated relative to the total RNA nano kit's ladder using computer software (Agilent 2100 Expert Software). Some experiments used 1.5% 4-morpholinepropane sulfonic acid–formaldehyde denaturing agarose gels stained with ethidium bromide and photographed, as described.[18]

Reverse Transcription and qPCR

A two-step method was used for reverse transcription and quantitative (q)PCR (qRT-PCR). cDNA samples were prepared from 1 ng of RNA primed with random hexamers using a commercially available kit (High Capacity Reverse Transcriptase kit; Applied Biosystems, Foster City, CA) in a total volume of 20 μL. An 84-base β_2-microglobulin amplicon was used for qPCR, as previously described.[19,20] Each 25-μL reaction contained 5 μL of cDNA (×1 TaqMan universal PCR master mix; Applied Biosystems), 0.3 μmol/L primer 5′-TGACTTTGTCACAGCCCAAGATA-3′, 0.3 μmol/L primer 5′-AATCCAAATGCGGCATCTTC-3′, and 0.2 μmol/L probe 5′-[4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein]TGATGCTGCTTACATGTCTCGATCCCA[6-carboxytetramethylrhodamine]-3′. Samples were incubated at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein fluorescence was monitored on a genetic analyzer (Bio-Rad 7500). Cycle threshold values were calculated by the instrument's software without alteration.

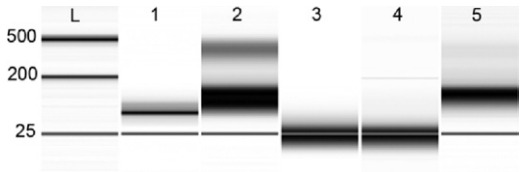

Figure 1. Postformaldehyde fixation processing of an RNA 50mer. Gel-simulated image (Agilent 2100 Bioanalyzer) of the RNA 50mer fixed in 5% buffered formaldehyde (pH 7.4) for 2 hours and then heated at 70°C for 30 minutes, where indicated, in the following buffers, without removing excess formaldehyde. **Lane L:** RNA ladder. **Lane 1:** Non–formaldehyde-treated RNA 50mer heated in TAE buffer (pH 4). **Lane 2:** Formaldehyde-fixed RNA 50mer. **Lane 3:** Fixed RNA 50mer heated in TAE buffer (pH 4). **Lane 4:** Fixed RNA 50mer heated in TAE buffer (pH 7). **Lane 5:** Fixed RNA 50mer heated in TAE buffer (pH 9).

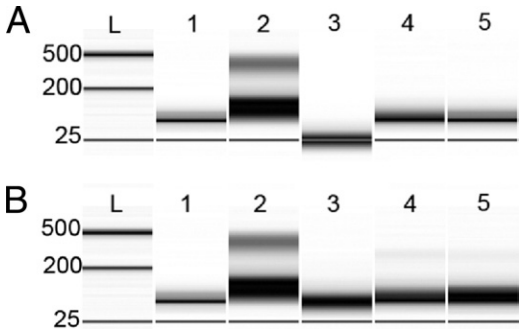

Figure 2. Postfixation processing of an RNA 50mer with excess formaldehyde removed. Gel-simulated image (Agilent 2100 Bioanalyzer) of the RNA 50mer fixed in 5% buffered formaldehyde (pH 7.4) for 2 hours. **A: Lane L:** RNA ladder. **Lane 1:** Non–formaldehyde-treated RNA 50mer heated in TAE buffer (pH 4). **Lane 2:** Formaldehyde-fixed RNA 50mer. **Lane 3:** Fixed RNA 50mer heated in TAE buffer (pH 4). **Lane 4:** Fixed RNA 50mer heated in TAE buffer (pH 7). **Lane 5:** Fixed RNA 50mer heated in TAE buffer (pH 9). **B: Lane L:** RNA ladder. **Lane 1:** Non–formaldehyde-treated RNA 50mer. **Lane 2:** Formaldehyde-fixed RNA 50mer. **Lane 3:** Fixed RNA 50mer heated in 20 mmol/L phosphate with 1 mmol/L EDTA (PE) buffer (pH 3). **Lane 4:** Fixed RNA 50mer heated in PE buffer (pH 7). **Lane 5:** Fixed RNA 50mer heated in PE buffer (pH 9). The excess formaldehyde was removed by repeated washing in a concentrator (Microcon), and the samples were heated at 70°C for 30 minutes, where indicated.

Results

Effect of Fixation and Postfixation Processing on an RNA 50mer

Exposure of the RNA 50mer to 5% formaldehyde for 2 hours demonstrated heterogeneous multimeric species in the range of 2× to 8× the size of the monomer, as assessed by electrophoresis (Figure 1, lane 2). Shorter fixation times did not reproduce the 8× species, and longer fixation times did not increase its intensity or produce additional bands (data not shown). To identify conditions that would return the formaldehyde-fixed 50mer to the electrophoretic migration pattern of native samples, we examined the effects of buffer composition, pH, temperature, and time on reversal of fixation (Table 1).

Fixed samples without the removal of excess formaldehyde were incubated in Tris buffers (with and without acetate and EDTA) in the pH range of 4 to 9 at 70°C for 30 minutes. Incubation at a pH of 4 or 7 resulted in low molecular weight RNA fragments and the disappearance of 2× and 8× multimeric species (Figure 1). Overnight heating in ×1 TAE (40 mmol/L Tris-acetate and 1 mmol/L EDTA; pH 4 or 7) at 42°C produced substantially the same results (Table 1). The 70°C treatment for 30 minutes at a pH of 9 produced a band corresponding to the ×2 species, some low molecular weight RNA, and no native RNA. When the fixed RNA 50mer was heated in phosphate buffers (with and without EDTA), pH 4 to 9, there were mostly high molecular weight species but nothing migrating in the range of the native oligomer (Table 1).

Similar incubations were performed at 70°C for 30 minutes with the formaldehyde-fixed RNA 50mer; excess formaldehyde was removed by repeated washing on membranes (Centricon). In TAE (pH 4), there was only low molecular weight RNA (Figure 2). However, in TAE (pH 7 or 9), there was substantial RNA in the migration range of the

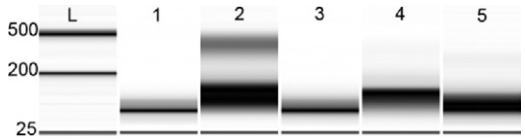

Figure 3. Effects of buffer composition on postfixation processing of an RNA 50mer: gel-simulated image (Agilent 2100 Bioanalyzer). **Lane L:** RNA ladder. **Lane 1:** Native RNA 50mer. **Lane 2:** RNA 50mer fixed in 5% buffered formaldehyde (pH 7.4) for 2 hours. Excess formaldehyde was removed by repeated washing in a concentrator, and the samples were heated at 70°C for 30 minutes in the following. **Lane 3:** ×1 TAE buffer (pH 9). **Lane 4:** 20 mmol/L ammonium bicarbonate (pH 9). **Lane 5:** 20 mmol/L phosphate buffer (pH 9).

native 50mer. In phosphate buffers at a pH of 3, there was some quickly migrating RNA, but most was in the range of the native 50mer (Figure 2, Table 1). In phosphate buffers at pH values of 7 and 9, there was some slowly migrating RNA, but most was in the range of the native 50mer. Ammonium acetate, pH 7 (Table 1), and ammonium bicarbonate, pH 9 (Figure 3), did not appear to reverse formaldehyde adducts. The data suggested that postformaldehyde fixation processing of RNA was most successfully performed in TAE (pH 7 to 9) at 70°C for 30 minutes (Table 1) and that TAE (pH 4) produced low molecular weight products (suggesting degradation). Phosphate, phosphate-glycine, and Tris buffers performed essentially the same at a pH of 8 (Table 2). Tris buffer consistently, but marginally, outperformed phosphate (Figures 2 and 3).

Effect of Fixation and Postfixation Processing on Total Cellular RNA

To examine the correlation between the RNA 50mer and a more biologically relevant system, we determined the effects of formaldehyde fixation and postfixation processing on total cellular RNA extracted from HeLa cells. Electrophoretic profiles of the RNA starting material were substantially the same with and without incubation at 70°C in TAE (pH 9; Figure 4). The sharp 28S and 18S bands suggested that the RNA was of high quality (Figure 4 and Figure 5). Much of the formaldehyde-fixed RNA migrated as two diffuse bands that were slower than the 28S and 18S ribosomes. After incubation at 70°C for 30 minutes in TAE (pH 9), the profile of formaldehyde-fixed RNA appeared similar to those of native RNA. After 70°C incubation in TAE (pH 4), formaldehyde-fixed RNA migrated between 100 and 1000 bases. Incubation in phosphate buffer

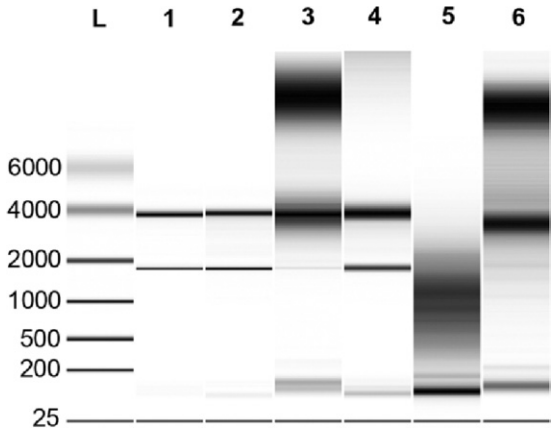

Figure 4. Effects of fixation and postfixation processing on total cellular RNA: gel-simulated image (Agilent 2100 Bioanalyzer) of total cellular RNA fixed in 5% buffered formaldehyde (pH 7.4) for 2 hours. **Lane L:** RNA ladder. **Lane 1:** Native non–formaldehyde-treated RNA. **Lane 2:** Non–formaldehyde-treated RNA heated in TAE buffer (pH 9). **Lane 3:** Total RNA fixed in 5% formaldehyde. **Lane 4:** Fixed total RNA heated in TAE buffer (pH 9). **Lane 5:** Fixed total RNA heated in TAE buffer (pH 4). **Lane 6:** Fixed total RNA heated in 20 mmol/L phosphate buffer (pH 9). The excess formaldehyde was removed by repeated washing in a concentrator, and the samples were heated at 70°C for 30 minutes, where indicated.

(pH 9) resulted in an electrophoretic migration pattern running just slower than native RNA. In the total RNA system, the low molecular weight species from TAE (pH 4) treatment; the nativelike species from TAE (pH 9) treatment; and the few high molecular weight species from phosphate buffer (pH 9) treatment were substantially reproduced from the 50mer data. This trend was also present when total cellular RNA was analyzed by qRT-PCR (Table 3).

The reversal of formaldehyde fixation closer to neutral pH by phosphate and Tris buffers was more closely examined. Heating in phosphate buffer (pH 6 to 7) was less successful at reversing formaldehyde adducts without hydrolyzing RNA than at a pH of 8 (Table 2). At a pH of 8, nearly complete reversal of formalin adducts with minimal hydrolysis was observed regardless of Tris, phosphate, or phosphate-glycine buffers (Table 2).

Discussion

Buffer conditions for the reversal of formaldehyde-fixed RNA, as measured by gel electrophoresis, had substan-

Table 2. Optimization of Formaldehyde Fixation Reversal Conditions on Total Cellular RNA as Measured by Electrophoretic Mobility

Formaldehyde fixation reversal conditions				RNA species recovered (range)		
Buffer	Temperature	Time (minutes)	pH	Low mol. wt.	Native	High mol. wt.
Formaldehyde Fixed, No Reversal						
–	–	–	–	–	–	++
With Excess Formaldehyde Removed						
P	70°C	30	6	+	+	+
P	70°C	30	7	+	+	–
P	70°C	30	8	–	++	–
PG	70°C	30	8	–	++	–
T	70°C	30	8	–	++	–

P, phosphate; PG, phosphate-glycine; T, Tris; –, none; +, some; ++, nearly all.

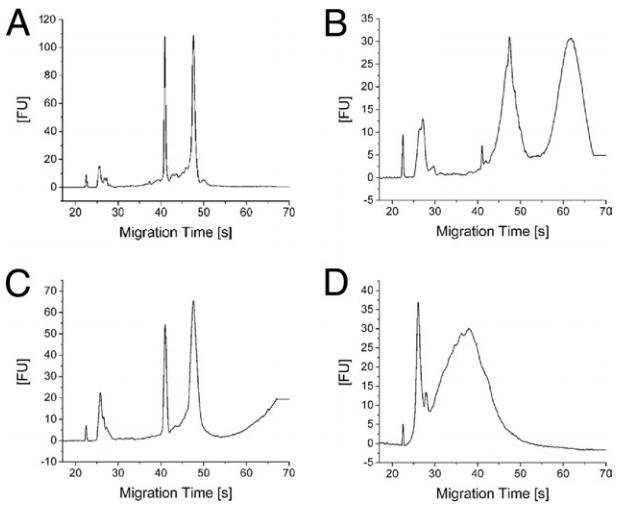

Figure 5. Effects of fixation and postfixation processing on total HeLa RNA. Electropherograms (Agilent 2100 Bioanalyzer) of total cellular RNA fixed in 5% buffered formaldehyde (pH 7.4) for 2 hours. FU denotes fluorescence units. **A:** Non–formaldehyde-treated RNA heated in TAE buffer (pH 9). **B:** Total RNA fixed in 5% formaldehyde. **C:** Fixed total RNA heated in TAE buffer (pH 9). **D:** Fixed total RNA heated in TAE buffer (pH 4). The excess formaldehyde was removed by repeated washing in a concentrator (Microcon), and the samples were heated at 70°C for 30 minutes, where indicated.

tial effects on the products. Products included high- and low molecular weight RNA, suggesting failure to reverse interstrand cross-linked formaldehyde adducts and chain fragmentation, respectively. Diffuse/broad bands were consistent with methylol-adducted and/or intrastrand cross-linked species. These results were not previously observed in other model systems.[11,12]

On formaldehyde fixation, the RNA 50mer spread out to species migrating at approximately ×2 and ×8 the size of the monomer, suggesting interstrand–cross-linked dimer and octamer multimers (Figure 1, lane 2). The monomer-dimer-octamer species are seen in some proteins, in which the equilibrium among these multimers is affected by solvent accessibility.[21] The protein octamer structures show four subunits forming a square that sits on top of the opposite square, like a cube.[22] Formaldehyde-fixed total RNA electrophoresed as diffuse bands at approximately the size of the 28S subunit and several times that size (Figure 4, lane 3). Others[12] reported no differences in the electrophoretic migration of formaldehyde-fixed and native total cellular RNA when analyzed by agarose gel electrophoresis. A possible explanation for this discrepancy is that typical 4-morpholinepropane sulfonic acid–formaldehyde agarose gels contain 6.7% v/v (approximately 2.2 mol/L) formaldehyde as a denaturing agent.[18]

In the presence of excess formaldehyde, 70°C incubation of fixed RNA in Tris buffers at pH 4 or 7 produced small RNA fragments, suggesting hydrolysis. The inability of Tris (pH 9) and phosphate buffers to reverse formaldehyde fixation could be attributed to excess formaldehyde rereacting with the RNA 50mer (Figure 1). Fixation reversal went more smoothly when the excess formaldehyde was removed (Table 1). Heating-fixed RNA in pH 3 to 4 buffers continued to produce low molecular weight species (Figures 2–4, Table 1). This suggested that acidic buffers produced hydrolysis/chain fragmentation. This is not surprising considering that, at pH values lower than 6, nonenzymatic RNA hydrolysis increases as pH decreases.[23] However, others[14] reported that optimal formaldehyde adduct demodification of RNA proceeded in 10 mmol/L citrate (pH 4) when heated at 70°C for 30 minutes. Our end point was electrophoretic migration, whereas the end point in the other study[14] was RT-PCR efficiency. This discrepancy could be reconciled by either of two possibilities: hydrolysis was of little importance for RT-PCR analysis of short amplicons, or electrophoretic mobility might not fully characterize formaldehyde adducts that do not produce cross-linking, such as methylols. To address these possibilities, we checked the correlation between formaldehyde adduct demodification and reverse transcription and qPCR amplification efficiency. The data show poor amplification of formaldehyde-fixed RNA compared with native RNA (Table 3). Adduct demodification treatments substantially agreed with electrophoretic mobility results. Amplification from fixed RNA reversed in TAE buffer (pH 9) was slightly better than that of phosphate buffer but not statistically significant. Both were substantially better amplified than fixed RNA reversed in TAE (pH 4). Thus, neither of the previously described hypotheses was supported. Nucleases in protein-contaminated RNA preparations were possibly less active at a pH of 4 than at physiological pH.

For the optimal pH for formaldehyde adduct demodification of RNA, our data are in better agreement with formaldehyde demodification in Tris-EDTA (pH 7.5)[12] and with the likely composition of proprietary proteinase K buffers.[3,15] We suspect that the proprietary buffers are not acidic because the most common proteinase K buffer is SDS in Tris-EDTA (pH 7.5),[18] and the optimal pH for this enzyme is 7.5 to 12.[23] Interestingly, heating in TAE (pH 4) did not produce substantial low molecular weight species from the native 50mer, only for the formaldehyde-fixed 50mer (Figure 2). This suggested the possibility that formaldehyde-fixed RNA was more labile than native RNA.

Because Tris at a pH of 9 compared favorably with phosphate at a pH of 9 in the reversal of formaldehyde

Table 3. Optimization of Formaldehyde Fixation Reversal Conditions on Total Cellular RNA as Measured by qRT-PCR

Formaldehyde fixation reversal conditions	qRT-PCR results (C_T)
Native RNA (none)	28.9 ± 0.1
Formaldehyde-fixed RNA (none)	34.7 ± 0.1
Recovered in TAE (pH)	
4	33.3 ± 0.1
9	29.3 ± 0.4
Recovered in phosphate (pH 9)	29.8 ± 0.4
NTC	35.1 ± 0.9

In the formaldehyde-fixed sample, excess formaldehyde was removed before analysis.
C_T, cycle threshold; TAE, Tris-acetate EDTA; NTC, no template control.

adducts, it seemed that the primary amine moiety of Tris buffer was involved. We hypothesized that Tris formed Schiff bases and methylols for more efficient formaldehyde demodification. This hypothesis was tested by comparing formaldehyde demodification of the RNA 50mer in Tris or phosphate with amine-containing buffers (ammonium acetate and ammonium carbonate). Figure 3 and Table 1 show that the ammonia-containing buffers were less efficient at reversing the effects of formaldehyde on the 50mer at a pH of 7 to 9 than were phosphate buffers. In addition, there were no obvious differences between the reversals of formaldehyde modification of total cellular RNA for phosphate, phosphate plus glycine (amine containing), and Tris at a pH of 8 (Table 2). This suggested that amines were not required for efficient formaldehyde demodification. We tried to isolate Tris-formaldehyde adducts, including reducing equimolar solutions with sodium cyanoborohydride, but were unable to isolate Tris-formaldehyde Schiff bases and methylols (data not shown). Thus, we found little to support the previously described hypothesis.

The pK_a and, thus, the pH of many biological buffers depends on temperature. The pH values of Tris and phosphate buffers are estimated to decrease by 1.5 and 0.15 units, respectively, by increasing the temperature by 50°C.[24] This explains some of the marginally different effects of Tris and phosphate buffers on formaldehyde adduct demodification. Room temperature Tris buffer at a pH of 7 to 9 becomes a pH of 5.5 to 7.5 at 70°C. There is an order of magnitude less change in the pH of phosphate buffer from room temperature to 70°C. If a pH from approximately 6 to 7 at 70°C is most desirable for demodification, then Tris buffer at a pH of 8 to 9 would be expected to outperform phosphate buffer at a pH of 8 to 9. In our experiments, basic Tris buffers consistently outperformed phosphate buffers at demodifying formaldehyde-adducted RNA. The apparent pH optimum for demodification appears to intersect with the pH of least nonenzymatic RNA hydrolysis.[25]

Our results cannot be applied directly to the recovery of RNA from FFPE tissues because they do not account for the effects of postfixation tissue dehydration that occurs during the dehydration and embedding process. Nevertheless, some laboratories may find practical value because specimens may arrive in the laboratory already completely or partially fixed in formaldehyde and/or a portion of a submitted specimen is not processed for embedding and sectioning.

In summary, it appears that conditions can be optimized for the reversal of formaldehyde-induced RNA modifications. The goal is to avoid "too weak" treatments that fail to demodify formaldehyde adducts and "too strong" treatments that cause RNA hydrolysis. Although conditions may require tuning to specific samples, heating in dilute buffers (pH 8) at 70°C for 30 minutes appears to be a good recommendation for initial treatments. Our data provide no evidence to support a role for the amine moiety of Tris base as a direct formaldehyde scavenger.

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