Article

Residual Humidity in Paraffin Embedded Tissue Reduces Nucleic Acid Stability

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Abstract: Molecular diagnostics in healthcare relies increasingly on genomic and transcriptomic methodologies and requires appropriate tissue specimens from which nucleic acids (NA) of sufficiently high quality can be obtained. Besides suration of ischemia and fixation type, NA quality depends on a variety of other pre-analytical parameters, like storage conditions and duration. It has been discussed that improper dehydration of tissue during processing influences quality of NAs and shelf-life of fixed tissue. Here we report on establishing a method to determine the amount of residual water in fixed, paraffin-embedded tissue (fixed by neutral buffered formalin, or a non-crosslinking fixative) and its correlation to the performance of NAs in qRT-PCR analyses. The amount of residual water depended primarily on the fixative type and the dehydration protocol and, to a lesser extent on storage conditions and time. Moreover, we found that these parameters were associated with the qRT-PCR performance of extracted NAs. Besides cross-linking of NAs and modification of nucleobases by formalin, hydrolysis of NAs by residual water was found to contribute to reduced qRT-PCR performance. The negative effects of residual water on NA stability are not only important for the design and interpretation of research, but must also be taken into account in clinical diagnostics where reanalysis of archived tissue from a primary tumour may be required, e.g., after disease recurrence. We conclude that improving shelf-life of fixed tissue requires meticulous dehydration and dry storage to minimize the degradative influence of residual water on NAs.

Keywords: fixed tissue; nucleic acid quality; next-generation sequencing,

1. Introduction

Diagnostics in healthcare increasingly relies on detailed molecular analyses of alterations of the genome and transcriptome, using tissue specimens harvested during surgery or biopsy. Such analyses have become invaluable for diagnosis and therapy selection, and eventually personalized treatment of the patient. qRT-PCR-based molecular diagnostics

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and gene-panel-based Next Generation sequencing are readily established in cancer diagnostics, and due to lowering of cost, whole-genome and whole-exome sequencing are increasingly used not only for research but also for diagnostics, which, however, places increasing demands on sample quality.

Tissue used for diagnostic purposes is usually formalin-fixed, paraffin-embedded (FFPE), where the fixative is 10% neutral buffered formalin (NBF, 4% formaldehyde v/v. in aqueous phosphate-buffered solution). This material is widely available and has been used for decades for pathomorphological characterization, but also for immunohistochemical, histochemical and in-situ hybridization methodologies [1]. The use of FFPE tissue for transcriptomic and genomic analyses, however, revealed severe shortcomings regarding quality and reliability of the results, in particular after prolonged storage at room temperature, showing a time-dependent aging effect [2-5]. This is a result of chemical modification of biomolecules, e.g., by crosslinking of nucleic acids (NA) and proteins [6,7], or by modification of nucleobases [8-10]. Alternative, non-modifying fixatives, such as PAXGene® Tissue System (PFPE) have shown to give much better results and longer shelf-life [11]; however, they are not routinely used in healthcare.

Both qRT-PCR-based and advanced analysis methodologies, such as next generation sequencing, require input material in which NAs are sufficiently well preserved to yield reliable results [5]: failing to detect a critical variant may invalidate the diagnosis with dire consequences for the patient's treatment. Consequentially, there is growing emphasis on the quality of biospecimens, in particular their suitability for the intended diagnostic purpose. It has long been recognized that the critical steps in producing highly reliable results often do not lie with the analysis itself, but rather with the pre-analytical workflow. Accordingly, a series of international standards for the pre-analytical processing of biological samples was developed [12-15].

Depending on the pre-analytical processing of fixed tissue, the quality of NAs and their shelf life may vary considerably, both for chemically modifying and non-modifying fixatives. Specifically for FFPE tissue, a variety of parameters was described to impair morphological representation and immunoreactivity [1,2,16]. Besides ischemia times during tissue harvesting (during and after surgery), fixative type, fixation temperature and duration, ratio of fixative and tissue volumes are important determinants affecting NA quality and shelf life [4].

Preparation of fixed tissue is a multi-step process: after immersing tissue into the fixative (at room temperature) for the appropriate time ('fixation'), the material is dehydrated in a series of ethanol baths with increasing concentration (ending with 100%, which is usually 99% plus 1% denaturing agent), followed by replacing ethanol with a nonpolar solvent ('clearing') and finally infiltration of tissue with paraffin ('impregnation') [17]. For PFPE tissue, only the 'fixation' step is different from FFPE tissue.

For a long time, it was suspected that the quality of NAs and their stability is not only affected by the fixation process alone, but also by the subsequent steps: residual water, resulting from improper dehydration after fixation, or from improper storage, may revert the fixation process [8], and lead to hydrolytic degradation of NAs. Improper dehydration

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might be a consequence of carryover of water along the ethanol series which may increase the water content of the last bath. This may be caused, e.g., by prolonged use of ethanol (often to reduce cost), especially in manual dehydration protocols. It should also be noted that ethanol forms an azeotropic mixture with water (3.5% water) which can form upon exposure of absolute ethanol to (humid) air. The experimental conditions we selected (95% and 90% ethanol) reflect these possibilities. Another is that fixed tissue can accumulate water from the environment over time; therefore we also investigated changes in residual humidity during different storage scenarios. Finally, FFPE and PFPE are not only different by their fixation chemistry (cross-linking and non-cross-linking), but also by way of other chemical modifications: formalin introduces oxidative modifications in nucleobases and the sugar-phosphate backbone, which make the material more polar, facilitating the uptake and retention of water.

In this study, we combined the measurement of temperature-dependent water release from FFPE and PFPE tissue, amplicon-length dependent quantitative real-time polymerase chain reaction (qRT-PCR) and nuclear magnetic resonance (NMR) spectroscopic analysis of the NAs to establish the causal link of residual humidity to reduced shelf life and compromised NA quality.

2. Results

2.1. Experimental design

Table 1 shows the different fixation, dehydration and storage protocols used with the tissue samples. While dehydration with 95% ethanol (EtOH) as the last dehydration bath already leads to significantly compromised NA quality and shelf life, we decided to reduce EtOH content even to 90%, to better illustrate the effects – NMR measurements also benefited from the larger amount of compromised NAs. For details of the experimental protocols see Materials and Methods, below. For clarity, we often show only the results for 100% (nominally) and 90% EtOH, supplementary data contain all data in a database.

Fixative	Dehydration protocol (concentration of last ethanol bath)	Storage duration	Storage condition
NBF	100%	0-1 week	4°C
PGTS	95%	3 months	Room temperature dry
	90%	6 months	Room temperature 100% r. h.
		1 year	

Table 1: Conditions of sample treatment and storage varied in the study. Mouse liver was divided into three parts that were subjected to the three different storage conditions. NBF, neutral-buffered formalin, PGTS, PAXgene Tissue System; r.h., relative humidity

2.2 Parameters affecting Residual Humidity (RsH) and Amplicon-length dependent qRT-PCR performance index (AL-PPI)

Residual humidity (RsH), i.e., the percentage of water remaining bound to tissue after dehydration) may be a major determinant of tissue NA quality. To unravel this com-

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plex interplay, we first showed how RsH is influenced by fixation type. We then investigated the effects of dehydration protocol as well as storage conditions and duration, and how RsH relates to the amplicon-length qRT-PCR performance index (AL-PPI, see Materials and Methods, below), reflecting the difference in amplification efficiencies of short and long amplicons (the lower AL-PPI, the more the RNA was modified and degraded). This was followed by a comprehensive analysis of all factors affecting the quality of NAs in fixed tissue. Note that although AL-PPI is derived from qRT-PCR results, we included amplicon lengths of up to 526 base pairs in its definition and measurement, to account for the fact that sometimes read lengths in NGS are much higher than in qRT-PCR. Moreover, this allows a more finely graded assessment of the differences.

Influence of fixation type (at 100% EtOH) on RsH and AL-PPI

In the first experimental series (Fig. 1), the last EtOH dehydration step was always performed with 100% EtOH (optimal dehydration).

After 1 week of storage, RsH was generally lower for PFPE than for FFPE samples (RsH_{PFPE} = 0.6% … 4%; RsH_{FFPE} = 1.5 … 4%). Prolonged storage for up to one year revealed that high ambient humidity (emulated by storage at RT and 100% r.h., in a humid chamber) led to an increase of RsH, while storage at ambient temperature in dry atmosphere or at 4°C left RsH largely unchanged over time. Interestingly, we found the increase of RsH at 6 months most pronounced both for FFPE and PFPE when stored at RT and 100% r. h. (RsH = 4%).

AL-PPI of PFPE tissue RNA was generally high and close to that of cryopreserved tissue, which served as a reference ($AL\text{-}PPI_{PFPE}$ = 0.88 ... 0.95; data were normalized so that $AL\text{-}PPI_{cryo}$ = 1.0) and did not appreciably vary with RsH (except for a slight dip after 6 months storage). Conversely, $AL\text{-}PPI_{FFPE}$ was much lower, approximately half of that of PFPE or cryopreserved material ($AL\text{-}PPI_{FFPE}$ = 0.43 ... 0.64) with increased loss of qRT-PCR performance over time.

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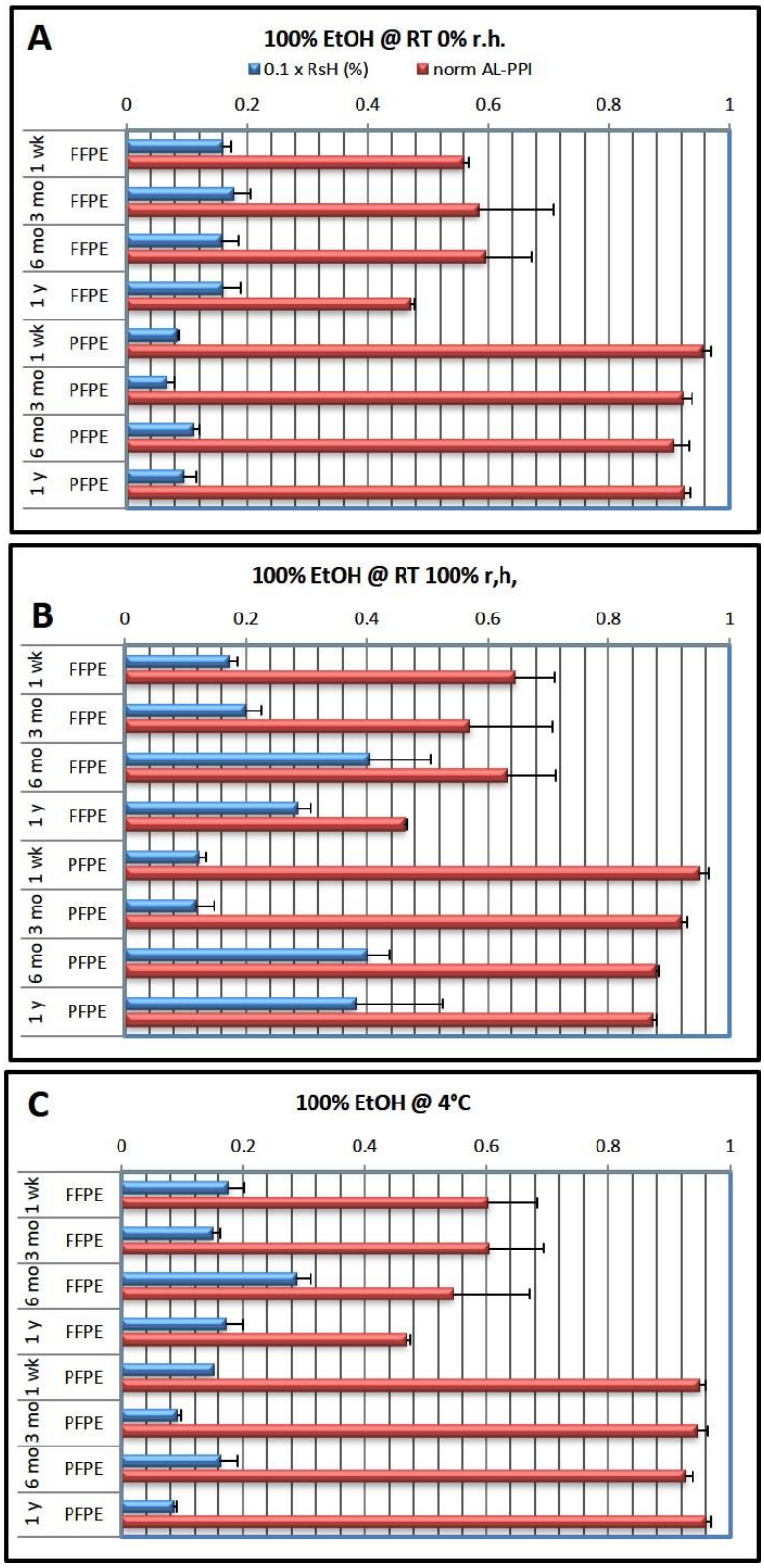

Fig. 1: Effects of fixation type and storage conditions over time using the 100% ethanol dehydration protocol. The last ethanol step in the dehydration protocol was 100% ethanol (cf. Table 2). Blue columns show residual humidity (0.1 x RsH, wt%; factor 0.1 chosen to allow one axis for both parameters), red columns show normalized qRT-PCR performance (AL-PPI; see Methods). Data are shown as mean +/- standard deviation (n=3).

Dependence of RsH and AL-PPI on dehydration protocol

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In the second experimental series (Fig. 2) the last EtOH dehydration step was always performed with 90% EtOH. Results showed that after 1 week of storage, RsH was much higher than after optimal dehydration, both for FFPE (RsH_{FFPE} = 6.1% … 10.0%) and PFPE (RsH_{PFPE} = 3.6% … 6.7%), FFPE tissue thus contained about twice as much residual water than PFPE tissue. RsH remained little changed at RT in dry atmosphere (0% r.h.), but increased over time both at 100% r.h. at RT and at 4°C, both for FFPE and PFPE tissue. Interestingly; a maximum of RsH could be observed after 6 months storage under these conditions.

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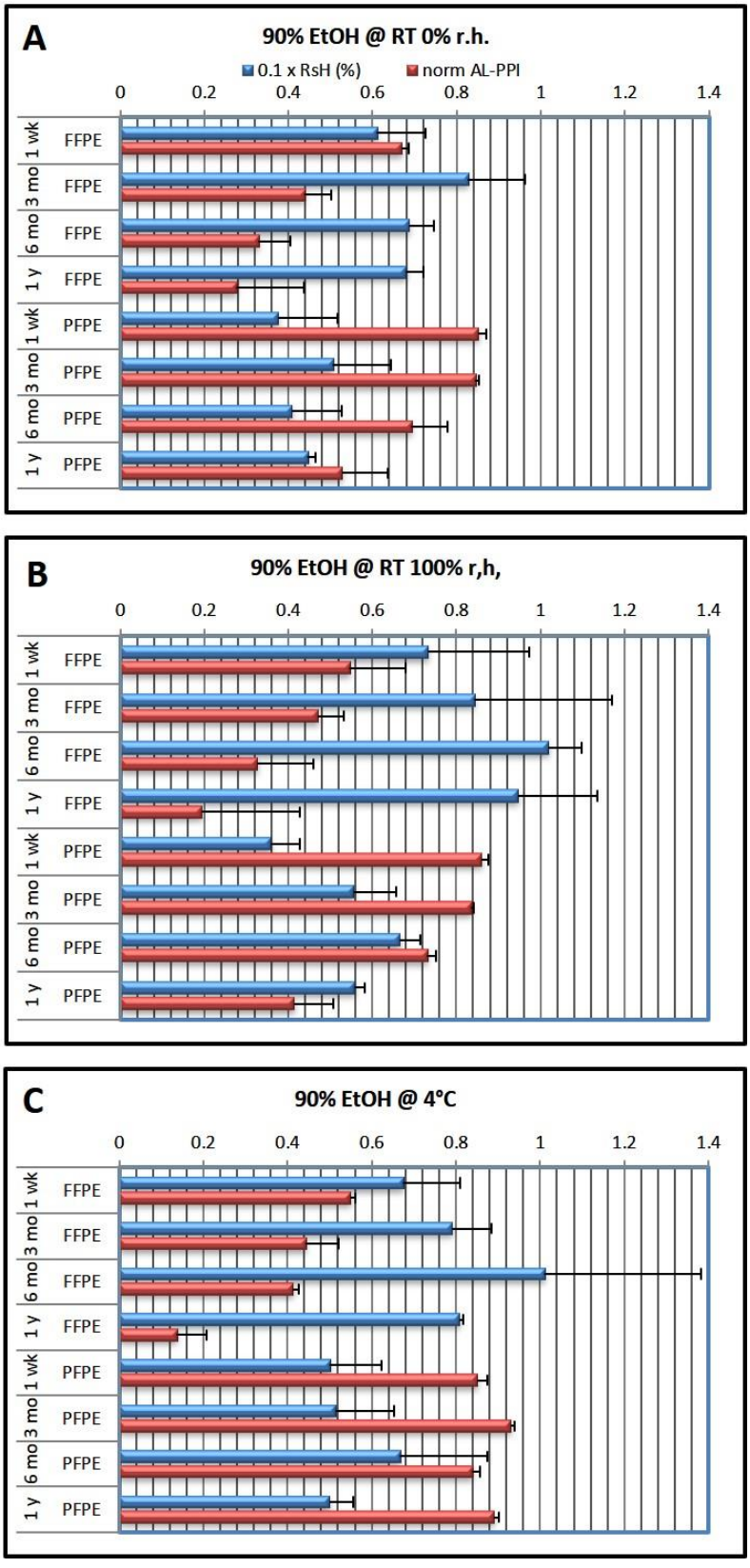

Fig. 2: Effects of fixation type and storage conditions over time using the 90% ethanol dehydration protocol. The last ethanol step in the dehydration protocol was 90% ethanol (cf. Table 2). Blue columns show residual humidity (0.1 x RsH, wt%; factor 0.1 chosen to allow one axis for both parameters), red columns show normalized qRT-PCR performance (AL-PPI; see Methods). Data are shown as mean +/- standard deviation (n=3).

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AL-PPI$_{FFPE}$ decreased strongly over time, from 0.54 … 0.67 at 1 week, to 0.13 … 0.28, nearly independent of storage conditions, and also between 6 months and 1 year of storage, where a decrease in RsH was observed, suggesting consumption of water through hydrolysis of tissue macromolecules, or reversal of fixation [9].

AL-PPI$_{PFPE}$ also decreased over time whenever tissue was stored at ambient temperature, from 0.85 … 0.86 to 0.41 … 0.53. This was not observed for PFPE tissue stored at 4°C, where AL-PPI$_{PFPE}$ remained between 0.85 and 0.93.

To describe better the complex interactions of tissue fixation, dehydration and storage protocols we used multivariate data analysis of RsH and AL-PPI. Principal Component (PC) Analysis (PCA), of RsH and (orthogonal) Partial Least Squares-Discriminant Analysis ((O)PLS-DA) of both parameters showed that RsH and AL-PPI are predominantly affected by storage duration, dehydration protocol, and fixation protocol. Temperature and humidity of storage did not significantly affect RsH, which is in line with the comparable qRT-PCR performance (AL-PPI) for samples stored under these conditions (Fig. 3).

Storage duration (Fig. 3A): PCA analysis (PC1 and PC3) shows a very clear separation of the short- and long-term stored samples – these principal components are clearly attributable to changes of RsH with storage duration. This is corroborated by OPLS-DA, showing that RsH increases significantly after 6 and 12 months of storage and AL-PPI is significantly reduced after 12 months of storage.

Dehydration protocol (Fig. 3B): PCA analysis shows no distinct separation of the different dehydration protocols, however, PC1 and PC2 show a distinct broadening of the RsH data with decreasing ethanol concentration in the last dehydration step (90% > 95% > 100%). OPLS-DA reveals highly significant differences between the RsH and AL-PPI values for the protocols.

Fixation type (Fig. 3C): PCA reveals good separation for all shown PCs, and a distinctly broader distribution of RsH for FFPE samples (PC1 vs. PC2). OPLS-DA shows significantly higher RsH, and significantly lower AL-PPI for FFPE compared to PFPE material.

Storage conditions (Fig. 3D) have no significant influence on RsH and AL-PPI, however, dry storage at room temperature shows a narrower distribution of RsH data (PC1 vs. PC2) than the other storage conditions.

We also performed univariate analyses for the different conditions, separately for FFPE and PFPE tissue (Supplementary Fig. 3), with similar results.

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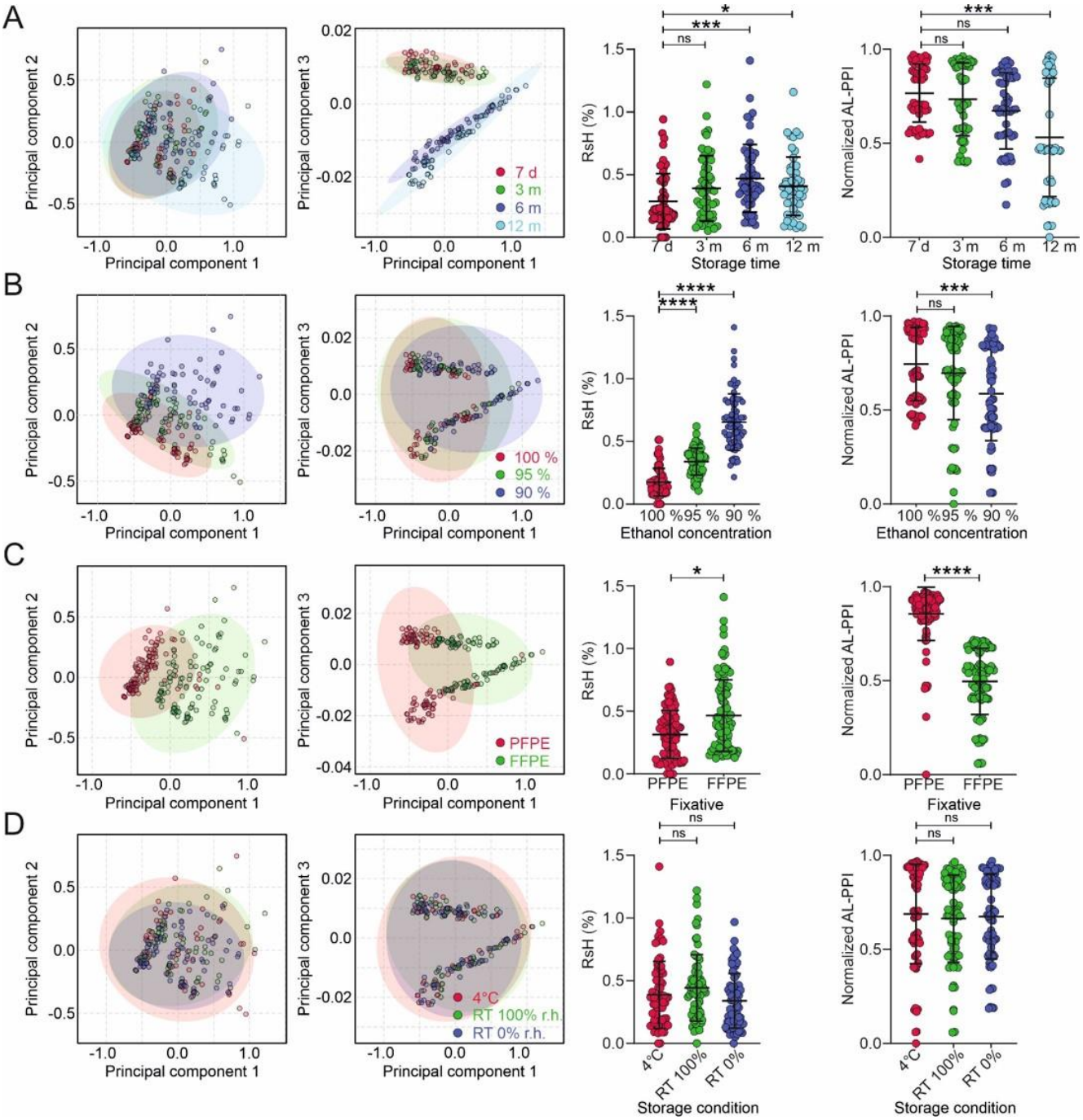

Fig. 3: Multivariate analysis of humidity and qRT-PCR performance. PCA plots of residual humidity (RsH) using storage time (A), ethanol concentration (B), fixation method (C), and storage temperature/humidity as groups (D). A univariate statistical analysis of RsH and normalized PCR performance index (AL-PPI; see supplementary methods) as function of the groups described in A, B, C, and D is shown next to the PCA plots. Data are represented as mean +/− standard deviation (SD). Statistical significance of the differences among multiple groups was determined using one-way ANOVA (Dunnett's multiple comparisons test) or unpaired t-test for 2 groups. p>0.05=ns; p<0.05=*; p<0.01=**; p<0.001=***; p<0.0001=****

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2.3 NMR results

Given the impact of tissue fixation protocols on qRT-PCR performance we hypothesized that (R)NA accessibility and modifications are causing these differences. To obtain insight into potential NA modifications in extracted samples, we used untargeted metabolomic analysis by NMR spectroscopy. Extracted DNA and RNA are large polymers and are difficult to study by solution NMR. To overcome this limitation and to obtain single nucleotides, we performed complete enzymatic digestion of nucleic acids using a mix of alkaline phosphatase, benzonase, and phosphodiesterase.

1D ^{1}H-NMR analysis of the digested nucleic acid samples showed the presence of several NMR signals characteristic for (modified) nucleotides and sugars at different concentrations. Assignment of these signals revealed adenosine, cytidine, guanosine and uridine as the main digestion products. In a subset of samples, additional ribose amplicons, 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, thymidine and base degradation products were detected (Supplementary Fig. 4).

To characterize the impact of tissue fixation and storage protocols on metabolite distribution we used again PCA, and (O)PLS-DA. Similar to RsH and AL-PPI, the metabolite profiles were mainly affected by storage duration, residual water, and the fixation protocol. (Fig. 4).

Storage for 12 months revealed increased heterogeneity (Fig. 4A), indicated by a broader distribution over PC1 and PC2. We observed large changes in two principal components, indicating degradation of the sugar and base moieties, respectively. OPLS-DA analysis of NMR-based metabolite profiles using storage duration as groups revealed a clustering with correlation coefficients R^2Y of 0.519, and a Q^2 of 0.351 ($p < 0.01$; Fig. 4A). Inspection of the OPLS-DA loading profiles revealed 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, and thymidine as significantly increased metabolites. We then compared the impact of the dehydration protocol on the NMR metabolite profiles. As shown in Fig. 4B, increased concentrations of residual water led to more heterogeneous metabolite profiles. OPLS-DA analysis of NMR-based metabolic profiles using 90% and 100% EtOH as groups revealed base and sugar degradation products as significantly increased degradation products in FFPE tissue ($R^2Y = 0.226$, $Q^2 = 0.067$, $p = 0.02$; Fig. 4B). In addition to storage duration and dehydration protocol, the fixation type shows an impact on metabolomic profiles. Using OPLS-DA analysis, we identified decreased concentrations of adenosine, cytidine, guanosine and uridine in FFPE samples ($R^2Y = 0.371$, $Q^2 = 0.310$, $p < 0.01$; Fig. 4C). This can be explained as significantly reduced nucleic acid yield in FFPE tissue, compared to PFPE, after hydrolysis, due to increased cross-linking of nucleic acids, resulting in lower accessibility for the enzymes.

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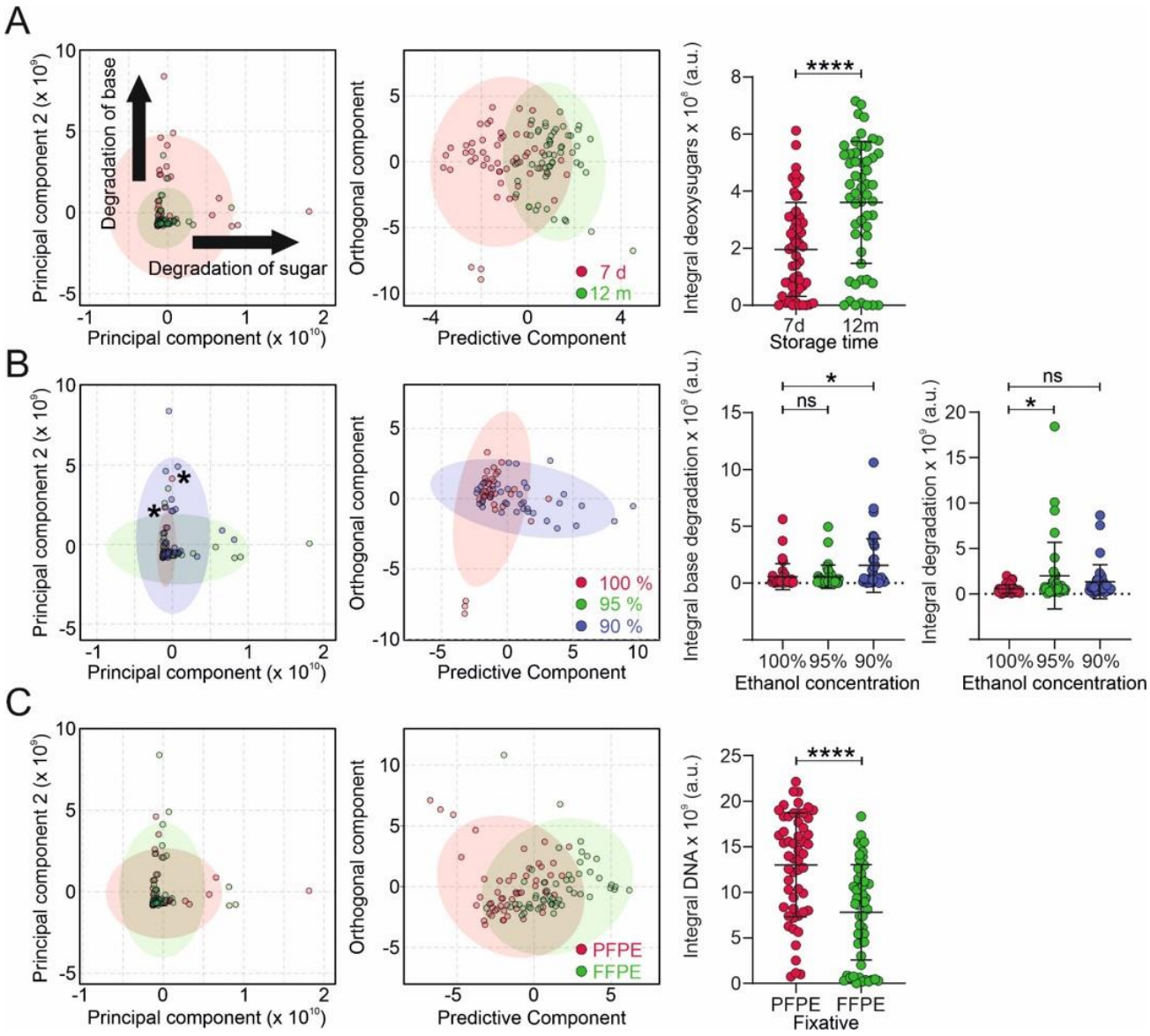

Figure 4. Multivariate NMR-based analysis of digested nucleotide extracts. PCA, PLS-DA and O-PLS-DA plots of NMR samples using storage time (A), ethanol concentration (B), and fixation method (C) as groups. A univariate statistical analysis of normalized metabolite group concentrations associated with significant changes observed in the O-PLS-DA analysis as function of the groups described in A, B, and C is shown next to the PCA/PLS-DA/O-PLS-DA plots. Data are represented as mean +/− standard deviation (SD). Statistical significance of the differences among multiple groups was determined using one-way ANOVA (Dunnett's multiple comparisons test) or unpaired t-test for 2 groups. p>0.05=ns; p<0.05=*; p<0.01=**; p<0.001=***; p<0.0001=****

3. DISCUSSION

In this study we have demonstrated that the type of tissue fixation, the amount of residual water, storage conditions and the duration of tissue storage impact on the quality of NA (DNA, RNA) in tissue. Residual water content is affected by the type of fixation (cross-linking, non-cross-linking) also through the reaction of formalin wit NAs, making them more polar, favoring increased water uptake and retention. Another influencing factor is the fixation protocol (100%, 95%, 90% final EtOH step) and storage conditions (humid, dry, 4°C) in a complex way. Residual water in turn correlates with the quality of RNA, regarding the performance in qRT-PCR, and the stability of nucleic acids in general,

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as shown by hydrolysis products in the NMR analysis. Apart from the fixation type, which affects NA quality strongest, residual humidity is the most prominent factor leading to NA degradation in stored samples.

Fixation with formalin is known to dramatically impair the quality of NA for Next-Generation sequencing, but also for qRT-PCR methods. Formalin oxidatively modifies NA, specifically nucleobases, which leads to sequencing artefacts and reduced performance of qRT-PCR-based techniques. Moreover, it leads to cross-linking of NAs with polynucleotides and polypeptides, which leads to reduced availability of NAs for analysis, which is reflected in the reduced yield in the NA digestion for NMR analysis. In addition to that, our results also show that FFPE tissue shows a high content of residual humidity, due to the higher polarity of the modified NAs and amino acids, and are more hydrophilic during storage in non-dry environment. On the other hand, non-cross-linking fixation methods, like PAXgene® TissueFix used in this study, do not modify NAs and amino acids, leading to lower residual humidity and lower hydrophilicity, resulting in better stability of NAs.

This highlights the importance of a robust tissue processing protocol by which the residual water content is as low as possible, specifically for achieving long-term stability of NAs. Such a protocol features an ethanol dehydration series that concludes with 100% ethanol, before the dehydrated tissue is transferred to the clearance step (usually immersion in xylene) that allows the final paraffin infiltration to proceed evenly and completely. In many routine pathology laboratories such protocols are well established, usually by automated tissue processing machines, and all dehydration and clearing bath solutions are regularly replaced to prevent water accumulating in the final (100%) EtOH bath(s). However, we show here the possible consequences and the dangers that may occur with manual tissue processing, where solvent baths may be changed less regularly (e.g., due to economic pressure or impaired supply chains), or become exposed to air humidity for a long time, leading to the well-known EtOH/water azeotrope.

Residual humidity drastically reduced the performance of qRT-PCR, in particular for FFPE tissue. From a previous study we know that this is mainly due to compromised performance of the reverse transcriptase reaction [5]. By comparison, PFPE tissue generally contained less residual water and showed better qRT-PCR performance. The main reason is that PFPE tissue is not chemically modified, as is the case with formalin. In PFPE tissue the hydration shells of protein and nucleic acid macromolecules are altered, which leads to coagulation *in situ*. This *per se* does not introduce chemical modifications that may increase the polarity of the side chains of amino acids and nucleobases and is the active principle of PAXGene® Tissue Fix and other agents that act by coagulation using water-miscible alcohols and organic acids. Formalin, on the other hand does not only lead to cross-linking, it increases the hydrophilicity by chemical modification [8,9]. Moreover, the major modifications that have been found previously in FFPE tissue (such as hydroxymethylation of nucleobases [9]) are reversible by hydrolysis and the reactive products formed in this process may lead over time to further modifications.

Owing to the specific modifications of the nucleic acids by formalin, the effect of storage time is particularly pronounced for FFPE tissue, while the storage conditions (i.e., temperature and humidity) have only little effect. Nucleic acids in PFPE tissue are generally more stable than in FFPE material, and they are less affected by storage time. Interestingly, we found that NAs from PFPE somewhat benefit from cold storage (4°C), while those from FFPE tissue seem to benefit a little from dry storage at room temperature. Overall, PFPE tissue preserves a much better quality of NAs, that are more stable, and give better yield, than FFPE material.

Comprehensive metabolomics data provide new insights into the mechanistic understanding of the link between tissue fixation protocols and sample quality (using qRT-PCR as read-out). Metabolomic characterization of digested NA samples obtained from differently prepared tissues revealed relevant metabolite markers for sample quality. Our analysis revealed alterations of NA yield, NA dehydration, sugar and base modification in extracted DNA samples. Moreover, we found that the tissue fixation type affects the

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metabolite profile of digested NA samples, and thus their degradation profiles. The related metabolites might be used, and further extended, in follow-up studies as markers in a clinical setting for quality control of fixed tissues and to optimize fixation protocols, which is a difficult task when relying on qRT-PCR only.

Using the residual water measurement principle based on a P_2O_5 sensor we could demonstrate in this study the correlation of residual water on NA stability in stored tissue samples, which can be explained by the fact that even under optimal conditions, not all the water is removed during tissue processing. A small fraction of water remains bound to membranes, NAs and proteins as an integral part of the secondary and tertiary structure that cannot be removed without collapsing it. Indeed, removing this 'structural water' requires comparatively harsh methods, such as heating to temperatures that may already lead to the chemical decomposition of the molecules. However, it is unlikely that structural water participates in widespread degradation processes since it is strictly localized. In addition, water forms a hydration shell around biological macromolecules and assemblies, such as cell membranes. In addition there is interstitial water (extracellular) whil is even is more mobile and can partake in hydrolysis reactions, similar to water in the hydration shell. These types of water are largely removed during tissue processing, completing the precipitation process that was initiated by the fixative, and to allow infiltration of the hydrophobic paraffin. In native tissue, more water is present that is not directly engaged in hydrating macromolecules (often not quite correctly termed 'bulk water'), but contributes to the mobility of the macromolecules and participates in exchanges with the hydration shell. Bulk water, too, is removed during tissue processing. During storage, water from the surrounding air is in equilibrium with the paraffinized tissue (there is likely exchange through micropores and –cracks) and becomes loosely adsorbed, and is in equilibrium with the residual water. That means that residual water in tissue may be composed of structural water, the hydration shell and bulk water catalyzing hydrolytic degradation of NAs and other biomolecules.

Based on our results we may give recommendations for the preparation of fixed tissue that contains high-quality NAs: (i) if possible, use non-crosslinking fixatives, (ii) ensure that the dehydration protocol is meticulously observed - specifically the last dehydration step must not be compromised by > 1% water in ethanol, and (iii) store the samples as briefly as possible, cool and in dry atmosphere. Furthermore, our study emphasizes the importance to consider and document in detail the tissue processing as well as the storage condition and duration of samples used in research projects. This is important, since integrated analysis of data generated from samples of different processing and storage conditions may result in major bias in the results obtained. We also recommend normalizing study groups for the storage duration of biobanked or archived samples.

Otherwise, in particular with FFPE tissue, setting up a study in which 'young' and 'old' samples are mixed, and where dehydration protocols were not precisely followed or not comparable may lead to high variation in NA analyses, and in the worst case to wrong results. In NGS analyses such samples will lead to a large dropout rate due to failed quality checks, favoring 'young' samples.

4. Materials and methods

Unless specified otherwise, all chemicals used were by Merck or Sigma-Aldrich (Vienna, Austria) and were of analytical grade or better.

4.1 Experimental design

Tissue samples were subjected to different fixation, dehydration and storage protocols as given in Table 1. For details of the protocols see below.

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Tissue collection

We used mouse liver tissue to have proper control over pre-analytical parameters like the interval between tissue harvesting and onset of fixation (cold ischemia). Furthermore, liver is a large organ with relatively homogenous composition that allows obtaining several aliquots from a single organ.

Mice (Swiss albino Him-OF-1) were kept on standard chow (Ssniff, Spezialdiäten GmbH, Soest, Germany) under identical standard conditions (controlled humidity and temperature, 12-hour day / night cycle) until sacrifice, by cervical dislocation followed by liver harvesting within 1-2 minutes. Isolated livers were rinsed in ice-cold phosphate-buffered saline and the lateral and medial lobes grossed into 3 equally sized portions which were immediately transferred to a labelled tissue cassette and immersed in the respective fixative. For reference, aliquots were snap-frozen in isopentane precooled with liquid nitrogen, and stored in liquid nitrogen until use. The whole workflow required 4-5 minutes for each animal.

Organ harvesting does not require a specific animal experimentation license by Austrian law, since approved conditions for housing, feeding and killing of the animals were in place (license for the facility BMWF-66.010/0147-V/3b/2018).

Tissue fixation, processing and storage

After 24 hours of fixation in either 10% neutral-buffered formalin (NBF; corresponding to 4% v/v formaldehyde in phosphate buffer) pH 6.8-7.2 or 4 hours in PAXgene Tissue Fixative (PGTS) followed by 24 hours of stabilization in PAXgene Tissue Stabilizer, the tissue was dehydrated in ethanol solutions, cleared in xylene and impregnated with low-melting paraffin in a Microm STP 120 tissue processor that was specifically reserved for this experiment – fresh solutions were prepared before processing the batches for a storage time-point.

To avoid contaminating the dehydration reagents with formalin, NBF tissue was immersed in a separate 70% ethanol bath for at least 1h prior to processing; processing was performed separately for NBF and PGTS tissue.

The three protocols used for dehydration, clearing and paraffin infiltration are shown in Table 2. They only differed in the ethanol concentration of the last two ethanol steps (7, 8) before clearing and infiltration with low-melting paraffin. After processing, the tissue was embedded completely in paraffin wax and stored for the appointed storage duration. For each storage duration (1 week, 3 months, 6 months and 1 year) three batches of 18 samples each were processed on three consecutive days. For each of the batches one of the final ethanol concentrations was used for steps 7 and 8 while the other steps (1-7, 9-12) remained unchanged (54 samples were processed in total).

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Table 2: Tissue dehydration, clearing and paraffin infiltration protocols used in this study.

Protocol:		*100% Ethanol*	*90% Ethanol*	*95% Ethanol*
Step	**Time**	**Media**		
1	15min	70% Ethanol	70% Ethanol	70% Ethanol
2	15min	70% Ethanol	70% Ethanol	70% Ethanol
3	30min	80% Ethanol	80% Ethanol	80% Ethanol
4	1h	90% Ethanol	90% Ethanol	90% Ethanol
5	1h	96% Ethanol	96% Ethanol	96% Ethanol
6	1h	96% Ethanol	96% Ethanol	96% Ethanol
7	1h	*100% Ethanol*	*90% Ethanol*	*95% Ethanol*
8	1h	*100% Ethanol*	*90% Ethanol*	*95% Ethanol*
9	1h	Xylene	Xylene	Xylene
10	1h	Xylene	Xylene	Xylene
11	1h30min	Paraffin (55°C)	Paraffin (55°C)	Paraffin (55°C)
12	1h30min	Paraffin (55°C)	Paraffin (55°C)	Paraffin (55°C)

After embedding, the FFPE and PFPE blocks were transferred to the appropriate storage environment. Blocks were kept in airtight polyethylene boxes either at room temperature (RT; monitored/controlled, 22 – 25°C) or at 4°C in the refrigerator, unopened until processing. Storage at RT was either under dry conditions (desiccant – MiniPax desiccant packages, Sigma Aldrich) or at 100% (humid chamber, not in direct contact with liquid). Storage at 4° was performed without specific humidity control. Table 1 gives an overview on storage conditions and duration. Since the storage boxes were not opened during the whole storage time we did not observe mold formation even after one year of storage at 100% r.h. (however, it became noticeable under these conditions several months after opening).

Tissue sectioning for humidity measurement and RNA extraction

For humidity measurement, paraffin wax surrounding the tissue was carefully removed and tissue blocks sectioned into 20 µm (nominal setting on the microtome; HM355/Histocom) flakes as a compromise between sufficiently fast water diffusion during heating (see below) and rapid handling. 20 mg flakes (approximately; precisely weighed) were placed into a 5 mL crimp-sealed vial with Teflon®-sealed stoppers immediately after sectioning and weighing (RotiLabo crimp-sealed, long-necked flasks, Roth, Vienna, Austria).

Immediately after sectioning for humidity measurement, 5-10 mg of 5 µm sections were prepared on the microtome for NA extraction. RNA was extracted using RNeasy FFPE Kit (QIAGEN GmbH, Hilden, Germany) for FFPE, the PAXgene Tissue RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) for PFPE, or the Invitrogen TRIzol procedure (Life Technologies, Darmstadt, Germany) for snap-frozen tissue controls according to the manufacturer's instructions.

The RNA concentration in the extracts was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies-ThermoFisher Scientific, Wilmington, DE), and electropherograms were obtained using an Agilent 2100 Bioanalyzer platform with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). Agilent 2100 Expert software version B.02.03.SI307 was used to calculate the RNA integrity number (RIN).

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Additionally, total nucleic acids were extracted using QIAamp™ DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Germany) for FFPE and the PAXgene Tissue DNA Kit (PreAnalytiX) for PFPE, without RNAse digestion. The nucleic acid concentration in the extracts was determined using a NanoDrop ND-1000 spectrophotometer.

Humidity measurement

Tissue humidity was measured in an instrument based on the EasyH_2O™ humidity measurement instrument that was provided by Berghof GmbH (Eningen, Germany). It was modified to avoid clogging of tubing and sensor by deposition of evaporated paraffin. The measurement principle is based on a P_2O_5 sensor [18], which detects water by hydrolysis of P_2O_5 to metaphosphoric acid, H_2PO_4, and subsequent electrolysis of the water bound in this fashion, which regenerates P_2O_5. The charge required for electrolysis is directly proportional to the water bound to the sensor and was recorded as electrolysis current over time. The instrument performance and accuracy were checked before and after each measurement series (9-15 measurements), with apura®Water Standard Oven 1% (Na_2WO_4 x 2 H_2O, Merck; 15 – 25 mg; 1% wt/wt water content). Deviations from the nominal value were always below 2%.

Water was released from the sample by raising the temperature and water vapour is transferred to the sensor by means of a stream of pressurized air, dried by passing through a molecular sieve bed (10 Å beads, Roth, Karlsruhe, Germany, bed volume 1 L). Temperature was controlled by heating the vial with the sample in an air bath heated by a resistance heater. By controlled increase of the temperature it is possible to distinguish between loosely adsorbed ('ambient') water (released below 70°C) and water more tightly bound in the hydration shell (released above 100°C) [19,20]. Water released was calculated as percentage of original tissue weight, including infiltrated paraffin (residual water, RsH). For a typical water release curve see Supplementary Figure 2.

Humidity data were evaluated using Origin Pro 2.0 (Origin Labs, Germany), using the specific curve fitting, extrapolation and integration functions. A detailed description of data evaluation is given in Supplementary Methods.

Fragment length dependent quantitative RT-PCR on mouse liver

We have previously described an amplicon-length dependent performance assay for qRT-PCR [5] in human tissue where we exploited the differences in amplification of GAPDH to rate the RNA quality. This method allows a finer graded assessment of RNA quality than the RNA integrity number does, in particular for FFPE tissue. However, the mouse genome contains several GAPDH iso- and pseudogenes which precluded using GAPDH as a reference gene. Instead, we used the mouse gene *ywhaz* that appeared well-suited to the task [21,22], using five amplicons of lengths from 84 to 526 bases. Details about the protocol are shown in the supplement, and primer sequences are shown in Supplementary Table 1.

Evaluating performance in a amplicon-length dependent qRT-PCR assay – amplicon-length performance index (AL-PPI)

We and others have previously demonstrated that qRT-PCR is quite sensitive to RNA quality [5,11,23] which is reflected by increased C_q values for longer amplicons, or even no result at all. Although these data give sufficient distinction between the quality of dif-

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ferent NAs, they are difficult to use in statistical analyses directly. We have therefore empirically designed an amplicon-length qRT-PCR performance index (AL-PPI) that integrates the relation of C_q with amplicon length. Details on the calculation of this index are given in the supplement, an illustration of the empirical match between this index and the amplicon length qRT-PCR data is shown as Supplementary Figure 2.

Data evaluation

PCR and humidity measurements were performed in triplicates for both humidity measurements and qRT-PCR performance. RsH and AL-PPI were the parameters used for evaluation. For each triplicate, the mean value and standard deviation were calculated to give a single parameter data point.

NMR sample preparation, data acquisition and analysis

Digestion of NA was necessary to make NAs accessible for NMR experiments as in NA polymers ^{1}H signals are broadened beyond detection. The protocol is described in detail in Supplementary Methods.

NMR spectra of digested NA samples were recorded at 310 K using Bruker Avance Neo 600 MHz NMR spectrometer equipped with a TXI probe head. The Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence was used to acquire ^{1}H 1D NMR spectra with presaturation for water suppression (cpmgpr1d, 512 scans, 73728 points in F1, 12019.230 Hz spectral width, 1024 transients, recycle delay 4 s). NMR spectral data were processed as previously described [24]. Quantification of metabolites was carried out by signal integration of NMR spectra. To account for different DNA concentrations in the extracts, integrals were normalized based on the amount of extracted DNA. Univariate statistical analysis was carried out using Graph Pad Prism 5.01. (GraphPad Software, La Jolla, CA, USA). Data are represented as mean +/− standard deviation (SD).

Statistical differences among multiple groups (one-way ANOVA) are indicated by P-values of < 0.05 (*), < 0.01 (**), or < 0.001 (***). A detailed description of the procedures is given in the Supplement.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1: Supplementary Methods: qRT-PCR measurements of *ywhaz* in mouse liver; Analysis of water release from fixed, paraffin-embedded tissue; Definition and calculation of the performance index for amplicon-length dependent qRT-PCR (AL-PPI); Preparation of nucleic acid samples for NMR analysis; Figure S1: Water Release from FFPE Tissue; Figure S2: Amplicon-length dependent qRT-PCR and the AL-PPI; Figure S3: Univariate analysis of humidity and qRT-PCR performance: Figure S4: NMR characterization of digested nucleotide extracts; Table ST1: Sequences, melt tables and length of the products.

Author Contributions: Conceptualization: PMA, ML, CU, RK, TM, KZ

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Investigation: PMA, DP, BB, ML, CU, IK, UF, RK, TM

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Project administration: PMA, ML, TM

Supervision: PMA

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Institutional Review Board Statement: Organ harvesting does not require a specific animal experimentation license by Austrian law, since approved conditions for housing, feeding and killing of the animals were in place (license for the facility BMWF-66.010/0147-V/3b/2018).

Data Availability Statement: All data used in this study appear in the paper's text, figures and supplementary material.

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