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Comparison of methods in the recovery of nucleic acids from archival formalin-fixed paraffin-embedded autopsy tissues

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ABSTRACT

Archival formalin-fixed paraffin-embedded (FFPE) human tissue collections are typically in poor states of storage across the developing world. With advances in biomolecular techniques, these extraordinary and virtually untapped resources have become an essential part of retrospective epidemiological studies. To successfully use such tissues in genomic studies, scientists require high nucleic acid yields and purity. In spite of the increasing number of FFPE tissue kits available, few studies have analyzed their applicability in recovering high-quality nucleic acids from archived human autopsy samples. Here we provide a study involving 10 major extraction methods used to isolate total nucleic acid from FFPE tissues ranging in age from 3 to 13 years. Although all 10 methods recovered quantifiable amounts of DNA, only 6 recovered quantifiable RNA, varying considerably and generally yielding lower DNA concentrations. Overall, we show quantitatively that TrimGen's WaxFree method and our in-house phenol–chloroform extraction method recovered the highest yields of amplifiable DNA, with considerable polymerase chain reaction (PCR) inhibition, whereas Ambion's RecoverAll method recovered the most amplifiable RNA.

Retrospective studies of infectious disease have become increasingly important during the past several years, giving us a clearer understanding of pathogen evolution and emergence [1,2]. Worldwide, formalin-fixed paraffin-embedded (FFPE)[1] human autopsy tissue samples provide a vast archive of pathologically disease-specific materials for potential use in biomolecular investigations [3–7]. However, using FFPE autopsy tissues for these investigations poses a myriad of challenges. The collections are often in a declining state and contain minute amounts of tissue per sample [8]. Although formalin is an excellent preservative for maintaining the integrity of tissues, the time since death, and the time to fixation, the paraffin-embedding process and subsequent storage lead to nucleic acid degradation and extensive modification that may affect the yield and quality of nucleic acids (DNA and RNA) [9–23]. More important, formaldehyde creates cross-links between nucleic acids and proteins [24,25], resulting in DNA and RNA species with average base pair lengths of approximately 200 bp or less [26–29]. As is the case with many archival tissue collections in developing countries, the sampling (autopsy vs. biopsy material), tissue type, and formalin fixation process (buffered vs. unbuffered) are often unknown or poorly documented.

A number of studies have addressed improvements in the process of isolating high-quality nucleic acids from FFPE tissues [3,21,30–34], rendering them suitable for downstream applications such as polymerase chain reaction (PCR) [35–37]. These improvements have now been harnessed by major biotechnology companies and included in commercial FFPE kits (see Table 1 for details of those assessed in this study). The scope of this article is not to enhance or alter the methods prescribed in these commercial kits but rather to determine, in the end, which kit consistently yields the highest amount of amplifiable DNA and RNA. Although most of these commercially available extraction kits have been tested on freshly fixed paraffin tissues [9,38–40], only a few studies have compared the relative performance of these methods on archival pathological or postmortem tissues, e.g. [41]. A careful

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[1] *Abbreviations used:* FFPE, formalin-fixed paraffin-embedded; PCR, polymerase chain reaction; mRNA, messenger RNA; RT, reverse transcription; PCE, phenol-chloroform extraction; EDTA, ethylenediaminetetraacetic acid; qPCR, quantitative PCR; BSA, bovine serum albumin; dNTP, deoxynucleoside triphosphate; β2 M, β2-microglobulin; cDNA, complementary DNA; C_T, cycle threshold; bp, base pair.

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Table 1
The 10 nucleic acid extraction methods analyzed in this study

Protocol	Nucleic acid	Manufacturer	Catalog number	Deparaffinization	Digestion method	Digestion time and temperature	Purification method
ABS	RNA	Stratagene (La Jolla, CA, USA)	400809	d-Limonene, ethanol	Digestion buffer, proteinase K	Overnight (55 °C)	Guanidine thiocyanate filtration
GEN	DNA	Sigma (St. Louis, MO, USA)	G1N10	Xylene, ethanol	Chaotropic salt buffer, proteinase K	Overnight (55 °C)	Silica-based membrane
HPP	RNA	Roche (Basel, Switzerland)	3270289001	Xylene, ethanol	Tissue lysis buffer, SDS, proteinase K	Overnight (55 °C)	High pure filter
HPM	RNA		4823125001			3 h (55 °C)	
PCE	DNA/RNA	In-house	Local	Xylene, ethanol	In-house buffer, proteinase K	Overnight (55 °C)	Phenol-chloroform and Microcon (YM-10)
RAR	RNA	Ambion (Austin, TX, USA)	1975	Xylene, ethanol	Proprietary buffer, protease	RNA–3 h (55 °C)	Glass-fiber filtration
RAD	DNA					DNA–48 h (55 °C)	
TRD	DNA	Ambion (Austin, TX, USA)	AM9738	Xylene, ethanol	Guanidinium thiocyanate	5 min (room temperature)	Phenol-chloroform and alcohol precipitation
TRR	RNA						
WXF	RNA	TrimGen (Sparks, MD, USA)	DE-50	Q-Solution	R-Resin, enzyme mix	Overnight (45 °C)	WR filtration

Note. These methods consisted of 1 in-house method and 7 commercial kits. Their abbreviations and respective companies are as follows: the in-house PCE (phenol-chloroform extraction) method, Ambion's TRR (TRI-Reagent solution–RNA) method, Ambion's TRD (TRI-Reagent solution–DNA) method, Sigma's GEN (GenElute Mammalian Genomic DNA Miniprep Kit) method, Ambion's RAR (RecoverAll Total Nucleic Acid Isolation Kit–RNA) method, Ambion's RAD (RecoverAll Total Nucleic Acid Isolation Kit–DNA) method, TrimGen's WXF (WaxFree Paraffin Sample RNA Preparation Kit) method, Roche's HPP (High Pure RNA Paraffin Kit) method, Roche's HPM (High Pure RNA Micro Kit) method, and Stratagene's ABS (Absolutely RNA FFPE Kit) method. The table also shows deparaffinization reagents, digestion times, and a summary of the respective purification methods as detailed in the protocols.

comparison between these methods (commercial and in-house) on archived specimens is lacking in the literature. For these reasons, we present a qualitative and quantitative comparison of the total nucleic acids (DNA and RNA) released by 7 commercially available extraction kits (2 of which split into two separate extractions) and 1 in-house method (for a total of 10 methods) on seven FFPE autopsy tissue blocks ranging from 1995 to 2005. For each method, we measured total DNA and RNA, the number of amplifiable human nuclear single-copy DNA molecules and messenger RNA (mRNA), and the level of PCR inhibition present in each of the extracts derived from the FFPE tissues. To maximize sample utility in downstream applications, the preferred method should offer both high overall nucleic acid recovery and amplifiability of both DNA and RNA.

Materials and methods

Sampling and laboratory work authentication

We randomly selected seven FFPE autopsy blocks, all visceral tissues involving random organs to mimic archival pathological tissues that would typically be found in most repositories in the developing world. All tissues, according to documentation, were fixed in 10% buffered formalin before paraffin embedment, with embedment dates ranging from 1995 to 2005. The tissues were obtained from the tissue repository at the Department of Pathology, School of Biomedical Sciences, College of Health Sciences at Makerere University in Uganda. Patient identifiers, except the serial identification numbers that correlate with the sample's year of fixation, were removed to maintain anonymity. Approval for use of these histopathological human tissues in our research was obtained from the ethical review boards of both the College of Health Sciences at Makerere University and the Faculty of Health Sciences at McMaster University.

Genetic studies on degraded samples, such as archival FFPE autopsy tissues, are at risk for contamination from exogenous sources, particularly when working with human DNA [42]. This type of study requires strict laboratory conditions with dedicated facilities designed to avoid contamination, thereby ensuring the authenticity of the data generated. For this reason, all work took place in the clean rooms of the McMaster Ancient DNA Centre within dedicated and physically separated work areas designed to avoid cross-contamination to the greatest extent possible.

Nucleic acid extraction methods

We used 10 different extraction methods on seven archival pathological blocks, yielding a total of 70 extracted samples. The methods involved 7 commercial kits (2 of which provided protocols for the separate isolation of DNA and RNA and, therefore, were regarded as separate methods, thereby totaling 9) and 1 in-house organic digestion method (see Table 1 for details and abbreviations). Notably, the Ambion's RecoverAll kit has both a shorter and longer incubation period, thereby enabling the comparison of different digestion times. Overall, we followed the extraction protocols in the commercial kits as supplied by their manufacturers, with the exception of the DNase treatment step that was performed only on aliquots of each extract prior to reverse transcription (RT) and PCR, to compare the effects of DNase digestion on downstream PCR and RT–PCR. The in-house phenol-chloroform extraction (PCE) method begins with proteinase K digestion, followed by purification using sequential phenol-chloroform extractions and concentration over YM-10 Microcon centrifugal filter units (Millipore, Billerica, MA, USA).

Sample preparation

Using a sterile scalpel blade, we removed 25 mg of tissue from each FFPE autopsy block after excess paraffin wax surrounding the embedded tissue was cut away. With the exception of TrimGen's WXF method and Stratagene's ABS method, where dewaxing was done with the Q-Solution and d-Limonene, respectively, as supplied with the kit, all remaining samples were dewaxed using xylene. We washed once with dewaxing solution and twice with 100% ethanol and then dried the sample over air prior to continuing with enzymatic digestion. We followed the manufacturers' instructions in all cases. RNA and DNA extracts were eluted in 200 μl of RNA Storage Solution (Ambion, Austin, TX, USA) and/or 1× TE buffer (Tris–ethylenediaminetetraacetic acid [EDTA], pH 7.5). The eluted nucleic acid extracts were immediately aliquoted (into 20-μl volumes) and stored at −80 °C until all extractions were completed. Subsequent analyses were carried out on all of the extracts in concert to avoid any potential variation between as-

says. For each of the 10 extraction methods, we processed the same seven FFPE tissue blocks and one negative control; this ensured that resulting trends across samples were attributable to the extraction methods rather than inter-sample variation. Although variations within the same tissue block are possible, we avoid this bias by averaging across all samples and all methods to ensure that any potential inter-sample variations are kept to a minimum.

Comparison of nucleic acid recovery

Total DNA concentrations in each extract were measured using the PicoGreen double-stranded DNA (dsDNA) Quantitation assay (Molecular Probes, Eugene, OR, USA) on a TBS-380 Mini-Fluorometer with a Minicell Adaptor Kit (Turner BioSystems, Sunnyvale, CA, USA). Total RNA was measured using a 2100 Agilent Bioanalyzer and RNA 6000 Pico Chip assay (Agilent Technologies, Palo Alto, CA, USA). As a comparative study, we also quantitated total RNA in each sample using the RiboGreen RNA assay (Molecular Probes) on a TBS-380 Mini-Fluorometer (see supplementary material for details).

qPCR comparisons of c-Myc and β2M genomic copies

All quantitative PCR (qPCR) assays were conducted using an MX3000P Real Time PCR System (Stratagene, La Jolla, CA, USA). Each 20-μl reaction contained 1× PCR Buffer II, 2.5 mM $MgCl_2$, 1.0 μg/μl bovine serum albumin (BSA), 250 μM of each deoxynucleoside triphosphate (dNTP), 250 nM of each primer, 0.167× SYBR Green I, 0.05 U/μl AmpliTaq Gold DNA polymerase, and 2 μl of template DNA extracts (or water for nontemplate controls). PCR conditions were as follows: initial denaturation at 95 °C for 7 min, 50 cycles of 95 °C denaturation for 30 s, 30 s of annealing at 60 or 59 °C for c-Myc and β2 M (β2-microglobulin) primers, respectively, and extensions at 72 °C for 30 s, and a final extension at 72 °C for 10 min.

Quantitation of nuclear DNA (c-Myc) copies

We estimated nuclear DNA copies in each extract using a qPCR assay designed to amplify an 81-bp fragment targeting the human *c-Myc* gene with primers CMYC_E3_F1 (5'-GCCAGAGGAGGAACGAGC-3') and CMYC_E3_R1 (5'-GGGCCTTTTCATTGTTTTCCA-3') [43]. For this assay, a synthetic oligonucleotide standard was designed with a base pair transition (bolded) that is not normally found in the human genome (5'-GTCTTGGAGCGCCAGAGGAGGAACGAGCTAAAACGG**C**GCTTTTTTGCCCTGCGTGACCAGATCCCGGAGTTGGAAAACAATGAAAAGGCCCCCAAGGTAGT-3'), enabling differentiation of target amplicons and potential contamination. A standard curve was derived from amplification of serial dilutions of the standard (2 to 2×10^4 copies) in replicates. Concentrations of the target molecules were estimated from their amplification plots in relation to the above standard, whose reaction efficiency and R^2 (coefficient of correlation) were 99.2% and 0.973, respectively.

qPCR quantitation of RNA (β2M) copies

To quantitate the number of mRNA copies in each of the extracts, a section of the *β2M* gene was amplified using B2 M F (5'-TGACTTTGTCACAGCCCAAGATA-3') and B2 M R (5'-AATCCAAATGCGGCATCTTC-3') primers [11] using a SYBR Green I-based qPCR assay. This assay amplifies over an exon/intron boundary, producing a 1.96-kb amplicon from genomic DNA templates but only an 85-bp amplicon from complementary DNA (cDNA); thus, DNA- and RNA-derived products are easy to differentiate. For comparative purposes, we used both DNase-treated (DNase+) and DNase-untreated (DNase–) extracts in the RT–PCR step. First-strand cDNA synthesis was done using the reverse primer above (B2 M R) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We used a gene-specific primer rather than random hexamers because previous studies have documented that the former is more robust in real-time amplifications than the latter [44]. As for the *c-Myc* genomic DNA quantitation above, amplifiable mRNA quantities were assessed by comparison with a standard curve generated from serial dilutions (2 to 2×10^4 copies) of a synthetic *β2M* DNA fragment (5'-GAACCATGTGACTTTGTCACAGCCCAAGATAGTTAAGTGGGATCGAGACATGTAAGCAGCATCATGG**C**GGTTTGAAGATGCCGCATTTGGATTGGATGA-3').

Inhibition assay

The presence of inhibitors in the extracts may lead to false-negative PCR results as well as an underestimation of the total DNA/RNA quantity. We assessed the level of inhibition in all extracts based on the measurement of the performance of an internal positive control in qPCR spiked with FFPE extracts. The control template for this assay was 1×10^3 copies of a purified cloned PCR product stemming from the cytochrome *b* of mammoth [45,46]. The cycle threshold (C_T) measured in the presence of each extract was compared with that of an unspiked control reaction. In the absence of inhibitors in the extract, the C_T should remain the same, whereas in more inhibited extracts, the C_T increases relative to the reference point. The level of inhibition in each sample extract, therefore, was measured as the shift in C_T relative to that of the unspiked control reaction.

Results

Total DNA recovery and c-Myc DNA quantitation

All samples and extraction methods recovered measurable amounts of DNA as assessed by the PicoGreen assay; however, the amounts varied considerably, ranging from 0.02 (0–0.04) ng/μl in Ambion's TRR method to 5.47 (2.03–7.73) ng/μl in the in-house PCE method (Table 2). Of the 10 methods tested, 6 yielded significantly higher amounts of DNA: (from highest to lowest) the in-house PCE, TrimGen's WXF, Ambion's RAD, Stratagene's ABS, Ambion's RAR, and Sigma's GEN (Table 2 and Figs. 1A and 1B). All methods except Ambion's TRR yielded amplifiable nuclear *c-Myc* copies that ranged from approximately 0.5 (0–3.0) copies/μl in Ambion's TRD to 425 (0–1636) copies/μl in TrimGen's WXF (Figs. 1C and 1D). Overall, there were four methods that consistently yielded high amounts of recoverable total DNA as well as amplifiable DNA copies: TrimGen's WXF, Ambion's RAD, the in-house PCE, and Sigma's GEN (Table 1 and Figs. 1A and 1B). There was no apparent correlation between the age of samples and total copy number.

Total RNA recovery and β2M RNA quantitation

Total RNA recovery was lower than DNA by approximately an order of magnitude and ranged from 0.002 (0–0.10) ng/μl in Sigma's GEN to 1.45 (0–4.88) ng/μl in TrimGen's WXF. Of the 10 methods tested, 6 yielded RNA, and only 4 of these gave consistent results: (from highest to lowest) TrimGen's WXF (1.45 [0.00–4.88]), Ambion's RAD (0.85 [0.16–2.66]), the in-house PCE (0.43 [0.06–0.67]), and Ambion's RAR (0.11 [0.00–0.35]). As with DNA recovery, RNA concentrations varied significantly across methods (Table 2) and samples, with both Stratagene's ABS and Roche's HPM yielding no measurable RNA quantities (Figs. 1E and 1F). Interestingly, as an exploratory analysis, we observed on average twice as much amplifiable RNA from extracts that had been treated

Table 2
Summary of the performance of the 10 nucleic acid extraction methods based on the FFPE autopsy tissues analyzed in this study.

Protocol	DNA (ng/μl)	*c-Myc* DNA copies	RNA–DNase+ (ng/μl)	*β2M* (DNase+) copies	*β2M* (DNase–) copies	C_T shift
ABS	2.77 (1.01–4.06)[4]	1 (0–9)[8]	0[9]	0[10]	0[7]	–0.28 (–0.51 to –0.17)[1]
GEN	2.42 (0.17–3.61)[6]	165 (0–828)[4]	0.02 (0.00–0.10)[6]	10 (0–40)[5]	14 (0–53)[5]	0.05 (–0.18 to 0.37)[6]
HPM	0.24 (0.06–0.57)[8]	7 (0–23)[7]	0[9]	3 (0–22)[6]	2 (0–13)[6]	–0.12 (–0.27 to –0.01)[2]
HPP	0.99 (0.31–2.59)[7]	24 (0–77)[6]	0.04 (0.00–0.11)[5]	85 (0–276)[3]	103 (0–314)[3]	–0.07 (–0.23 to 0.45)[3]
PCE	5.47 (2.03–7.73)[1]	342 (14–981)[2]	0.43 (0.06–0.67)[3]	64 (0–292)[4]	46 (0–174)[4]	1.96 (0.26 to 4.20)[10]
RAD	4.17 (2.35–6.49)[3]	307 (0–994)[3]	0.85 (0.16–2.66)[2]	7813 (41–28,380)[1]	4449 (65–15,920)[1]	0.66 (–0.14 to 2.33)[8]
RAR	2.44 (0.87–4.20)[5]	36 (0–38)[5]	0.11 (0.00–0.35)[4]	643 (0–2307)[2]	343 (0–1257)[2]	0.07 (–0.20 to 0.33)[7]
TRD	0.17 (0.02–0.73)[9]	0.5 (0–3)[9]	0.003 (0.00–0.022)[7]	3 (0–16)[8]	0[7]	0.02 (–0.35 to 0.02)[5]
TRR	0.02 (0.00–0.04)[10]	0[10]	0.002 (0.00–0.015)[8]	0.6 (0–4)[9]	0[7]	–0.01 (–0.20 to 0.12)[4]
WXF	4.93 (3.03–6.87)[2]	425 (0–1636)[1]	1.45 (0.00–4.88)[1]	3 (0–19)[7]	0[7]	1.63 (0.22 to 5.97)[9]

Note. In the table, average concentrations of total DNA (based on *TBS*-380 PicoGreen assay) and RNA (based on Agilent RNA Pico Chip assay) from each method are compared with amplifiable DNA (*c-Myc*) and RNA (*β2M*) copies, respectively. Also included for comparative purposes are the *β2M* RNA copies estimated from DNase-treated extracts and the average levels of inhibition in each method as quantitated by the qPCR C_T shift of a mammoth DNA standard amplified in a PCR spiked with the FFPE extract. The relative ranks across the parameters as used to evaluate the performance of the methods compared in this study are indicated in superscripted numbers.

with DNase prior to RT than from the undigested ones (see Table 2 and Fig. S1 in the supplementary material for more details).

Total recovered RNA appears to be degraded to short fragment sizes of approximately 200 bp or less (see Figs. S2 and S3 in the supplementary material); however, the total RNA concentration remains correlated with RNA amplifiability ($R^2 = 0.591$, $P < 0.001$). Despite lower amounts of total RNA than DNA, there are more amplifiable RNA *β2M* copies (2.74×10^3) than *c-Myc* DNA copies (2.41×10^2) from samples yielding both (mean values not statistically different from each other, $t = 1.756$, $P > 0.05$). Overall, Ambion's RAD yielded the highest amount of amplifiable RNA copies (7813 [41–28,380]) followed closely by Ambion's RAR method (643 [0–2307]).

PCR inhibition

Our assessment of the levels of PCR inhibition via a qPCR-based inhibition assay [45,47] showed that only 3 of the 10 methods analyzed had detectable levels of PCR inhibition (Figs. 1I and IJ). FFPE samples extracted using the PCE method were the most inhibited with an average C_T shift of 1.96, followed by TrimGen's WXF with an average C_T shift of 1.63 and RAD with an average C_T shift of 0.66 (Table 2).

Effect of incubation time on total nucleic acid yields

Comparison of the nucleic acid recoveries from Ambion's RAR and RAD methods suggested that a longer incubation time resulted in a greater recovery of total DNA ($t = 5.02$, $P = 0.001$) and RNA ($t = 2.34$, $P > 0.05$) and an increase in the number of amplifiable DNA and RNA copies (not statistically significant) (Table 2). However, in addition, a prolonged incubation led to an overall increase in the level of inhibition.

Discussion

Total DNA, amplifiable nuclear DNA copies, and inhibition

Not all 10 methods tested here were originally designed for the extraction of DNA. Despite this, all methods except Ambion's TRR recovered DNA as measured by the *TBS*-380 *PicoGreen assay*. Of these 9 remaining methods, 6 had between 2- and 32-fold more DNA than the other 3 methods. It is important to note that despite detectable DNA within the extracts, this itself was not a guarantee of the successful amplification of single-copy nuclear DNA. In fact, only 4 of the 6 methods yielded more than 100 amplifiable DNA copies (Table 2).

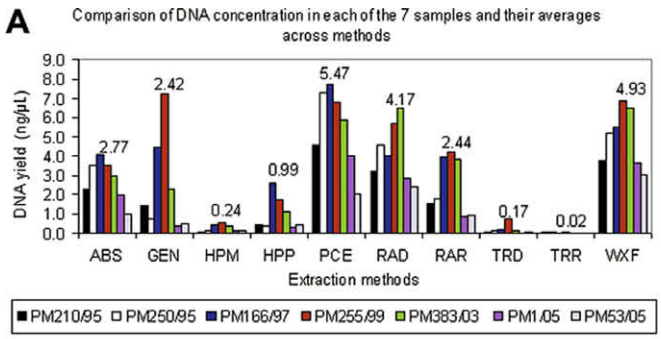
Although the in-house PCE, Ambion's RAD, and TrimGen's WXF yielded the most DNA, these methods also contained the highest amounts of PCR inhibition as judged by the shift in C_T of an internal standard during qPCR. The standard shifted by 1.96, 0.66, and 1.63 cycles for the in-house PCE, Ambion's RAD, and TrimGen's WXF, respectively (Table 2 and Fig. 2). The efficiency of the internal standard assay was quite high at 99.2%. Because we expect a doubling of template molecules for each cycle of PCR with 100% efficient reactions, total DNA copies in these extracts were slightly underestimated. If we correct for the level of inhibition, the in-house PCE, Ambion's RAD, and TrimGen's WXF should contain up to 3.9-, 1.3-, and 3.2-fold more *c-Myc* DNA copies than were measured (Table 1).

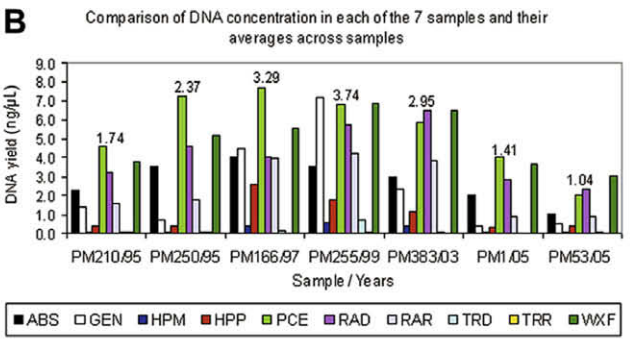
Exploring alternative methods to overcome inhibition was not the focus of this study. Nonetheless, if inhibition could be overcome by using a combination of PCR facilitators [47] or potentially eliminating small nucleic acids, which might account for the inhibition, the three methods resulting in the highest DNA copies (the in-house PCE, Ambion's RAD, and TrimGen's WXF) would theoretically yield 1526, 1091, and 400 *c-Myc* DNA copies in each microliter extract. Based on our experience, small nucleic acid fragments, such as those copurified in ancient DNA, may themselves inhibit PCRs. A positive correlation was observed between total DNA recovered and a C_T shift ($R^2 = 0.464$, $P < 0.001$).

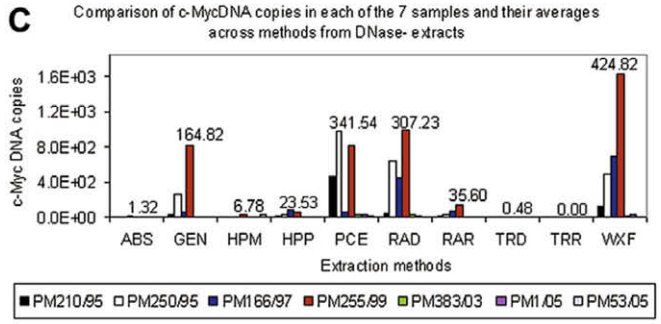
Total RNA and amplifiable nuclear RNA copies

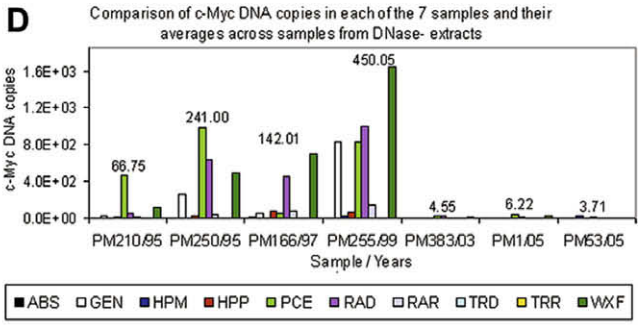
Total RNA recovered from all samples via the 10 extraction methods yielded short RNA fragment sizes of approximately 200 bp or less (Figs. S2 and S3). These results are in agreement with previous work [4,48–50] showing that RNA recovered from such archival tissues is degraded to less than 200 bp in length. Nonetheless, we found a positive correlation between amplifiable DNA and

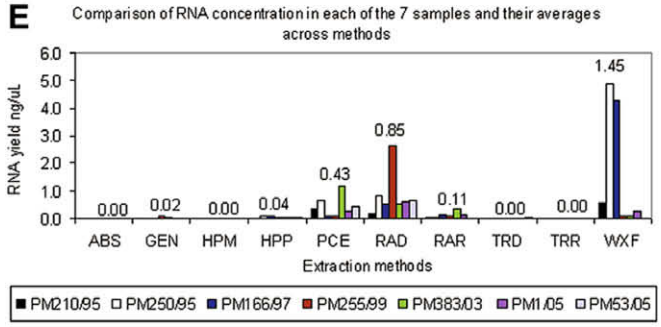
Fig. 1. Total nucleic acids recovered and their corresponding qPCR-based amplifiable genomic copies from FFPE tissues. (A–F) Comparison across samples (ranging from the year 1995 to 2005) and methods of average recovered DNA quantity within each sample and method studied based on *TBS*-380 PicoGreen assays in comparison (A and B), respective *c-Myc* nuclear DNA copies as a measure of amplifiable genomic DNA from each sample and method (C and D), and recovered RNA quantity across each sample and method based on the Agilent Bioanalyzer RNA Pico Chip assay (E and F). (G and H) Amplifiable RNA as measured by *β2M* cDNA copies across methods. (I and J) Estimated levels of inhibition in each sample and across as deduced from the shift in qPCR cycle threshold (C_T) numbers that involved amplifications of a known standard (cloned mammoth mitochondrial cytochrome *b* DNA fragment) spiked with the FFPE nucleic acid extracts. Inset numbers above bars are average estimates across samples and within each method of the different parameters tested.

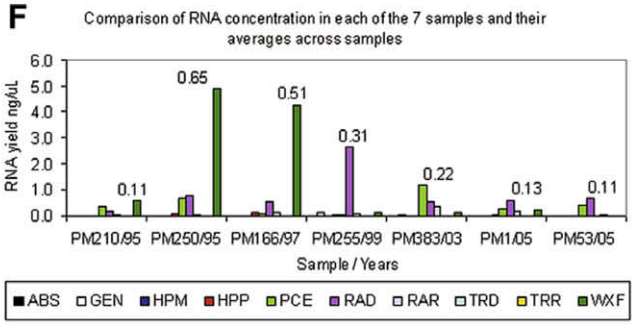

A
Comparison of DNA concentration in each of the 7 samples and their averages across methods
DNA yield (ng/µL)
2.77
2.42
0.24
0.99
5.47
4.17
2.44
0.17
0.02
4.93
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF
Extraction methods
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05

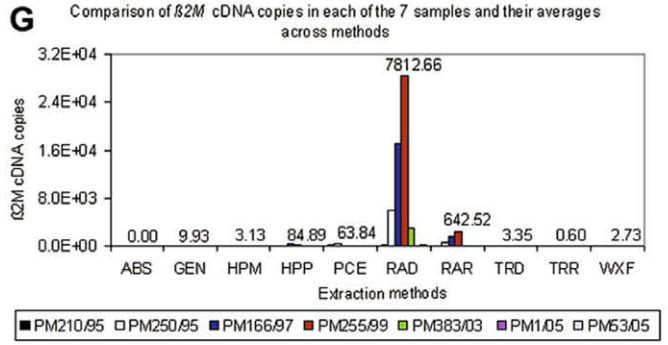

B
Comparison of DNA concentration in each of the 7 samples and their averages across samples
DNA yield (ng/µL)
1.74
2.37
3.29
3.74
2.95
1.41
1.04
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05
Sample / Years
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF

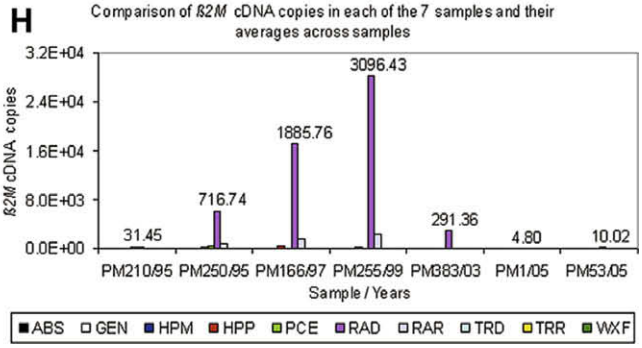

C
Comparison of c-MycDNA copies in each of the 7 samples and their averages across methods from DNase- extracts
c-Myc DNA copies
1.32
164.82
6.78
23.53
341.54
307.23
35.60
0.48
0.00
424.82
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF
Extraction methods
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05

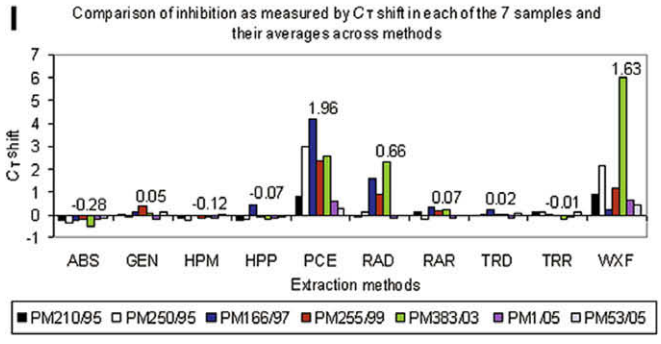

D
Comparison of c-Myc DNA copies in each of the 7 samples and their averages across samples from DNase- extracts
c-Myc DNA copies
66.75
241.00
142.01
450.05
4.55
6.22
3.71
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05
Sample / Years
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF

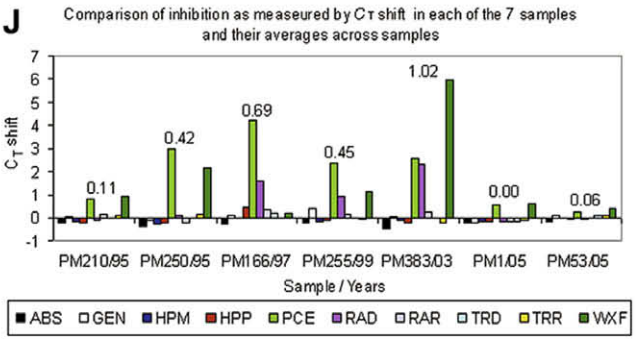

E
Comparison of RNA concentration in each of the 7 samples and their averages across methods
RNA yield ng/uL
0.00
0.02
0.00
0.04
0.43
0.85
0.11
0.00
0.00
1.45
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF
Extraction methods
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05


F
Comparison of RNA concentration in each of the 7 samples and their averages across samples
RNA yield ng/uL
0.11
0.65
0.51
0.31
0.22
0.13
0.11
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05
Sample / Years
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF


G
Comparison of ß2M cDNA copies in each of the 7 samples and their averages across methods
ß2M cDNA copies
0.00
9.93
3.13
84.89
63.84
7812.66
642.52
3.35
0.60
2.73
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF
Extraction methods
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05


H
Comparison of ß2M cDNA copies in each of the 7 samples and their averages across samples
ß2M cDNA copies
31.45
716.74
1885.76
3096.43
291.36
4.80
10.02
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05
Sample / Years
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF


I
Comparison of inhibition as measured by Cτ shift in each of the 7 samples and their averages across methods
Cτ shift
-0.28
0.05
-0.12
-0.07
1.96
0.66
0.07
0.02
-0.01
1.63
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF
Extraction methods
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05


J
Comparison of inhibition as measeured by Cτ shift in each of the 7 samples and their averages across samples
Cτ shift
0.11
0.42
0.69
0.45
1.02
0.00
0.06
PM210/95 PM250/95 PM166/97 PM255/99 PM383/03 PM1/05 PM53/05
Sample / Years
ABS GEN HPM HPP PCE RAD RAR TRD TRR WXF

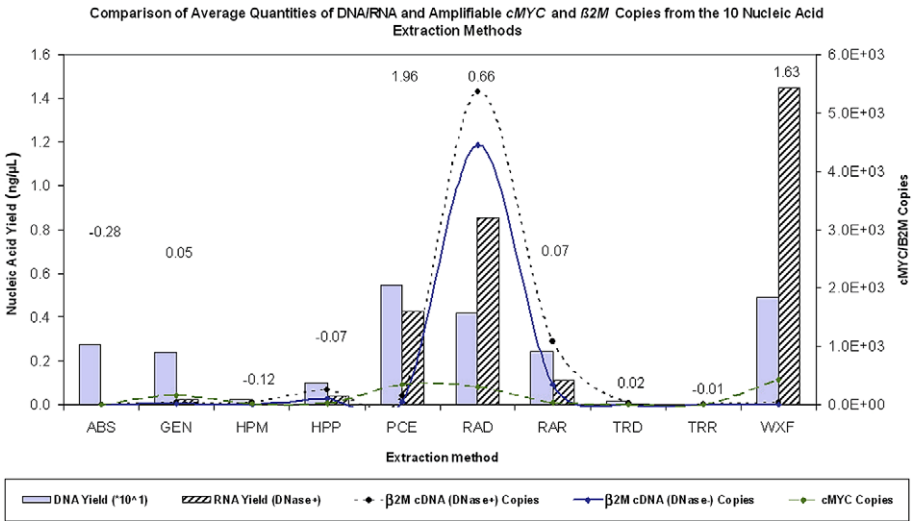


Fig. 2. Summary of average nucleic acid recovery (DNA/RNA concentrations), amplifiable genomic DNA (*c-Myc*). and RNA (*β2M*) copies from the nucleic acid extraction methods tested. Grayish bars indicate the estimated total DNA quantity as measured using PicoGreen assay. Forward-slashed bars represent the respective average quantities of RNA in DNase-treated (DNase+) extracts estimated based on RiboGreen assays. The solid and short-dashed lines show qPCR quantitated *β2M* copies from DNase+ and straight (DNase−) extracts, respectively, whereas the long-dashed line shows the trend in the average amplified copies of *c-Myc* DNA in the extracts. Inset numbers are the average inhibition levels as measured by C_T shift based on spiked amplifications of mammoth mitochondrial DNA.

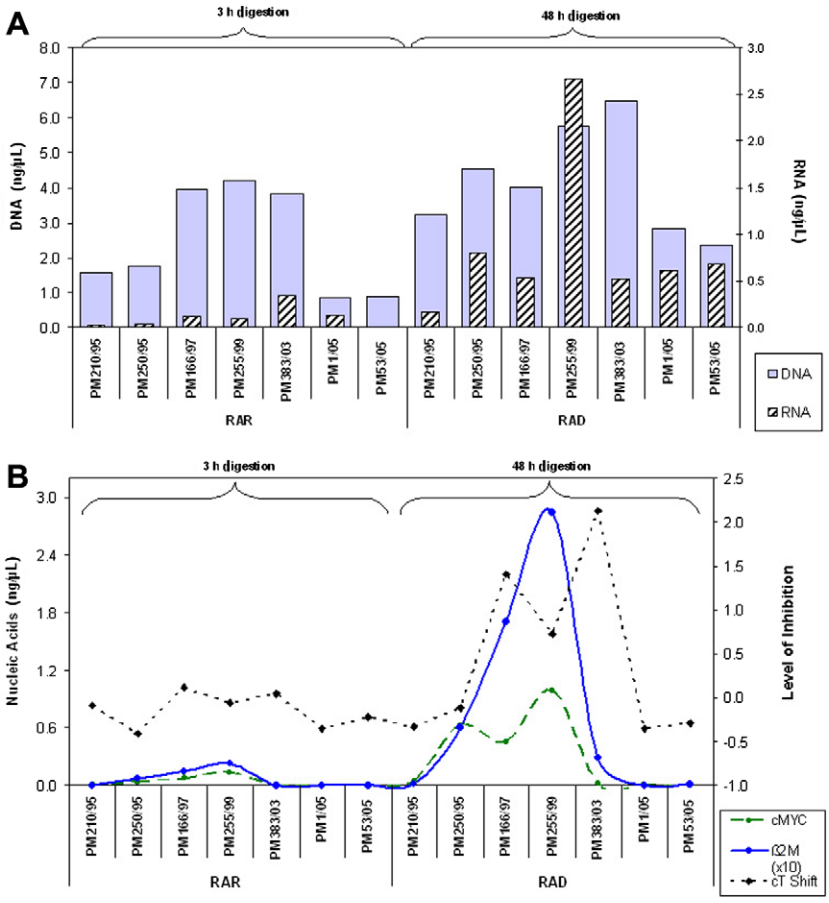


Fig. 3. DNA and RNA recovered from RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues as tested following the RNA extraction protocol (RAR, 3 h digestion time) and DNA protocol (RAD, 48 h digestion time) in all seven FFPE tissues studied. (A) Comparison of the DNA concentrations (in ng/µl) based on PicoGreen assay (grayish bars) with the corresponding RNA quantities based on Agilent Bioanalyzer RNA Pico Chip assay (superimposed forward-slashed bars). (B) Comparison of amplifiable genomic DNA inferred from *c-Myc* gene copies (blue solid line) with *β2M* cDNA copies (green solid line). The respective estimates of inhibition levels (C_T shift) in each sample are represented by the black dashed line. Although the plots show average increase in amplifiable nucleic acids (based on both *c-Myc* and *β2M* copies) with the longer incubation time of 48 h, only nucleic acid concentrations were significantly greater with longer digestion (DNA, $P < 0.05$, and RNA, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

RNA copies across all methods ($R^2 = 0.302$, $P < 0.01$). We assume that the apparent anomaly that a greater number of RNA copies are amplified in comparison with single-copy nuclear DNA copies is simply due to the ubiquitous expression of *β2M* as opposed to the single-copy *c-Myc* gene. The observation that twice as much amplifiable RNA was present in extracts treated with DNase prior to RT (Table 2 and Fig. S1) suggests that DNase treatment allows more specific binding of the reverse transcriptase to the RNA template given that potential DNA–RNA hybrids that might interfere with RT are reduced by the DNase digestion step [51]. The most preferred method for total RNA amplifiability was Ambion's RAD (Table 2 and Fig. 2), which produced an order of magnitude more copies than Ambion's RAR, the second best method, followed by the in-house PCE and Roche's HPP (see details across methods and samples in Figs. 1G and 1H).

Nucleic acid preservation and age

It is generally assumed that older samples contain quantitatively fewer and more damaged nucleic acids [50,52]. However, in the field of ancient DNA, this has been shown not to always be the case; rather, it is correlated with the conditions of preservation such as temperature, humidity, and pH, to mention but a few [42]. The surprising observation that older samples, stemming from the years 1995, 1997, and 1999, yield relatively higher copy numbers of both *c-Myc* DNA (Fig. 1D) and *β2M* RNA (Fig. 1H) than the relatively recent ones (2003 and 2005) suggests that the ability to amplify DNA and RNA might not necessarily diminish with aging of poorly stored FFPE autopsy tissue samples. This contradictory observation has been demonstrated in other studies [10,49,53,54], showing that the long-term storage of FFPE samples appears to have no significant negative effect on downstream applications such as PCR. There are many possible explanations for this finding, including the rate of tissue fixation and embedment [54], which may affect the long-term preservation of formalin-fixed DNA and RNA; however, without further detailed studies, it is impossible to comment specifically on this observation.

Effect of incubation time

Ambion's two methods, RAR and RAD, had 3- and 48-h digestion times, respectively. There is an average increase in both recoverable and amplifiable nucleic acids quantities with the longer digestion of FFPE tissues (Table 2 and Figs. 2 and 3). This observation corroborates the fact that the formalin-induced protein–nucleic acid cross-linkages are reversible by thermal energy [24] as well as extended chemical digestion [11]. The increased nucleic acid recovery resulting from a longer incubation time did, however, lead to a slight increase in PCR inhibition (Fig. 3).

Which paraffin method worked best for autopsy tissues?

In assessing which of the 10 tested paraffin methods works best for a range of autopsy tissue samples, we ranked them according to those recovering both the highest concentrations of total nucleic acids as well as the highest amounts of amplifiable quantities of both DNA and RNA while taking inhibition into account. Based on these general criteria, we found that Ambion's RAD, the in-house PCE, TrimGen's WXF, and Ambion's RAR methods performed superior to the rest of the methods compared in this study (Table 2). Although the WXF kit was designed for RNA only, elimination of the DNase treatment step during extraction enabled this method to yield high amounts of both DNA and RNA. Despite the high nucleic acid recovery from the 4 methods listed above, we were able to amplify just a few RNA copies (148 and 3) from 2 of the methods (the in-house PCE and TrimGen's WXF, respectively). Thus, while these methods recovered good total nucleic acids, they do not guarantee RNA amplification success. Ambion's RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (RAD, the 48-h digestion protocol for DNA) performed best in terms of total amplifiable RNA recovery, yielding an average of 7813 copies, followed by its 3-h digestion version (RAR) at 643 copies/μl extract (see Figs. 2 and 3 for details).

Conclusion

The recovery and amplification of nucleic acids from archived formalin-fixed autopsy human tissues is a growing field in retrospective genetic studies. Scientists are faced with the problem of choosing methods that not only are able to recover high amounts of nucleic acids but also yield amplifiable copies. In this study, we have provided a careful test comparison of 10 major FFPE extraction methods on seven randomly collected archival human pathological tissues. Whereas we found that TrimGen's WXF, the in-house PCE, and Ambion's RAD are the preferred methods for the recovery of amplifiable DNA copies, Ambion's RAD and RAR are the preferred methods for the recovery of amplifiable RNA copies for such ancient human pathological tissue collections. These results should serve as a guide to molecular pathologists interested in using these valuable tissues for retrospective epidemiological studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.01.014.

References

[1] M. Worobey, M. Gemmel, D.E. Teuwen, T. Haselkorn, K. Kunstman, M. Bunce, J.-J. Muyembe, M.J.-M. Kabongo, R.M. Kalengayi, E. Van Marck, M.T.P. Gilbert, S.M. Wolinsky, Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960, Nature 455 (2008) 661–664.
[2] J.K. Taubenberger, A.H. Reid, A.E. Krafft, K.E. Bijwaard, T.G. Fanning, Initial genetic characterization of the 1918 "Spanish" influenza virus, Science 275 (1997) 1793–1796.
[3] M. Abramovitz, M. Ordanic-Kodani, Y. Wang, Z. Li, C. Catzavelos, M. Bouzyk, G.W. Sledge Jr., C.S. Moreno, B. Leyland-Jones, Optimization of RNA extraction from FFPE tissues for expression profiling in the DASL assay, BioTechniques 44 (2008) 417–423.
[4] U. Lehmann, H. Kreipe, Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies, Methods 25 (2001) 409–418.
[5] H. Puchtler, S.N. Meloan, On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions, Histochem. Cell Biol. 82 (1985) 201–204.
[6] M. Srinivasan, D. Sedmak, S. Jewell, Effect of fixatives and tissue processing on the content and integrity of nucleic acids, Am. J. Pathol. 161 (2002) 1961–1971.
[7] R. Romero, A. Juston, J. Ballantyne, B. Henry, The applicability of formalin-fixed and formalin-fixed paraffin-embedded tissues in forensic DNA analysis, J. Forensic Sci. 42 (1997) 708–714.
[8] A. Okwi, W. Byarugaba, The prevalence of Karposi's sarcoma (KS) in the Uganda Biopsy Service before and during the AIDS era, 1965–1995, Uganda Med. J. 17 (2000) 5–10.
[9] P. Dedhia, S. Tarale, G. Dhongde, R. Khadapkar, B. Das, Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues, Asian Pac. J. Cancer Prev. 8 (2007) 55–59.

[10] M. Doleshal, A.A. Magotra, B. Choudhury, B.D. Cannon, E. Labourier, A.E. Szafranska, Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues, J. Mol. Diagn. 10 (2008) 203–211.
[11] M.T.P. Gilbert, T. Haselkorn, M. Bunce, J.J. Sanchez, S.B. Lucas, L.D. Jewell, E.V. Marck, M. Worobey, The isolation of nucleic acids from fixed, paraffin-embedded tissues: which methods are useful when?, PLoS ONE 2 (2007) e537.
[12] S.E. Goelz, S.R. Hamilton, B. Vogelstein, Purification of DNA from formaldehyde fixed and paraffin embedded human tissue, Biochem. Biophys. Res. Commun. 130 (1985) 118–126.
[13] G. Rupp, J. Locker, Purification and analysis of RNA from paraffin-embedded tissues, BioTechniques 6 (1988) 56–60.
[14] Y. Sato, R. Sugie, B. Tsuchiya, T. Kameya, M. Natori, K. Mukai, Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues, Diagn. Mol. Pathol. 10 (2001) 265–271.
[15] S.-R. Shi, R.J. Cote, L. Wu, C. Liu, R. Datar, Y. Shi, D. Liu, H. Lim, C.R. Taylor, DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: Heating under the influence of pH, J. Histochem. Cytochem. 50 (2002) 1005–1011.
[16] S.-R. Shi, R. Datar, C. Liu, L. Wu, Z. Zhang, R.J. Cote, C.R. Taylor, DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution, Histochem. Cell Biol. 122 (2004) 211–218.
[17] G. Stanta, S. Bonin, R. Perin, in: R. Rapley, D.L. Manning (Eds.), RNA Isolation and Characterization Protocols (Methods in Molecular Biology), Humana, Totowa, NJ, 1998, pp. 23–26.
[18] L. Wu, N. Patten, C. Yamashiro, B. Chui, Extraction and amplification of DNA from formalin-fixed, paraffin-embedded tissues, Appl. Immunohistochem. Mol. Morph. 10 (2002) 269–274.
[19] V.K. Rait, Q. Zhang, D. Fabris, J.T. Mason, T.J. O'Leary, Conversions of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration, J. Histochem. Cytochem. 54 (2006) 301–310.
[20] F. Castiglione, D. Degl'Innocenti, A. Taddei, F. Garbini, A. Buccoliero, M. Raspollini, M. Pepi, M. Paglierani, G. Asirelli, G. Freschi, P. Bechi, G. Taddei, Real-time PCR analysis of RNA extracted from formalin-fixed and paraffin-embedded tissues: Effects of the fixation on outcome reliability, Appl. Immunohistochem. Mol. Morph. 15 (2006) 338–342.
[21] Y.F. Chaw, L.E. Crane, P. Lange, R. Shapiro, Isolation and identification of cross-links from formaldehyde-treated nucleic acids, Biochemistry 19 (1980) 5525–5531.
[22] M. Feldman, Reactions of nucleic acids and nucleoproteins with formaldehyde, Prog. Nucleic Acid Res. Mol. Biol. 13 (1973) 1–49.
[23] C. Williams, F. Ponten, C. Moberg, P. Soderkvist, M. Uhlen, J. Ponten, G. Sitbon, J. Lundeberg, A high frequency of sequence alterations is due to formalin fixation of archival specimens, Am. J. Pathol. 155 (1999) 1467–1471.
[24] J. Finke, R. Fritzen, P. Ternes, W. Lange, G. Dolken, An improved strategy and a useful housekeeping gene for RNA analysis from formalin-fixed, paraffin-embedded tissues by PCR, BioTechniques 143 (1993) 448–453.
[25] M. Solomon, A. Varshavsky, Formaldehyde-mediated DNA–protein cross-linking: a probe for in vivo chromatin structures, Proc. Natl. Acad. Sci. USA 82 (1985) 6470–6474.
[26] N. Masuda, T. Ohnishi, S. Kawamoto, M. Monden, K. Okubo, Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples, Nucleic Acids Res. 27 (1999) 4436–4443.
[27] S.A. Johnson, D.G. Morgan, C.E. Finch, Extensive postmortem stability of RNA from rat and human brain, J. Neurosci. Res. 16 (1986) 267–280.
[28] J. Lee, A. Hever, D. Willhite, A. Zlotnik, P. Hevezi, Effects of RNA degradation on gene expression analysis of human postmortem tissues, FASEB J. 19 (2005) 1356–1358.
[29] C.L. Wickham, M. Boyce, M.V. Joyner, P. Sarsfield, B.S. Wilkins, D.B. Jones, S. Ellard, Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies, Mol. Pathol. 53 (2000) 19–23.
[30] R. Coura, J.C. Prolla, L. Meurer, P. Ashton-Prolla, An alternative protocol for DNA extraction from formalin fixed and paraffin wax embedded tissue, J. Clin. Pathol. 58 (2005) 894–895.
[31] N.J. Coombs, A.C. Gough, J.N. Primrose, Optimisation of DNA and RNA extraction from archival formalin-fixed tissue, Nucleic Acids Res. 27 (1999) e12.
[32] J. Chen, G.E. Byrne, I.S. Lossos, Optimization of RNA extraction from formalin-fixed, paraffin-embedded lymphoid tissues, Diagn. Mol. Pathol. 16 (2007) 61–72.
[33] A. Shedlock, M. Haygood, T. Pietsch, P. Bentzen, Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens, BioTechniques 22 (1997) 394–400.
[34] S. Banerjee, W. Makdisi, A. Weston, S. Mitchell, D. Campbell, Optimisation of DNA and RNA extraction from archival formalin-fixed tissue, BioTechniques 18 (1995) 768–773.
[35] F. Lewis, N.J. Maughan, V. Smith, K. Hillan, P. Quirke, Unlocking the archive–gene expression in paraffin-embedded tissue, J. Pathol. 195 (2001) 66–71.
[36] M.R. Emmert-Buck, R.L. Strausberg, D.B. Krizman, M.F. Bonaldo, R.F. Bonner, D.G. Bostwick, M.R. Brown, K.H. Buetow, R.F. Chuaqui, K.A. Cole, P.H. Duray, C.R. Englert, J.W. Gillespie, S. Greenhut, L. Grouse, L.W. Hillier, K.S. Katz, R.D. Klausner, V. Kuznetzov, A.E. Lash, G. Lennon, W.M. Linehan, L.A. Liotta, M.A. Marra, P.J. Munson, D.K. Ornstein, V.V. Prabhu, C. Prange, G.D. Schuler, M.B. Soares, C.M. Tolstoshev, C.D. Vocke, R.H. Waterston, Molecular profiling of clinical tissue specimens: feasibility and applications, Am. J. Pathol. 156 (2000) 1109–1115.
[37] G. Stanta, C. Schneider, RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification, BioTechniques 11 (1991) 304–308.
[38] M. Gilbert, J. Sanchez, T. Haselkorn, L. Jewell, S. Lucas, E. Van Marck, C. Børsting, N. Morling, M. Worobey, Multiplex PCR with minisequencing as an effective high-throughput SNP typing method for formalin-fixed tissue, Electrophoresis 28 (2007) 2361–2367.
[39] J. Benavides, C. García-Pariente, D. Gelmetti, M. Fuertes, M.C. Ferreras, J.F. García-Marín, V. Pérez, Effects of fixative type and fixation time on the detection of Maedi Visna virus by PCR and immunohistochemistry in paraffin-embedded ovine lung samples, J. Virol. Methods 137 (2006) 317–324.
[40] F. Miething, S. Hering, B. Hanschke, J. Dressler, Effect of fixation to the degradation of nuclear and mitochondrial DNA in different tissues, J. Histochem. Cytochem. 54 (2006) 371–374.
[41] S. Bonin, F. Petrera, B. Niccolini, G. Stanta, P. CR, PCR analysis in archival postmortem tissues, Mol. Pathol. 56 (2003) 184–186.
[42] M. Hofreiter, D. Serre, H.N. Poinar, M. Kuch, S. Paabo, Ancient DNA, Nat. Rev. Genet. 2 (2001) 353–359.
[43] S. Smith, L. Vigilant, P.A. Morin, The effects of sequence length and oligonucleotide mismatches on 5' exonuclease assay efficiency, Nucleic Acids Res. 30 (2002) e111.
[44] S.A. Bustin, T. Nolan, Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction, J. Biomol. Tech. 15 (2004) 155–166.
[45] C. Schwarz, R. Debruyne, M. Kuch, E. McNally, H. Schwarcz, A.D. Aubrey, J. Bada, H. Poinar, New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains, Nucleic Acids Res. 37 (2009) 3215–3229.
[46] H.N. Poinar, C. Schwarz, J. Qi, B. Shapiro, R.D.E. MacPhee, B. Buigues, A. Tikhonov, D.H. Huson, L.P. Tomsho, A. Auch, M. Rampp, W. Miller, S.C. Schuster, Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA, Science 311 (2006) 392–394.
[47] C. King, M. Kuch, R. Debryune, H. Poinar, A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts, BioTechniques 47 (2009) 941–949.
[48] T.E. Godfrey, S.H. Kim, M. Chavira, D.W. Ruff, R.S. Warren, J.W. Gray, R.H. Jensen, Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription–polymerase chain reaction, J. Mol. Diagn. 2 (2000) 84–91.
[49] S. von Ahlfen, A. Missel, K. Bendrat, M. Schlumpberger, Determinants of RNA quality from FFPE samples, PLoS ONE 2 (2007) e1261.
[50] M. Cronin, M. Pho, D. Dutta, J.C. Stephans, S. Shak, M.C. Kiefer, J.M. Esteban, J.B. Baker, Measurement of gene expression in archival paraffin-embedded tissues: Development and performance of a 92-gene reverse transcriptase–polymerase chain reaction assay, Am. J. Pathol. 164 (2004) 35–42.
[51] D.H. Sutton, G.L. Conn, T. Brown, A.N. Lane, The dependence of DNase I activity on the conformation of oligodeoxynucleotides, Biochem. J. 321 (1997) 481–486.
[52] D. Bresters, M.E.I. Schipper, H.W. Reesink, B.D.M. Boeser-Nunnink, H.T.M. Cuypers, The duration of fixation influences the yield of HCV cDNA–PCR products from formalin-fixed, paraffin-embedded liver tissue, J. Virol. Methods 48 (1994) 267–272.
[53] M.R. Schweiger, M. Kerick, B. Timmermann, M.W. Albrecht, T. Borodina, D. Parkhomchuk, K. Zatloukal, H. Lehrach, Genome-wide massively parallel sequencing of formaldehyde fixed-paraffin embedded (FFPE) tumor tissues for copy-number- and mutation-analysis, PLoS ONE 4 (2009) e5548.
[54] R.A. Coudry, S.I. Meireles, R. Stoyanova, H.S. Cooper, A. Carpino, X. Wang, P.F. Engstrom, M.L. Clapper, Successful application of microarray technology to microdissected formalin-fixed, paraffin-embedded tissue, J. Mol. Diagn. 9 (2007) 70–79.