Experimental and Molecular Pathology 110 (2019) 104289

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Optimization of RNA extraction protocol for long-term archived formalin-fixed paraffin-embedded tissues of horses

Gisele Silva Boos[a,*], Daniel Nobach[a], Klaus Failing[b], Markus Eickmann[c], Christiane Herden[a,d]

[a] Institute of Veterinary Pathology, Justus-Liebig-University, Gießen 35392, Germany
[b] Unit for Biomathematics and Data Processing, Justus-Liebig-Universität, Gießen 35392, Germany
[c] Institute of Virology, Philipps-University, Marburg 35043, Germany
[d] Center of Mind, Brain and Behavior, Justus-Liebig-University Gießen, Gießen, Germany

ARTICLE INFO

Keywords:
RNA extraction
Proteinase K
Formalin fixation
Paraffin embedding
Molecular analysis

ABSTRACT

A suitable RNA extraction protocol was established to gain high quality RNA from formalin-fixed paraffin-embedded tissues to perform reliable molecular assays either applicable for using FFPE tissue archives or tissues with harsh formalin-fixation. Eighteen FFPE samples from the central nervous system of horses, stored up to 11 years, were used as archive cases. To test the influence of the fixation period, brain, liver, kidney, and skeletal muscle tissue fragments from another horse, were treated either with water or tris-acetate-EDTA buffer after fixation under different timepoints with 10% unbuffered formalin. Two deparaffinization methods and three proteinase K-based lysis step were tested and translated into three protocols. After detailed statistical analysis it was determined that a longer period and increase in volume of proteinase K incubation provide higher yields and purity of RNA ($P < 0.01$) of archived samples. Alongside, amplification of equid-housekeeping gene up to 298 bp was successful with the protocol adaptations. For different formalin-fixation timepoints, it was demonstrated that the right choice for treatment and formalin-fixation period is organ-related ($P \leq 0.05$). Essentially, little alterations to pre-existing extraction protocols unwound the RNA of up to 11-year-old samples, enabling the use of FFPE tissue archives or e.g. harshly fixed material needed in infection research under high biosafety levels for a variety of molecular analysis.

1. Introduction

Routine fixation in 10% formalin from biopsies and autopsies is still the most common type of sample archive in pathology facilities. Its usefulness is especially appealing to pilot and retrospective studies for the understanding of biomarkers, pathogen evolution and emergence, allied to lower risks of contamination (Abed and Dark, 2016; Gruber et al., 1993; Taubenberger et al., 1997; Worobey et al., 2008). Moreover, in animal experiments using pathogens of high biosafety levels harsh formalin fixation and paraffin embedding is needed for destruction of infectivity before histological evaluation and enable long storage of a panel of tissues (Thacker and Waters, 2017). In the recent past, interest in using the vast archives of formalin-fixed paraffin-embedded (FFPE) tissue has increased due to the development of molecular techniques for research and diagnostic purposes (Iwamoto et al., 1996; Lewis et al., 2001; Magdeldin and Yamamoto, 2012). However, FFPE material yield higher ranges of low quality nucleic acids, as fixation and embedding further interfere with molecular, genetic, and genomic analysis, through protein cross-linking and RNA fragmentation (Avraham et al., 2016; Mubemba et al., 2017; Nolan and Bustin, 2008). Hence, reports have increased detailing the development and improvement of techniques that efficiently recover RNA and DNA from said material (Dijkstra et al., 2012; Doleshal et al., 2008; Huijsmans et al., 2010; Okello et al., 2010; Sengüven et al., 2014) or adding steps to improve the quality of the FFPE material for a variety of analyses (Ciotti et al., 2009; Li et al., 2008; Vermeulen et al., 2009), for example by adding a heat-treatment after fixation (Evers et al., 2011).

Therefore, the aim of this study was to establish an easily applicable and reliable protocol to isolate RNA of appropriate quality from long term storage or very stringent FFPE tissue such as gained from experiments with highly pathogenic agents and from tissues submitted to different formalin fixation periods that allow all current molecular and

Abbreviations: cDNA, complimentary DNA; CNS, central nervous system; FFPE, formalin-fixed paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, room temperature; TAE, tris-acetate-EDTA buffer

* Corresponding author at: Institute of Veterinary-Pathology, Justus-Liebig-University, Gießen 35392, Germany.
E-mail address: gisele.s.boos@vetmed.uni-giessen.de (G.S. Boos).

https://doi.org/10.1016/j.yexmp.2019.104289
Received 29 March 2019; Received in revised form 24 May 2019; Accepted 19 July 2019
Available online 23 July 2019

Table 1
Formalin-fixation and paraffin-embedding conditions from the archived tissues available from the CNS of 18 horses from Brazil.

ID	Geographic region	Formalin fixation		Paraffin embedding	Tissue available
		Period	Type	Year	
1	Midwest	up to 48 h	10% buffered	2008	Spinal cord
2	Midwest	up to 48 h	10% buffered	2008	Spinal cord
3	Midwest	up to 48 h	10% buffered	2009	Cerebral cortex, cerebellum
4	Midwest	up to 48 h	10% buffered	2009	Cerebral cortex, cerebellum
5	Midwest	up to 48 h	10% buffered	2009	Spinal cord, cerebral cortex, trigeminal ganglion
6	South	up to 72 h	10% unbuffered	2013	Spinal cord, trigeminal ganglion
7	South	up to 72 h	10% unbuffered	2013	Spinal cord, cerebral cortex, cerebellum
8	South	up to 72 h	10% unbuffered	2013	Spinal cord, cerebral cortex, cerebellum
9	South	up to 72 h	10% unbuffered	2013	Spinal cord, trigeminal ganglion, obex, striatum
10	South	up to 72 h	10% unbuffered	2014	Spinal cord, mesencephalon
11	South	up to 72 h	10% unbuffered	2014	Spinal cord, cerebral cortex
12	South	up to 72 h	10% unbuffered	2014	Spinal cord, cerebral cortex, bulb
13	South	up to 72 h	10% unbuffered	2014	Spinal cord, regional ganglion
14	Midwest	NA	NA	2007	Spinal cord
15	Midwest	NA	NA	2011	Spinal cord, hippocampus, third ventricle (choroid plexus)
16	North	NA	10% formalin	2009	Spinal cord, cerebral cortex, cerebellum, obex
17	North	NA	10% formalin	2009	Hippocampus, cerebellum, mesencephalon, lateral ventricle
18	Northeast	NA	10% buffered	2009	Mesencephalon

NA: information not available.

genomic analyses.

2. Materials and methods

2.1. Archive samples

FFPE tissue derived from the central nervous system (CNS) of 18 horses were obtained from different locations in Brazil. These samples consisted of unresolved cases of nonsuppurative encephalitis and encephalopathies, maintained archived at room temperature (RT) and protected from light over the years (Table 1).

2.2. Archive tissue preparation

Two approaches were carried out to obtain the desired tissue sections (Table 2). (I) For the first one, pools with CNS fragments containing 10 sections with 8 μm thickness from each case, obtained by trimming the paraffin block with microtome, were placed at SuperFrost® Plus slides (R. Langenbrinck GmbH, Emmendingen, Germany). Afterwards, they were dried at 60 °C for 30 min in a heat-incubator and placed in glass cuvettes. Deparaffinization and tissue rehydration was performed in a two-row immersion in xylol for 30 min each, followed by immersion in descendent ethanol row of 96%, 70%, and 50% for 2 min each, finalized by an immersion of 2 min in RNase-free water. Tissue was then left to dry under a flow-hood. Finally, the clean tissue was scratched off the slides with sterile scalpel, which was changed with every sample, and placed in a sterile 2 mL collection tube.

(II) The second approach consisted of trimming 10 sections from the CNS fragments available from each case with 8 μm thickness, placing them directly into 2 mL collection tubes. Deparaffinization and rehydration was performed by pipetting 1.8 mL xylol into each tube and incubating at 37 °C for 20 min. Right afterwards, the tubes were centrifuged at 14,000 rpm for 2 min and the supernatant discarded. Subsequently, 1.8 mL absolute ethanol was pipetted, centrifuged at 14,000 rpm for 2 min, and supernatant discarded. This step was performed twice, before letting the tubes dry under a flow-hood.

Regardless of the method employed, every step was performed while wearing disposable gloves, and Molecular BioProducts™ RNase™ AWAY (Fisher Scientific GmbH, Schwerte, Germany) used as surface decontaminant.

2.3. RNA extraction

The protocols used for RNA extraction were adapted from the RNeasy FFPE kit (Qiagen GmbH, Hilden, Germany) and applied to the 18 CNS samples. To establish the maximum yield and purity of RNA, the tissue lysis' step was modified into three variations (A, B, C). (A) In the first, 150 μL PKD buffer was added to 10 μL proteinase K with concentration of 20 mg/mL (> 600 mAU/mL, solution, Brand) and incubated in a thermomixer at 56 °C for 15 min with tissues obtained with preparation (I). (B) In the second approach, 240 μL PKD buffer and 60 μL proteinase K concentration of 20 mg/mL (> 600 mAU/mL, solution, Brand) were incubated in a thermomixer at 56 °C with the tissues obtained from (II) for three overnights (72 h), adding 60 μL proteinase K (20 mg/mL) every 24 h. (C) In the third approach, 240 μL PKD buffer and 60 μL proteinase K 20 mg/mL (> 600 mAU/mL, solution,

Table 2
Tissue preparation and lysis methods tested for each RNA extraction protocol.

Prot.	Tissue preparation	Deparaffinization	Lysis	Lysis time and temperature
A	(I) On-slide	2× xylol for 30 min, 3× ethanol (96%, 70%, 50%) for 2 min, RNase-free water for 2 min	150 μL buffer PKD + 10 μL proteinase K	15 min at 56 °C
B	(II) Direct on collection tube	Xylol 37 °C for 20 min, xylol at RT, 100% ethanol at RT,	240 μL buffer PKD + 60 μL proteinase K	3× overnights at 56 °C (72 h)
C	(II) Direct on collection tube	Xylol 37 °C for 20 min, xylol at RT, 100% ethanol at RT	240 μL buffer PKD + 60 μL proteinase K	Overnight at 56 °C (24 h)

Prot.: protocol.

Table 3
Tissue preparation for different formalin fixation timepoints.

Tissue fragment	Aliquot	Additional treatment (after formalin fixation before paraffin embedding)	Fixation time in 10% unbuffered formalin			
Brain	Aliquot 1 to 4	H_2O, RT	24 h	7 days	14 days	21 days
	Aliquot 5 to 8	TAE buffer, 70 °C	24 h	7 days	14 days	21 days
	Aliquot 9*	H_2O, RT	†	†	†	†
	Aliquot 10*	TAE buffer, 70 °C	†	†	†	†
	Aliquot 11*	Frozen for 21 days at −80 °C	†	†	†	†
Liver	Aliquot 1 to 4	H_2O, RT	24 h	7 days	14 days	21 days
	Aliquot 5 to 8	TAE buffer, 70 °C	24 h	7 days	14 days	21 days
	Aliquot 9*	H_2O, RT	†	†	†	†
	Aliquot 10*	TAE buffer, 70 °C	†	†	†	†
	Aliquot 11*	Frozen for 21 days at −80 °C	†	†	†	†
Kidney	Aliquot 1 to 4	H_2O, RT	24 h	7 days	14 days	21 days
	Aliquot 5 to 8	TAE buffer, 70 °C	24 h	7 days	14 days	21 days
	Aliquot 9*	H_2O, RT	†	†	†	†
	Aliquot 10*	TAE buffer, 70 °C	†	†	†	†
	Aliquot 11*	Frozen for 21 days at −80 °C	†	†	†	†
Skeletal muscle	Aliquot 1 to 4	H_2O, RT	24 h	7 days	14 days	21 days
	Aliquot 5 to 8	TAE buffer, 70 °C	24 h	7 days	14 days	21 days
	Aliquot 9*	H_2O, RT	†	†	†	†
	Aliquot 10*	TAE buffer, 70 °C	†	†	†	†
	Aliquot 11*	Frozen for 21 days at −80 °C	†	†	†	†

† no formalin fixation required.
*naïve controls.

Brand) were incubated in a thermomixer at 56 °C with the tissues obtained from (II) for one night (24 h) (Table 2).

RNA extraction was then completed by following the manufacturer's instructions. Measurements concerning RNA yield (ng/μL), purity regarding proteins (260/280 nm) and contaminants (260/230 nm) were obtained by adding 2 μL extracted RNA to NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) socket.

2.4. Samples for comparison of formalin fixation time

Tissue samples of brain, liver, kidney and skeletal muscle for different formalin fixation time were obtained from one fresh equine carcass from leftovers of diagnostic necropsy (Table 3).

2.5. Preparation of samples for formalin-fixation time comparison

Tissue fragments of 1 × 1 × 1 cm^3 were fixed in unbuffered 10% formalin solution for 24 h, 7 days, 14 days and 21 days, respectively. Before further processing, for each time point one aliquot per tissue was kept in H_2O at room temperature while the other aliquot was washed with H_2O to remove the formalin solution and then with tris-acetate-EDTA (TAE) buffer (40 mmol/L Tris/acetate + 1 mmol/L EDTA; 50× stock solution made of 242 g Tris base (FW = 121.14; Merck, Darmstadt, Germany) in 1000 mL deionized water with 57.1 mL glacial acid (Roth, Karlsruhe, Germany) and 100 mL 0.5 M EDTA (Roth, Karlsruhe, Germany) for 30 min at 70 °C pH 7 (Table 3). Tissues were routinely processed for final paraffin embedding by dehydration in ascending alcohol immersions and clearing in xylol, carried out with Tissue-Tek® VIP® 2000 (Sakura, Torrance, USA).

2.6. RNA extraction from samples formalin-fixed in different time points

RNA was isolated from these FFPE material by protocol C. Control RNA was isolated in replica from naïve tissues of the same animal. These naïve tissues were separated in H_2O-treated (Aliquot 9), TAE-treated (Aliquot 10) and − 80 °C-frozen for 21 days (Aliquot 11) aliquots (Table 3), and RNA extraction carried out with Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instruction.

RNA yield and purity regarding proteins and contaminants were measured by NanoDrop 2000™ spectrophotometer (Thermo Scientific, Wilmington, USA).

2.7. Equid-GAPDH PCR

To investigate the RNA accessibility, six equid-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sets were tested ranging from 46 bp to 517 bp (Table 4). GAPDH lengthening 103 bp was carried out with archive samples that eventually produced 64 bp products, but not 170 bp. Oligonucleotide sequences were obtained with the Basic Local Alignment Search Tool (BLAST®) and primers were purchased at Biomers (Ulm, Germany).

Reverse transcription reaction (RT) was performed ahead, following the manufacturer's instructions using oligo-dT and random hexamers from the QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). A final RNA volume of up to 1000 ng in 12 was established for each sample within the 20 μL master mix, and 12 μL RNase-free water used as no-template control (NTC). Cycling was carried out in the Multicycler®PTC 200 (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and cycling conditions were followed according to manufacturer's instructions.

For the GAPDH PCR, a 15 μL master mix was prepared with the Qiagen® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany). Reagents consisted of 7.8 μL from 2× Qiagen Multiplex PCR master mix, 1.3 μL RNase-free water, 5 μL cDNA, 0.78 μL (10 μM) forward primer and 0.78 μL (10 μM) reverse primer. Amplification was carried out at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing temperature according to primer set for 60 s (Table 4), elongation at 72 °C for 90 s, and a single final elongation at 72 °C for 10 min. Additionally, cDNA obtained from fresh equid brain was used as positive control. Meanwhile, cDNA obtained from cat and squirrel brains was established as negative controls for each primer set (Table 4). The PCR was performed in duplicate or triplicate, depending on the amount of FFPE tissue available.

Amplification products were separated in horizontal gel systems, with pattern voltage of 7 V/cm for 1.5 h. Gel concentration was based upon amplified product's length, being 4% gel used for products up to 300 bp long, and 2% gel for products above that. The bands were detected by UV fluoroscopy (Vilber Lourmat, Torcy, France) and visualized at a wavelength of 254 nm. Gel images were acquired with the gel documentation system Kodak 1.0 “Digital Imaging” (Eastmann Kodak Inc., Rochester, USA).

Table 4
Sequences of equid-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers.

Target gene	Sequence 5′-3′	Position*	A.t.	Product	Negative control**
GAPDH eq421 F	TCTGCTGATGCCCCAATGTT	421–440	55 °C	46 bp	Cat
GAPDH eq447 R	ATTTCTCGTGGTTCACGCCC	466–447			
GAPDH eq214 F	TTCCATGGCACAGTCAAGGC	214–233	60 °C	64 bp	Cat
GAPDH eq277 R	AGATGGTGATGGCCTTTCCG	277–258			
GAPDH eq300 F†	CATCAAATGGGGCGATGCTG	300–319	60 °C	103 bp	Cat
GAPDH eq402 R	CCTTTTGGCTCCACCCTTCA	402–383			
GAPDHeq170 F§	ATCCCTGCTTCTACTGGT	667–685	55 °C	170 bp	Squirrel
GAPDHeq170 R	TTCACCACCTTCTTGATCTC	875–855			
GAPDHeq298 F§	TCTTCCAGGAGCGAGATC	275–293	55 °C	298 bp	Squirrel
GAPDHeq298 R	ATGAGTCCCTCCACGATG	609–591			
GAPDHeq517 F§	AAAGGCCATCACCATCTTC	262–280	55 °C	517 bp	Squirrel
GAPDHeq517 R	CACGACTGACACGTTAGG	812–794			

A.t.: annealing temperature. F: forward primer. R: reverse primer.
† GAPDH 103 bp carried out only with samples that amplified the 64 bp product, but failed at 170 bp.
§ Alignments by Kristine Kehr, Institute of Veterinary Pathology, Gießen.
*Reference gene NM001163856.1 for *Equus caballus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.
**Formalin fixation in 10% unbuffered formalin, Institute of Veterinary Pathology, Gießen.

Table 5
Yield and purity rates of RNA obtained from archive samples with three extraction protocols ($n = 18$).

	Protocol A			Protocol B			Protocol C		
	RNA ng/μL	260/280	260/230	RNA ng/μL	260/280	260/230	RNA ng/μL	260/280	260/230
Mean	12.36	1.647	1.23	63.09	1.73	1.13	268.07	1.85	1.80
Std. D	10.91	0.19	0.34	113.48	0.18	0.48	229.54	0.08	0.15
Min	1.30	1.22	0.25	4.40	1.33	0.36	24.60	1.65	1.39
Max	46.20	1.90	1.75	459.90	1.98	1.95	786.00	1.95	2.00

Max: maxima. Min: minima. Std. D: standard deviation.

2.8. Statistical analysis

The statistical evaluations were made using the statistical program package BMDP/Dynamic, Release 8.1 (Dixon, 1993). The creation of graphic illustrations in the context of the results presentation were generated through data transfer to MS Excel format.

To describe the data obtained with NanoDrop measures for RNA yield (ng/μL), purity in relation to proteins (260/280 nm) and contaminants (260/230 nm), mean values ($\bar{x}$), standard deviations (s), minima (min), maxima (max) and sample sizes (n) were calculated and tabulated for quantitative variables with approximately normal distribution, and logarithmic transformation carried out when the distribution was skewed to the right.

To determine which RNA extraction protocol would be more suitable for the archived samples, descriptive computations was followed by a pairwise correlation analysis between the protocols using the program BMDP6D. For the protocol's influence in the statistical significance, a one-factorial analysis of variance with repeated measures (one-way ANOVA) was carried out with the program BMDP2V for the approximately normally distributed variables. If this comparison showed significant differences ($p \leq .05$), the protocols were then compared pairwise using the Student-Newman-Keuls method. Additionally, data description was carried out by presentation in form of the box and whisker plot.

Naïve controls treatment suitability for each organ tested was assessed through one- and two-way ANOVA. While for the tests carried out with different formalin-fixation time and treatments, three-way ANOVA was performed (program BMDP2V) to assess RNA yield and purity obtained with each organ fragments.

3. Results

3.1. Archive samples

A variety of fragments referring from the CNS of 18 horses from Brazil were acquired for this study. Fixation and embedding year ranged from 2007 to 2014, collected in the country's Midwestern, Northeastern, Southern, and Northern regions (Table 1).

3.2. RNA extraction and statistical analysis of archive samples

RNA measurements obtained with NanoDrop 2000 spectrophotometer showed that RNA yields of the 18 samples tested with protocols using longer periods of proteinase K incubation (Protocol B and C) were higher when compared with the protocol established by the company's RNA extraction kit (Table 5, A). The purity rate of the isolated material concerning proteins (260/280 nm) and contaminants (260/230 nm) was also higher when protocols B and C were applied, considered satisfactory when they ranged between 1.9 and 2.1 (Table 5).

The bivariate correlation analysis was built to demonstrate pairwise association of each sample's result in relation to the variables RNA ng/μL, 260/280 nm, and 260/230 nm. The parameter RNA ng/μL was not normally distributed and therefore logarithmic transformation had to precede other tests. At last it was demonstrated that there was an association in the purity rate 260/280 nm only between protocols A and C ($R = 0.551$, $p = 0.018$) (Fig. 1). There were no statistically significant associations for the protein absorbance ratio between protocols A and B ($R = 0.324$, $p = 0.190$) and between B and C ($R = 0.388$, $p = 0.170$).

Analysis of variance (ANOVA) with repeated measures for the parameters, with logarithmic transformation if necessary, intended to compare the means obtained between the protocols (Table 6). The trials are represented by the same variables previously described at the descriptive statistics, concerning the RNA yield, protein absorption and

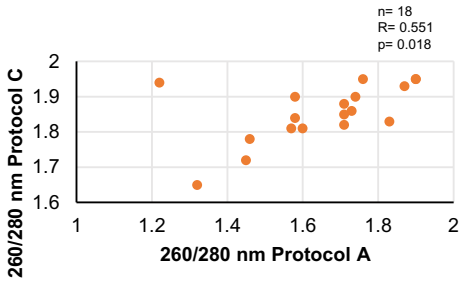

Fig. 1. Bivariate correlation between the 260/280 ratio of RNA extracted with Protocol A and C.

Table 6
Analysis of variance (ANOVA) with repeated measures. Statistical significance between variables of three RNA extraction Protocols.

Parameter	Sum of squares	df	Mean square	F-value	P-value
RNA ng/μL	15.18144	2	7.59072	41.12	< 0.0001
260/280	0.39196	2	0.19598	11.18	0.0002
260/230	4.724	2	2.362	19.85	< 0.001

df: degree of freedom. F: F-distribution.

purity of the three protocols assessed. Pairwise comparison between all groups assessed with Student-Newman-Keuls-test showed that the means of RNA yield obtained with each RNA extraction protocol were statistically significantly different between all groups (A, B, C) with $p < 0.01$ (Fig. 2A). When concerned to the mean of purity of the material extracted with each protocol in relation to proteins, there was statistically significant difference between protocols A and C ($p < 0.01$) and protocols B and C ($p < 0.01$) (Fig. 2B). The same pattern was observed for the purity of the material in relation to contaminants ($p < 0.01$) (Fig. 2C).

3.3. Equid-GAPDH PCR from archive samples

Ahead of the equid-GAPDH PCR, transcription of template RNA into cDNA was possible with all samples from the three extraction protocols (A, B, C). The maximum amount of RNA achieved for RT-PCR were 1000 ng. When using extraction protocol A, RNA total volumes varied from 15.6 ng to 554.4 ng, for protocol B, the amount varied from 52.8 ng to 1000 ng, and for protocol C the volumes ranged between 295.6 and 1000 ng (Supplemental file 1).

When using complimentary DNA (cDNA) obtained with RNA extraction protocol A amplification of the 46 bp and 64 bp products failed, and therefore, amplification applying the other primers were not further tested. When using protocol B, the 46 bp GAPDH product was obtained regularly, but amplification of the 64 bp GAPDH product was inconsistently possible when repetition of the method was carried out. Meanwhile, when protocol C was applied, for all 18 tested samples amplification of the 46 bp and 64 bp products was constantly possible (Fig. 3A) and for 17/18 samples also for the 170 bp amplicon (Fig. 3B). For one sample, also the 298 bp product was obtained (Fig. 3C).

3.4. RNA extraction and statistical analysis of naïve controls

In general, higher RNA yields were obtained with naive controls in all kinds of organs tested compared to formalin fixation (Table 7). Two-way ANOVA from naïve controls showed that the interaction organ and treatment was statistically significant regarding the RNA yield ($F = 121.06$, $P < 0.0001$), 260/280 nm ($F = 3.95$, $P = 0.0206$), and 260/230 nm ($F = 98.83$, $P < 0.0001$) from all tissues and treatments tested (Table 7). The best mean of RNA yield and purity ratios were achieved when fresh samples were frozen for 21 days at −80 °C (except

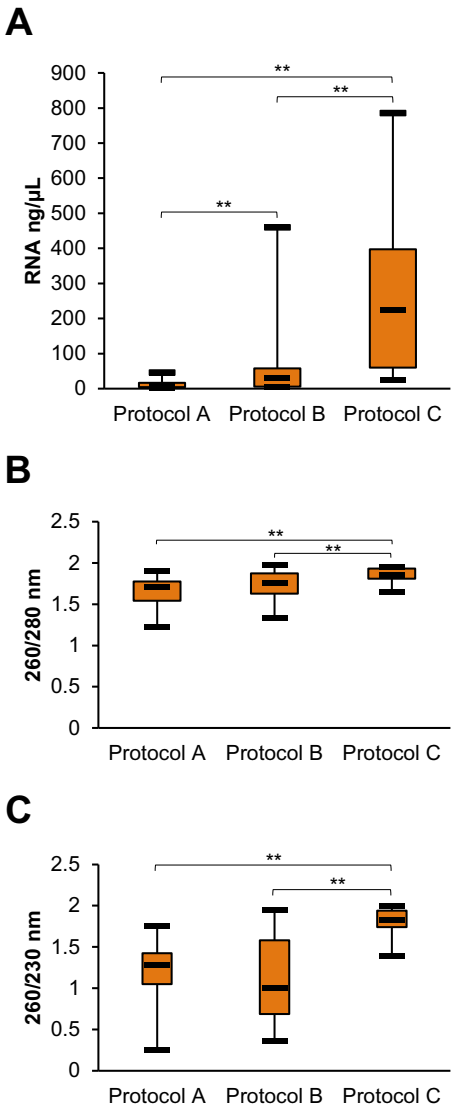

Fig. 2. Comparison of means between the RNA qualities obtained with three extraction Protocols. A: RNA yield. B: Purity regarding proteins. C: Purity regarding contaminants. ** $P < 0.01$.

liver), followed by the H_2O washing (except liver), and TAE buffer washing (Fig. 4, Table 7). RNA isolation results for each organ tested was statistically significant different ($P < 0.05$) with highest RNA yield from liver and kidney followed by brain and last skeletal muscle (Fig. 4A). TAE buffer washing achieved similar (kidney, liver) or lower results (brain, kidney, muscle) in 260/280 mm and 260/230 mm measurements indicating a similar or lower quality (Fig. 4B and C).

3.5. RNA extraction and statistical analysis of samples for comparison of formalin fixation time

For the RNA yield of FFPE material there were variable statistically significant differences between organs, treatment and formalin fixation time ($F = 87.35$, $P < 0.001$) (Table 8) albeit on a lower level than for the naïve controls. In general, H_2O washing after formalin-fixation achieved the best results for RNA yield ($F = 64.98$, $P < 0.0001$, Fig. 5A) and protein purity ratio ($F = 14.20$, $P < 0.001$, Fig. 5B). Interestingly, longer paraffin-fixation periods (14d and 21d) yielded in general the best results for brain fragments, afterwards washed only with H_2O (Fig. 5). Although there were differences between the treatments, in some organs and time points washing with TAE buffer led to

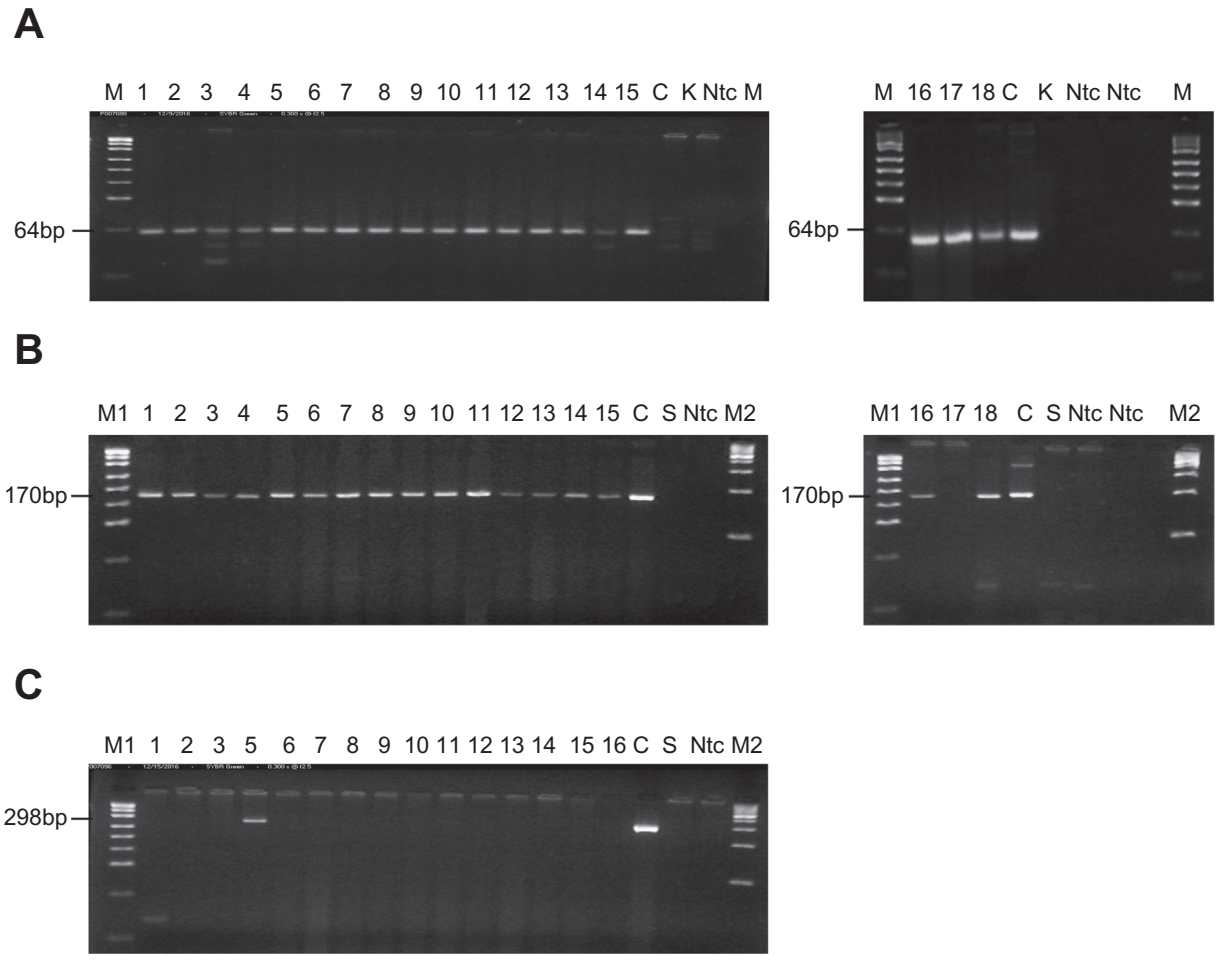

Fig. 3. Equid glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, 4% agarose gel. PCR carried out with RNA from extraction Protocol 3. Samples from the study are numbered from 1 to 18, C refers to equine cDNA used as positive control, Ntc is the no-template control. A: amplification of 64 bp in 18/18 samples. M refers to marker pUC19, S refers to squirrel cDNA used as negative control. B: amplification of 170 bp in 17/18 samples. M1 is the marker pUC19, M2 is the marker GR100. C: amplification of 298 bp in 1/18 samples. Sample 4 is missing due to material scarcity. M1 is the marker pUC19, M2 is the marker GR100.

Table 7
Two-way analysis of variance (ANOVA) with data screening. Statistical significance between RNA yield and purity of equine organs (brain, liver, kidney, skeletal muscle), and treatments (H_2O, TAE-buffer, 21 days frozen at −80 °C) used as naïve controls.

		Main effects (P-value)		Simple main effects (*P*-value)
Parameter	n	Organ	Treatment	Organ x Treatment
RNA ng/µL[a]	7	< 0.0001	< 0.0001	< 0.0001
260/280	7	0.0001	0.0015	0.0206
260/230	7	< 0.0001	< 0.0001	< 0.0001

[a] After logarithmic transformation.

higher RNA yields (e.g. liver, 14d fixation time; muscle, 7d fixation time, Fig. 5A), higher 260/280 (muscle, 24 h fixation, Fig. 5B) and 260/230 ratios (muscle, 14d fixation, Fig. 5C). For details about the best formalin-fixation time and treatment for each tested organ, see Fig. 6.

3.6. Equid-GAPDH PCR from naïve controls and from samples for formalin fixation time comparison

In naïve controls, amplicons from 64 bp to 517 bp were obtained. In formalin-fixed organs, regardless of treatment longest amplicons were 298 bp in kidney and skeletal muscle after 24 h formalin fixation and in brain after 21d. In brain and skeletal muscle most amplicons were longer than 170 bp, in kidney and liver only one amplicon after formalin fixation was longer than 103 bp (Fig. 6 and Fig. 7).

4. Discussion

Recovery and amplification of nucleic acid from FFPE material is of increasing interest for pilot and retrospective studies for a variety of objectives, e.g. the establishing of biomarkers, pathogen evolution and emergence, accompanied by lower risks of contamination (Abed and Dark, 2016; Gruber et al., 1993; Taubenberger et al., 1997; Worobey et al., 2008). Moreover, it is needed for destruction of infectivity before histological evaluation and enable long storage of a panel of tissues in experiments using pathogens of high biosafety levels (Thacker and Waters, 2017; PAHO, n.d). Polymerase-chain reaction assays have also been broadly used as reliable tools in the same settings for diseases occurring in developing countries (Bhatnagar et al., 2012; D'Andrea et al., 2012), and molecular studies have been increasingly adopting FFPE material for e.g. pathogen screening (Mubemba et al., 2017).

Opposed to the highly cost-demanding techniques that have been suggested as alternatives to unravel archives, local researchers frequently require sensitive tests at lower costs to support groundwork (Bonin et al., 2003). Nonetheless, the greater challenge is to optimize a technique that is also applicable to FFPE material since this allows simultaneous morphological and molecular analyses and is also affordable for different laboratory settings (Bhudevi and Weinstock, 2003; Lewis et al., 2001; Masuda et al., 1999).

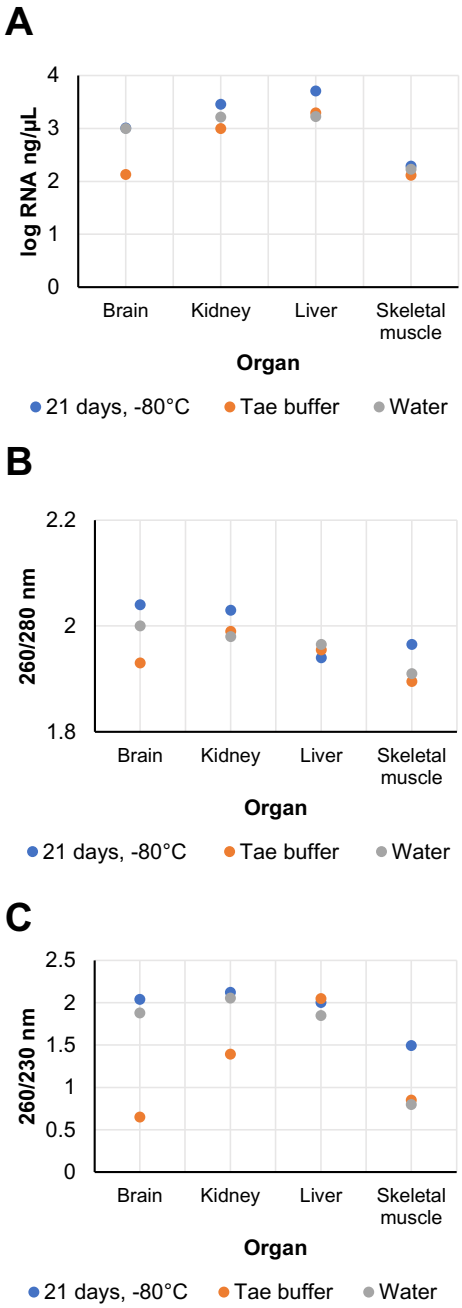

Fig. 4. Two-way analysis of variance (ANOVA) for naïve controls during RNA extraction. A: RNA yields after logarithmic transformation show higher amounts of RNA extracted after samples are frozen for 21 days at −80 °C ($F = 571.337$, $P < 0.001$). B: Assessment of RNA purity regarding protein cross-links (260/280 nm) shows higher measurements when brain, kidney, and skeletal muscle fragments are frozen for 21 days at −80 °C before RNA extraction, and when liver fragments are H_2O treated. C: Assessment of contaminants that could possibly inhibit or interfere in future PCR assays (260/230 nm) shows that brain, kidney, and skeletal muscle fragments are optimal for RNA extraction after frozen for 21 days at −80 °C, and liver fragments when washed with TAE-buffer.

In this study, by applying three different RNA extraction protocols, an easy and feasible methodology was established that was able to reverse most of the cross-linking subjected to FFPE material. This was further accompanied by an increase in total extraction yield of RNA and satisfactory purity rates concerning contaminants. Thus, also higher amounts of RNA were available for further analyses. Best results regarding length of amplicons were obtained when applying protocol C with constant amplification of a 170 bp GAPDH product. This indicates that treatment of at least 24 h with proteinase K significantly contributes to an increase not only in the length of RNAs but also in the total amount. In general, protocol C comprises only simple features that were added to the deparaffinization step, followed by a moderately harsh lysis, with increased digestion time and volume of the reagent.

Mostly, the attempt to obtain nucleic acids of good quality out of archive material is hindered by the fact that fixation and embedding procedures tend to highly degrade RNA (Bhudevi and Weinstock, 2003; Masuda et al., 1999; Nolan and Bustin, 2008). However, in experiments with highly pathogenic agents harsh formalin fixation represents an essential step for inactivation of infectivity and safety of further use without any biosafety regulations for any person dealing with this material (Hartman et al., 2012; PAHO and WHO, n.d.). For outcome of RNA isolation, adapting the first step of deparaffinization by incubating tissue fragments in xylol at 37 °C for 20 min, already yielded significant improvement in the RNA measurements when using protocol B and C compared to protocol A. This heating step needed for an efficient paraffin melting was already previously described in other successful molecular studies, and is involved in dissolving the heavy hydrocarbons forming the paraffin so that the tissues become liberated (Godfrey et al., 2000; Sengüven et al., 2014). Furthermore, xylol was kept as melting reagent for all methods tested, to maintain the similarities with in-house extraction methods. On the other hand, tests with other melting methods like by hot mineral oil and water are promising alternatives for an adequate xylol-free extraction, denotating lower toxic laboratory waste and improving laboratory staff health (Kalantari et al., 2016; Lagheden et al., 2016). Therefore, combination of the present protocol with these adaptations should be tested in further studies.

As a next step, tissue digestion tests with the horse brains were carried out with proteinase K (20 mg/mL) added to PKD lysis buffer provided in the isolation kit. There are several reports that describe different buffers and reagents, as well as variations in incubation time and temperature (Okello et al., 2010). In our study, treatment with proteinase K was used for its adequate performance demonstrated by a vast number of researches (Godfrey et al., 2000; Gruber et al., 1993; Okello et al., 2010; Wakamatsu et al., 2007), due to its availability in almost all laboratories, relative low cost, and non-toxicity when for instance compared to the chloroform-containing protocols. The incubation temperature of 56 °C was kept, as it ensures the adequate activation of proteinase K added to a lower risk of disintegrating the already damaged RNA (Brisco and Morley, 2012). In this study, incubation time of proteinase K was used as variable and revealed best results with a volume of 60 µL and an elongated treatment of 24 h. By applying this protocol, statistically significantly higher RNA quality and length of amplicons (170 bp) were obtained when compared to protocol A and B. In one sample (ID 5, Table 1), amplifying up to 298 bp long fragments was possible. In contrast, when using the original RNA isolation protocol for FFPE material with a brief proteinase K incubation even the smallest housekeeping gene products tested (48 bp and 64 bp) could not be amplified. This suggests that archived FFPE material require adjustments in proteinase K volume and incubation time to be able to overcome cross-linking. This was already achieved using protocol B but although the RNA yields were statistically higher in comparison to protocol A ($P = .01$), RNA purity concerning proteins (260/280 nm) and contaminants (260/230 nm) was not significantly increased when compared to protocol A. Similar results were obtained by Godfrey et al. (2000) who carried out a similar protocol as protocol B. They suggested that impurities and/or carry over of proteinase K from the RNA extraction could be responsible for the inadequate purity of the RNA which could not overcome by subsequent heating to inactivate the enzyme activity. Furthermore, the range value obtained with protocol C for 260/230 nm was significantly higher ($P < 0.001$), demonstrating that more samples were close to the pattern that can be considered free from contaminants. Results were reproducible with all 18 samples indicating that protocol C represents a suitable tool to achieve RNA of

Table 8
Three-way analysis of variance and covariance with repeated measures. Statistical significance between equine organs (brain, liver, kidney, and skeletal muscle), treatment (H2O, TAE-buffer, frozen 21 days at −80 °C), and formalin fixation time (24 h, 7–14–21 – days) for RNA extraction parameters (RNA ng/μL, 260/280, and 260/230).

		Main efects (*p*-value)			Dual interactions (p-value)			Triple interactions (p-value)
Param.	n	Organ	Treat	Fix. time	Organ x treat	Organ x fix. Time	Treat x fix. Time	Organ x treat x fix. Time
RNA ng/μL[a]	10	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
260/280 nm	10	< 0.0001	0.0096	< 0.0001	< 0.0001	< 0.0001	0.0033	0.1314
260/230 nm	10	< 0.0001	0.5413	< 0.0001	0.1313	< 0.0001	0.0465	0.0003

Fix.: fixation. Param.: parameter. Treat: treatment.
[a] After logarithmic transformation.

comparable quality which contribute to avoid possible false negative results due to PCR-inhibition (Nolan and Bustin, 2008).

However, the time span of proteinase K treatment could depend on the duration of formalin fixation (Granato et al., 2014; Groelz et al., 2018; Okello et al., 2010). As these informations are frequently missing, protocol C was tested with defined formalin fixation periods in four different organs. RNA yield was different between organs, and for each organ a "perfect" formalin fixation time in combination with a treatment to reduce formalin-fixation effects can be given. However, regardless of tested organ, higher RNA-yields were gained from controls without formalin fixation than from formalin-fixed material. Three-way ANOVA demonstrated that neither duration of formalin fixation nor treatment lead to similar results in tested organs. Therefore, other conditions like kind of tissue seem to be more relevant. A pre-trial with control material is therefore strongly advisable to reveal the best treatment for each experiment/tissue.

In summary, protocol C represents a reliable and easy to handle alternative for many laboratories where RNA from FFPE material is used ensuring to obtain comparable amounts and quality of RNA from samples that differ in duration of storage. It is important to emphasize

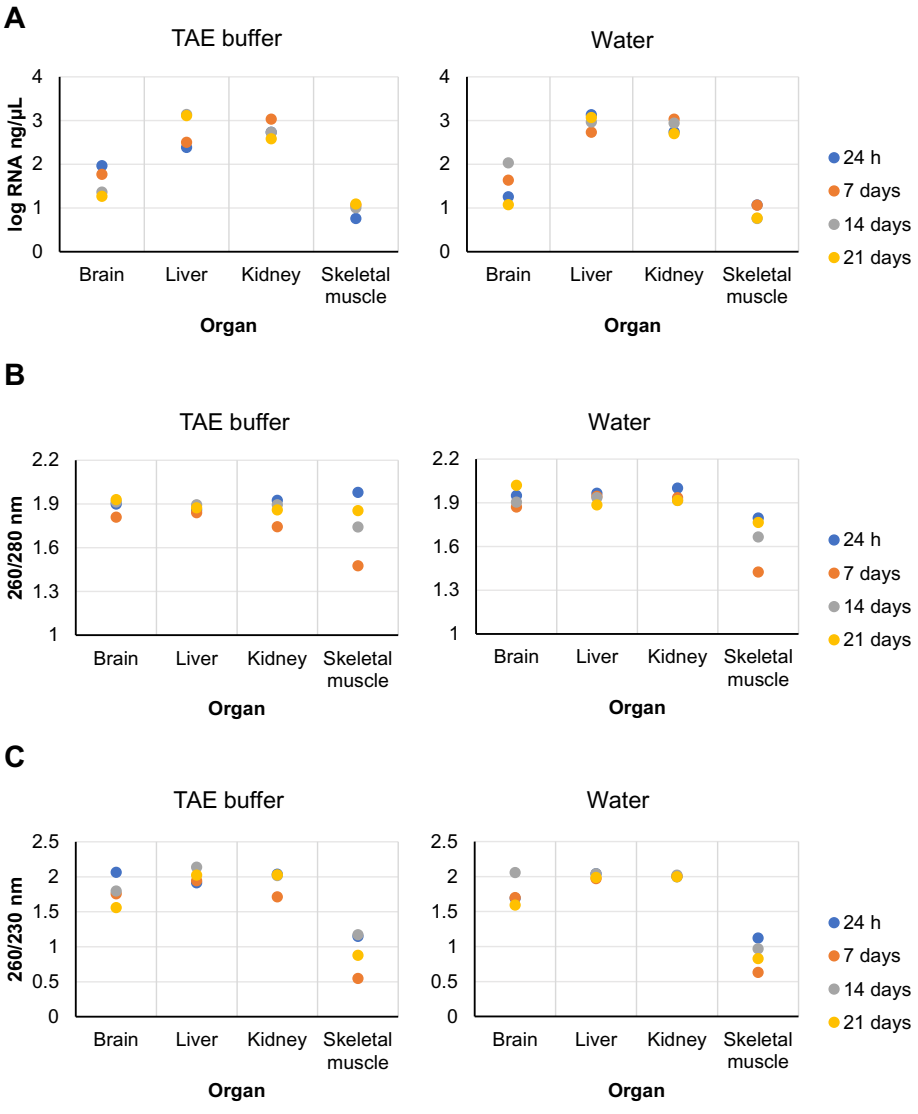

Fig. 5. Three-way analysis of variance (ANOVA) of RNA measurements carried out with organ fragments pre-treated with TAE-buffer or water, and then formalin-fixed for different time periods. A: RNA yields after logarithmic transformation are statistically significant different between the organs tested (F = 5626.071, P < 0.001), the amount of extracted RNA is also statistically significant different when different pre-treatments are carried out (F = 31.534, P < .001) and in different formalin-fixation periods (F = 26.377, P < 0.001). There are statistically significant differences in two-way and three-way interactions between organs, pre-treatments, and formalin fixation time (P < 0.001). B: For RNA protein purity (260/280 nm) there are statistically significant differences between the purity of material between organs (F = 92.469, P < 0.001), pre-treatments (F = 7.590, *P* = 0.01), and between the different formalin fixation periods (F = 55.881, P < 0.001). There are two-way interactions between organs, pre-treatments, and formalin fixation time showing statistically significant differences (*P* < 0.05), but there is no three-way interaction between these cases. C: The parameter showing purity of RNA regarding inhibitory contaminants (260/230 nm) was statistically significant different between the organ fragments (F = 596.793, P < 0.001) and between the formalin fixation periods (F = 29.528, P < .001). There is three-way interaction statistically significant different between organs, pre-treatments, and formalin fixation periods (F = 5.052, P < 0.001), and two-way interactions between organ x formalin fixation period (F = 8.309, P < 0.001), and pre-treatment x formalin fixation period (F = 2.968, *P* = 0.047).

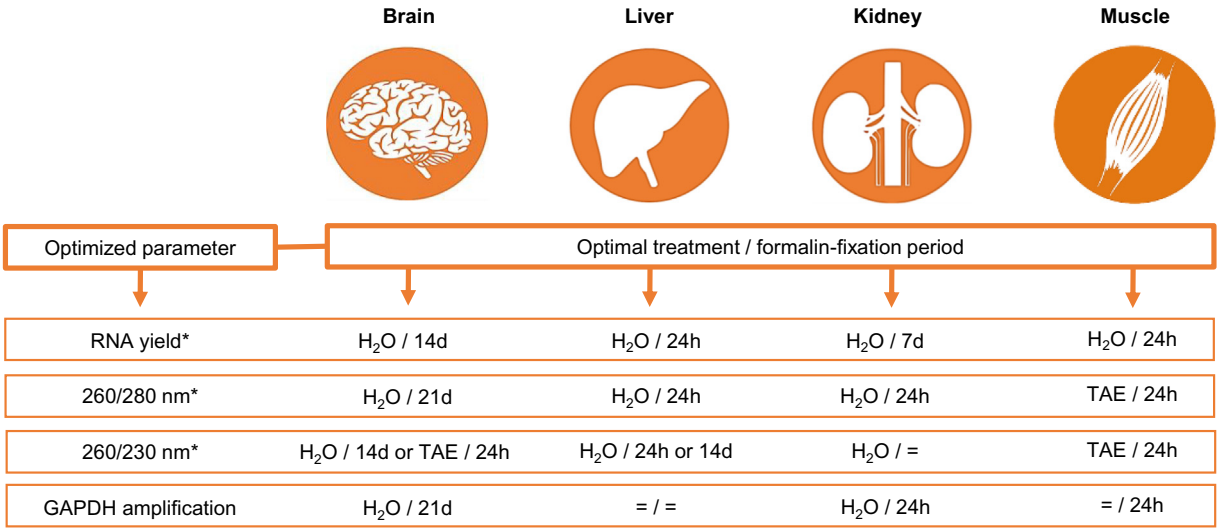

Fig. 6. Optimal treatment for tissue fragments washing after formalin-fixation and formalin-fixation time obtained for different organs with protocol C as RNA extraction method. * $P \leq 0.05$. = / = treatment and formalin-fixation period are unimportant. = formalin-fixation period is unimportant.

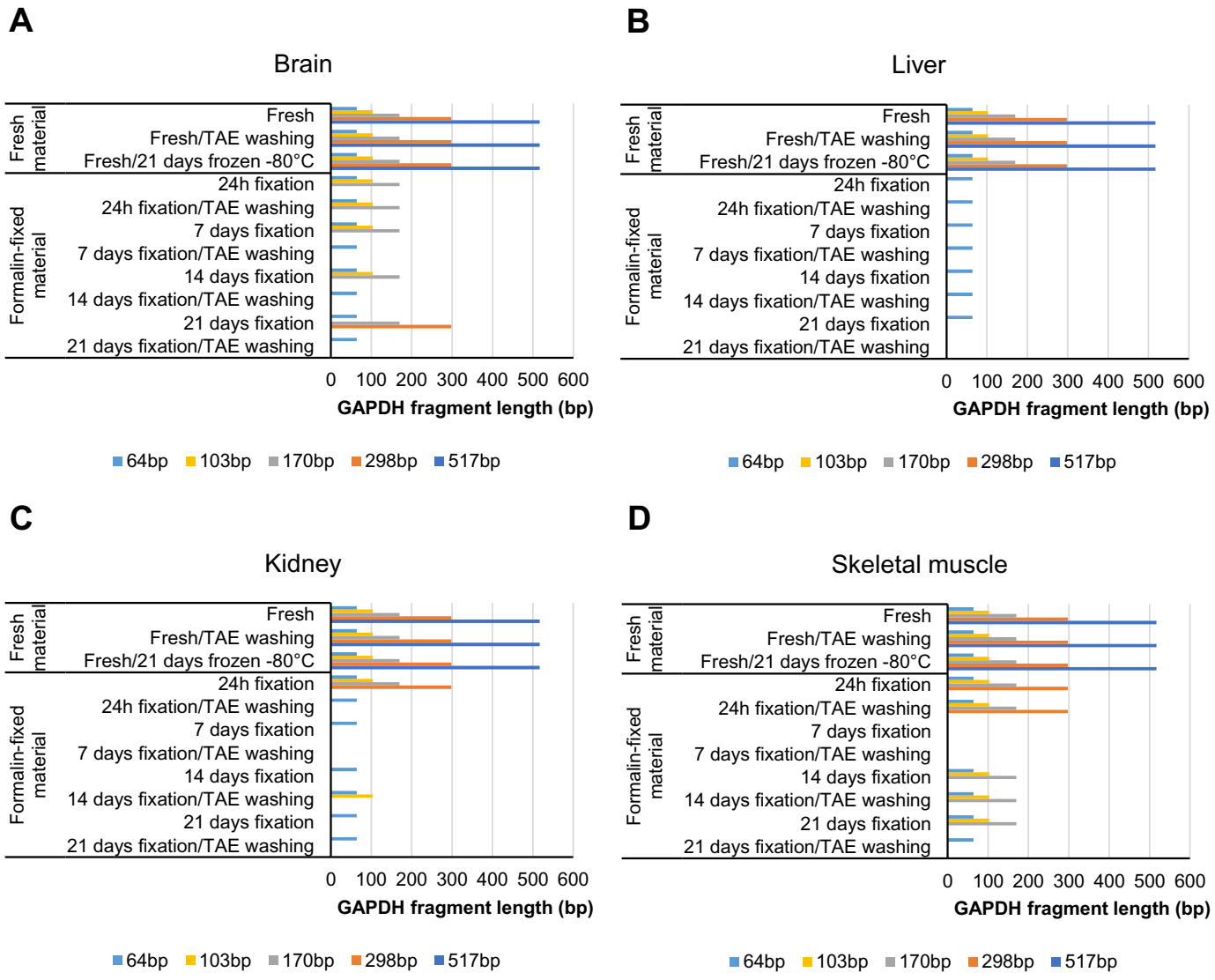

Fig. 7. Amplification products obtained after equid glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR. Comparison between amplified products using fresh material and formalin-fixed tissues at different time points. A: Central nervous system (CNS). B: Liver. C: Kidney. D: Skeletal muscle. Fresh material, independently from treatment, amplified the maximal length product tested of 517 bp. Length of amplifiable products decrease after formalin-fixation.

that the commercial extraction kits are adequate tools when fresh frozen or freshly generated FFPE material is available, but organ-specific adaptations are needed when FFPE material of longer storage will be applied.

Funding sources

This work was supported by the Brazilian Coordination of Superior Level Staff Improvement (CAPES)/National Council for Scientific and Technological Development (CNPq) and the German Academic Exchange Service (DAAD) (process number 490235/2013-1); and the German Federal Ministry of Education and Research (BMBF) - Nationales Forschungsnetz zoonotische Infektionskrankheiten - ZooBoCo (project number 01KI1722E).

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would like to thank Werner Hecht for fruitful discussion, Unit for Biomathematics and Data Processing Uni-Gießen (Marion Sparenberg) for statistical advice, Silke Engel for the excellent technical assistance, Daniele Mariath Bassuino, David Driemeier, Edson Moleta, Fabiana Marques Boabaid, José Diomedes Barbosa, Luciana Sonne, and Márcio Botelho Castro for sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104289.

References

Abed, F.M., Dark, M.J., 2016. Determining the utility of veterinary tissue archives for retrospective DNA analysis. PeerJ 4, e1996. https://doi.org/10.7717/peerj.1996.

Avraham, R., Haseley, N., Fan, A., Bloom-Ackermann, Z., Livny, J., Hung, D.T., 2016. A highly multiplexed and sensitive RNA-seq protocol for simultaneous analysis of host and pathogen transcriptomes. Nat. Protoc. 11, 1477–1491. https://doi.org/10.1038/nprot.2016.090.

Bhatnagar, J., Blau, D.M., Shieh, W.J., Paddock, C.D., Drew, C., Liu, L., Jones, T., Patel, M., Zaki, S.R., 2012. Molecular detection and typing of dengue viruses from archived tissues of fatal cases by RT-PCR and sequencing: diagnostic and epidemiologic implications. Am. J. Trop. Med. Hyg. 86, 335–340. https://doi.org/10.4269/ajtmh.2012.11-0346.

Bhudevi, B., Weinstock, D., 2003. Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J. Virol. Methods 109, 25–30. https://doi.org/10.1016/S0166-0934(03)00040-5.

Bonin, S., Petrera, F., Niccolini, B., Stanta, G., 2003. PCR analysis in archival postmortem tissues. Mol. Pathol. 56, 184–186. https://doi.org/10.1136/mp.56.3.184.

Brisco, M.J., Morley, A.A., 2012. Quantification of RNA integrity and its use for measurement of transcript number. Nucleic Acids Res. 40, e144. https://doi.org/10.1093/nar/gks588.

Ciotti, P., Garuti, A., Ballestrero, A., Cirmena, G., Chiaramondia, M., Baccini, P., Bellone, E., Mandich, P., 2009. Reliability and reproducibility of a RNA preamplification method for low-density array analysis from formalin-fixed paraffin-embedded breast cancer samples. Diagn. Mol. Pathol. 18, 112–118. https://doi.org/10.1097/PDM.0b013e3181831320.

D'Andrea, A., Martinez, Y.Z., Alduina, R., Monteverde, V., Molina, C.F., Vitale, M., 2012. Comparison of two PCR methods for detection of Leptospira interrogans in formalin-fixed and paraffin-embedded tissues. Mem. Inst. Oswaldo Cruz 107, 85–88. https://doi.org/10.1590/S0074-02762012000100012.

Dijkstra, J.R., Mekenkamp, L.J.M., Teerenstra, S., De Krijger, I., Nagtegaal, I.D., 2012. MicroRNA expression in formalin-fixed paraffin embedded tissue using real time quantitative PCR: the strengths and pitfalls. J. Cell. Mol. Med. 16, 683–690. https://doi.org/10.1111/j.1582-4934.2011.01467.x.

Dixon, W.J., 1993. BMDP Statistical Software Manual 1992: BMDP Release 7 v.1. University of California Press, Los Angeles.

Doleshal, M., Magotra, A.A., Choudhury, B., Cannon, B.D., Labourier, E., Szafranska, A.E., 2008. Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 10, 203–211. https://doi.org/10.2353/jmoldx.2008.070153.

Evers, D.L., Fowler, C.B., Cunningham, B.R., Mason, J.T., O'Leary, T.J., 2011. The effect of formaldehyde fixation on RNA: optimization of formaldehyde adduct removal. J. Mol. Diagn. 13, 282–288. https://doi.org/10.1016/j.jmoldx.2011.01.010.

Godfrey, T.E., Kim, S.H., Chavira, M., Ruff, D.W., Warren, R.S., Gray, J.W., Jensen, R.H., 2000. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5′ nuclease quantitative reverse transcription-polymerase chain reaction. J. Mol. Diagn. 2, 84–91. https://doi.org/10.1016/S1525-1578(10)60621-6.

Granato, A., Giantin, M., Ariani, P., Carminato, A., Baratto, C., Zorzan, E., Vascellari, M., Bozzato, E., Dacasto, M., Mutinelli, F., 2014. DNA and RNA isolation from canine oncologic formalin-fixed, paraffin-embedded tissues for downstream "-omic" analyses: possible or not? J. Vet. Diagn. Investig. 26, 117–124. https://doi.org/10.1177/1040638713509378.

Groelz, D., Viertler, C., Pabst, D., Dettmann, N., Zatloukal, K., 2018. Impact of storage conditions on the quality of nucleic acids in paraffin embedded tissues. PLoS One 13, e0203608. https://doi.org/10.1371/journal.pone.0203608.

Gruber, A.D., Greiser-Wilke, I.M., Haas, L., Hewicker-Trautwein, M., Moennig, V., 1993. Detection of bovine viral diarrhea virus RNA in formalin-fixed, paraffin-embedded brain tissue by nested polymerase chain reaction. J. Virol. Methods 43, 309–319. https://doi.org/10.1016/0166-0934(93)90149-L.

Hartman, A.L., Cole, K.S., Homer, L.C., 2012. Verification of inactivation methods for removal of biological materials from a biosafety level 3 select agent facility. Appl. Biosaf. 17, 70–75. https://doi.org/10.1177/153567601201700204.

Huijsmans, C.J., Damen, J., van der Linden, J.C., Savelkoul, P.H., Hermans, M.H., 2010. Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effect on downstream molecular applications. BMC Res. Notes 3, 239. https://doi.org/10.1186/1756-0500-3-239.

Iwamoto, K.S., Mizuno, T., Ito, T., Akiyama, M., Takeichi, N., Mabuchi, K., Seyama, T., 1996. Feasibility of using decades-old archival tissues in molecular oncology/epidemiology. Am. J. Pathol. 149, 399–406.

Kalantari, N., Bayani, M., Ghaffari, T., 2016. Deparaffinization of formalin-fixed paraffin-embedded tissue blocks using hot water instead of xylene. Anal. Biochem. 507, 71–73. https://doi.org/10.1016/j.ab.2016.05.015.

Lagheden, C., Eklund, C., Kleppe, S.N., Unger, E.R., Dillner, J., Sundström, K., 2016. Validation of a standardized extraction method for formalin-fixed paraffin-embedded tissue samples. J. Clin. Virol. 80, 36–39. https://doi.org/10.1016/j.jcv.2016.04.016.

Lewis, F., Maughan, N.J., Smith, V., Hillan, K., Quirke, P., 2001. Unlocking the archive–gene expression in paraffin-embedded tissue. J. Pathol. 195, 66–71. https://doi.org/10.1002/1096-9896(200109)195:1<66::AID-PATH921>3.0.CO;2-F.

Li, J., Smyth, P., Cahill, S., Denning, K., Flavin, R., Aherne, S., Pirotta, M., Guenther, S.M., O'Leary, J.J., Sheils, O., 2008. Improved RNA quality and TaqMan pre-amplification method (PreAmp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials. BMC Biotechnol. 8, 10. https://doi.org/10.1186/1472-6750-8-10.

Magdeldin, S., Yamamoto, T., 2012. Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. Proteomics 12, 1045–1058. https://doi.org/10.1002/pmic.201100550.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., Okubo, K., 1999. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 27, 4436–4443. https://doi.org/10.1093/nar/27.22.4436.

Mubemba, B., Thompson, P.N., Odendaal, L., Coetzee, P., Venter, E.H., 2017. Evaluation of positive Rift Valley fever virus formalin-fixed paraffin embedded samples as a source of sequence data for retrospective phylogenetic analysis. J. Virol. Methods 243, 10–14. https://doi.org/10.1016/j.jviromet.2017.01.014.

Nolan, T., Bustin, S., 2008. Procedures for quality control of RNA samples for use in quantitative reverse transcription PCR. In: Keer, J.T., Birch, L. (Eds.), Essentials of Nucleic Acid Analysis: A Robust Approach. RSC Publishing, Cambridge, pp. 189–207.

Okello, J.B.A., Zurek, J., Devault, A.M., Kuch, M., Okwi, A.L., Sewankambo, N.K., Bimenya, G.S., Poinar, D., Poinar, H.N., 2010. Comparison of methods in the recovery of nucleic acids from archival formalin-fixed paraffin-embedded autopsy tissues. Anal. Biochem. 400, 110–117. https://doi.org/10.1016/j.ab.2010.01.014.

PAHO, WHO, n.d. General Procedures for Inactivation of Potentially Infectious Samples with Ebola Virus and Other Highly Pathogenic Viral Agents 1 [WWW Document]. Pan Am. Heal. Organ. Heatlh Organ. URL https://www.paho.org/hq/dmdocuments/2014/2014-cha-procedures-inactivation-ebola.pdf (accessed 11.5.18).

Sengüven, B., Baris, E., Oygur, T., Berktas, M., 2014. Comparison of methods for the extraction of DNA from formalin-fixed, paraffin-embedded archival tissues. Int. J. Med. Sci. 11, 494–499. https://doi.org/10.7150/ijms.8842.

Taubenberger, J.K., Reid, A.H., Krafft, A.E., Bijwaard, K.E., Fanning, T.G., 1997. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science (80-.) 275, 1793–1796. https://doi.org/10.1126/science.275.5307.1793.

Thacker, T.C., Palmer, M.V., Waters, W.R., 2017. Evaluation of tissue fixation methods to inactivate Mycobacterium bovis under routine laboratory conditions. Appl. Biosaf. 22, 152–155. https://doi.org/10.1177/1535676017737315.

Vermeulen, J., Derveaux, S., Lefever, S., De Smet, E., De Preter, K., Yigit, N., De Paepe, A., Pattyn, F., Speleman, F., Vandesompele, J., 2009. RNA pre-amplification enables large-scale RT-qPCR gene-expression studies on limiting sample amounts. BMC Res. Notes 2, 235. https://doi.org/10.1186/1756-0500-2-235.

Wakamatsu, N., King, D.J., Seal, B.S., Brown, C.C., 2007. Detection of Newcastle disease virus RNA by reverse transcription-polymerase chain reaction using formalin-fixed, paraffin-embedded tissue and comparison with immunohistochemistry and in situ hybridization. J. Vet. Diagnostic Investig. 19, 396–400. https://doi.org/10.1177/104063870701900410.

Worobey, M., Gemmel, M., Teuwen, D.E., Haselkorn, T., Kunstman, K., Bunce, M., Muyembe, J.-J., Kabongo, J.-M.M., Kalengayi, R.M., Van Marck, E., Gilbert, M.T.P., Wolinsky, S.M., 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature 455, 661–664. https://doi.org/10.1038/nature07390.