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Setting out the frame conditions for feasible use of FFPE derived RNA

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ABSTRACT

Introduction: The usage of formalin-fixed paraffin embedded (FFPE) tissue is characterized by its long shelf-life and simple handling. Therefore it is the most commonly available tissue specimen in routine diagnostics and histological studies. Formaldehyde fixation may result in RNA degradation and cross linking with proteins, while storage conditions also affect RNA integrity. The present study was designed to investigate the influence of these factors on RNA analysis.
Design: FFPE-derived RNA from sections of 23 patients with spontaneous pneumothoraxes was used. Unstained sections of FFPE tissue were stored at various temperatures (−80 °C, −20 °C, 4 °C, 24 °C) prior to RNA extraction. The potential impact on RNA quality of semi-automatic and manual RNA isolation and three different deparaffinization agents (mineral oil, xylene and d-limonene) were compared.
Results: The storage temperature of FFPE sections affects RNA concentration and fragmentation, with the optimal storage temperature below -20 °C. The RNA extracted with d-limonene shows equivalent quality to the RNA extracted using more toxic standard agents. The manual isolation provides a higher RNA yield compared to the semi-automatic isolation. However, no differences in the amount of longer RNA fragments were observed. Furthermore, the semi-automatic isolation showed an enhanced RNA quality.
Conclusion: FFPE sections not directly used for RNA extraction should be stored below -20 °C to increase quality and yield of the RNA. Usage of semi-automatic isolation produces superior results and simplifies routine processes by having less hands-on-time. Replacement of toxic xylene by d-limonene may contribute to improved occupational safety while not influencing analytical results.

1. Introduction

Fixation in formaldehyde followed by paraffin embedment is the main method for preservation of human tissue specimens intended for use in medical diagnostics. Formalin-fixed paraffin embedded (FFPE) tissue is characterized by convenient and cost-effective storage at room temperature, minimal logistical and energetic effort [1]. They preserve tissue integrity and are thus suitable for immunohistochemical staining, morphology analyses [2] as well as for nucleic acid preparation including RNA.

Nowadays, RNA is an important tool, not only in research but also in diagnostics, enabling precise and detailed statements of cellular gene expression pattern, e.g. of cancer driving genes. There are various RNA isolation methods for FFPE. Most commonly used are silica bead-based ion exchange and paramagnetic beads based RNA-extraction methods [3]. Pure chemical precipitation is a rare.

Nevertheless, the feasibility and reliability of RNA acquired from formaldehyde fixated tissue is discussed controversially [2,4,5]. Formaldehyde fixation leads to massive degradation, cross-linking with proteins and alteration of the RNA [4], while storage conditions and

Abbreviations: AATI, Advanced Analytical Fragment Analyzer; D-Lim, d-limonene; FFPE, Formalin-fixed, paraffin-embedded; RNA, ribonucleic acid; RIN, RNA integrity number; RQN, RNA quality number; RT, reverse transcription; RT, PCR - reverse transcription polymerase chain reaction; qPCR, quantitative polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; Ct, cycle threshold; RFU, relative fluorescence units; MAK, threshold limit value ("Maximale Arbeitsplatz-Konzentration")

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exposure to oxygen also effect the RNA integrity [6]. The RNA integrity is often determined electrophoretic via Bioanalyser™, TapeStation™ or Fragment Analyzer™, as it is important information for further analyzes like RNA sequencing. Additionally, to formalin-dependent chemical modifications, the storage of the thin FFPE sections can reduce the integrity by increasing the surface and thereby exposing the RNA even more to environmental impacts. This can result in additional oxidation on the RNA. However, no distinct core set of rules is figured out for feasibly and reliable use of FFPE derived nucleic acids, especially for routine diagnostic purposes. The aim of the study was

1 to determine the optimal storage conditions for the FFPE tissue sections
2 to compare two different RNA isolation methods
3 to compare the standard deparaffinization agents with the more environmental friendly D-limonene

2. Materials and methods

Retrospectively collected formalin-fixed, paraffin-embedded (FFPE) tissues from twenty-three patients with spontaneous pneumothorax were used for the present study. These specimens are particularly well suited for this type of study since they are derived from mostly young, healthy patients without further pathological changes and neither show necrosis nor other changes. Another selection criteria was the minimal to none lymphocyte infiltration. Three of the samples were processed comparing different temperature profiles and durations of the already prepared tissue sections as well as different RNA storage conditions. Additionally, 20 patient samples for each method as well as RNA derived from a human fibroblast cell line (MRC-5) were selected for the method comparison, checking two different RNA isolation methods for their feasibility and reliability in routine procedures as well as the integrity of the resulting RNA, respectively.

2.1. Patient and public involvement

No patients or the public were involved in the development of the study questions or design and conduction of the study.

2.2. Study design

From each tissue block, two sections of each 10 µm thickness per sample were cut by a microtome (HM340E, Microm International GmbH – Thermo Fisher Scientific, Waltham, MA, USA) for further usage. Prepared sections were stored for one week at four different temperatures (−80 °C, −20 °C, 4 °C, 24 °C) previous to the RNA isolation in closed tubes. Triplicates were processed for each sample and temperature condition. After the most appropriate storage condition was identified, the samples for the second part of the study were stored at the determined optimal storage temperature (−20 °C). Subsequently, combinations of three different deparaffinization (d-limonene; xylene; mineral oil) and two RNA purification methods (Promega Maxwell RSC RNA FFPE KIT (Promega, Fitchburg, USA), Qiagen RNeasy FFPE KIT (Qiagen GmbH, Hilden, Germany)) were compared. An overview of the used methods is depicted in Fig. 1. FFPE tissue was compared to fresh samples, which were derived from a human fibroblast cell line (MRC-5). Cell culturing and harvesting was performed as described elsewhere [7]. Patient samples were recruited between 2013 and 2014 from the Biobank of the Institute of Pathology, University Hospital Essen, University Duisburg-Essen, Germany. The study was approved by the ethics committee of the Medical Faculty University Hospital Essen, University Duisburg-Essen, Germany (Ethics approval 13-5382-BO). The investigation conforms the principles outlined in the Declaration of Helsinki, and all patient samples were anonymized.

2.3. d-limonene and mineral oil deparaffinization for Promega Maxwell

1 ml d-limonene (SigmaAldrich, Merck, St. Louis, USA) was added to each sample. The samples were vortexed (VWR, Pennsylvania, USA) for 15 s and incubated for 2 min at 24 °C, then centrifuged (Eppendorf 5430, Hamburg, GER) for 2 min, 18.213 g (max speed) at 24 °C. The d-limonene was discarded and 1 ml ≥ 99.5% ethanol (Sigma-Aldrich) was added per sample. The samples were gently shaken for 15 s, 300 rpm and incubated for 2 min at 24 °C, subsequently the ethanol was discarded. The samples were then incubated with open lid for 7 min, 56 °C and 300 rpm (ThermoMixerC, Eppendorf). 1 ml of 3% 1, 2, 3-propanetriol (SigmaAldrich) was added per sample and incubated for 30 s at 24 °C. The samples were centrifuged for 2 min, 18.213 g at 24 °C and the propanetriol was discarded. Afterwards the isolation was performed as described by the manufacturer. Mineral oil deparaffinization for Maxwell was performed as described by the manufacturer (Technical manual TM436 12/14).

2.4. Xylene and d-limonene deparaffinization Qiagen RNeasy

1 ml isomers of xylene (AppliChem, A0663, 2500, Darmstadt, GER) and 1 ml d-limonene (Sigma-Aldrich) was added per sample, respectively, then vortexed for 10 s and incubated for 2 min and centrifuged for 2 min, 18.213 g at 24 °C. The xylene was subsequently discarded. 1 ml ≥ 99.5% ethanol was added per sample and centrifuged for 2 min, 18.213 g at 24 °C. Afterwards the ethanol was discarded. The remaining tissue pellet was dried with open lid for 7 min, 56 °C and 300 rpm. All further steps were performed as recommended by the supplier (RNeasy FFPE Handbook 12/2014). All RNA samples were stored at -80 °C until further use.

2.5. RNA measurement and qPCR

RNA concentrations were measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, WA, USA) with the appertaining RNA broad-range assay (Thermo Fisher Scientific, WA, USA) using 2 µl sample input volume. RNA integrity was assessed using a Fragment Analyzer and appertaining Standard Sensitivity RNA Analysis Kit (DNF-471-1 000 (15 nt)) (Advanced Analytical Inc., Ames, IA, USA). cDNA synthesis was performed with the RT RevertAidRT Kit (Thermo Fisher Scientific) using up to 5 µg total RNA per reaction and random hexamer primers as recommended by the supplier. Samples were run on a Mastercycler nexus (Eppendorf) instrument. For the subsequent reactions, cDNA was diluted to 50 ng/µl in nuclease-free water (Promega). The cDNA was stored at -20 °C until further use.

Relative cDNA quantification of *PSMA5*, *CCL18, ACTB and GAPDH* was analyzed by the $2^{-\Delta Ct}$ method. The known tumor markers *CCL18* and *PSMA5* were used as biological negative controls. For normalization purposes, gene expression values were normalized for reference gene expression in each sample. *ACTB* and *GAPDH* were selected as reference genes [1,8]. TaqMan Gene Expression Assays for *ACTB* (Hs01060665_g1); *GAPDH* (Hs02758991_g1); *PSMA5* (Hs00932059_m1) and *CCL18* (Hs00268113_m1) were purchased from Thermo Fischer Scientific. The TaqMan Universal PCR Master Mix - No Amp Erase (Thermo Fisher Scientific) was applied for all qPCR reactions. A set of primers with small amplicon size (< 100bp - ACTB 63bp; GAPDH – 93bp; CCL18 – 83bp; PSMA5 – 84bp) was used, to overcome the limits of RNA degradation [1]. All chosen assays exceed exon-exon boundaries to exclude genomic DNA contaminations. All cDNA samples were diluted to 12.5 ng/µl before qPCR. qPCR and data analysis was performed on a Roche LightCycler II 480 (Roche Applied Sciences, Penzberg, Germany). Each sample was measured as a triplicate. The efficiency of all assays used was calculated by their standard curves using five different concentrations from a pool of all isolated RNAs. qPCR analysis was performed in concordance to the MIQE-guidelines [9,10].

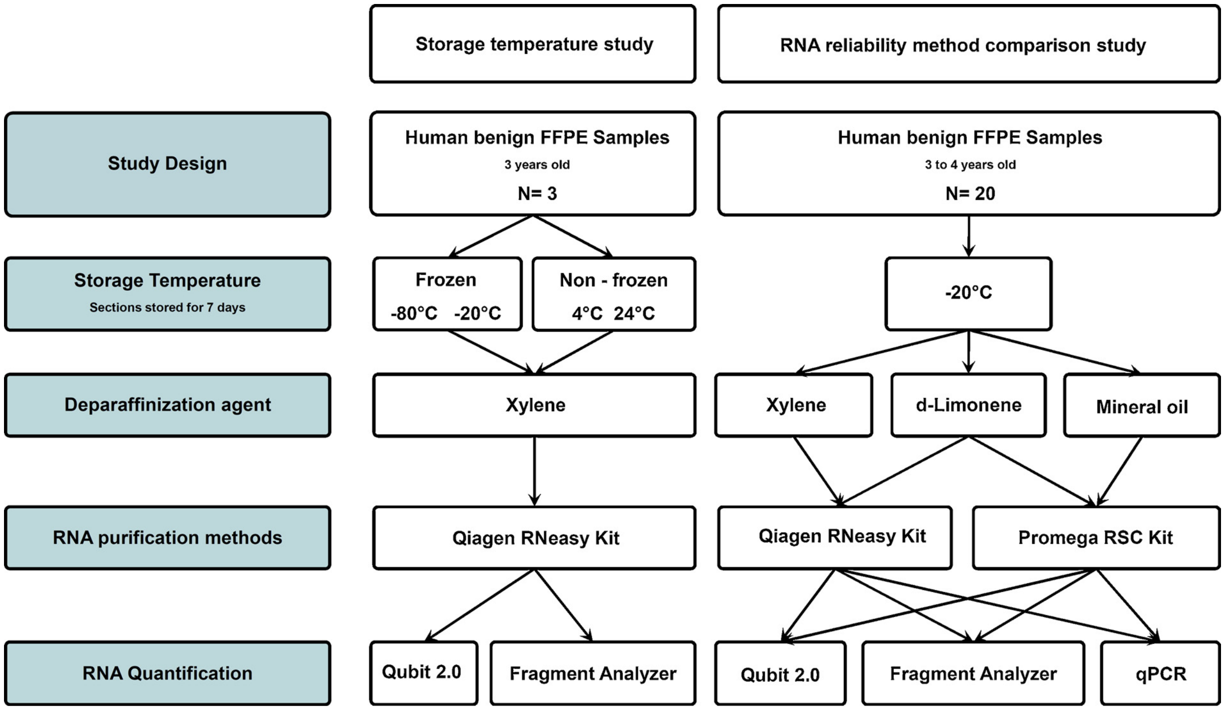

Fig. 1. Method overview flowchart: The two different study layers, storage temperature study (left) and the RNA reliability method comparison study (right) are shown separately. The storage temperature −20 °C was chosen from the storage temperature study for RNA reliability method comparison study.

2.6. Statistical analysis

All statistical analyses were performed using the R statistical programming environment (v3.3.0). The Shapiro–Wilk test was performed to distinguish between gaussian and non-gaussian distribution. For dichotomous factors and ordinal variables, the Wilcoxon signed-rank test, paired for non-gaussian distribution was applied, for gaussian distribution the paired student´s t – test was applied. For variance analysis of variables with more than two categories and an ordinal variable, the Kruskal-Wallis test for non-gaussian distribution was performed. For gaussian distribution, the analysis of variance (ANOVA) test was performed. The level of statistical significance was defined as $p \leq 0.05$.

3. Results

In order to analyze the effect of storage temperature on the quantity and quality of the RNA isolated from human tissue sections from FFPE blocks sections were stored at different temperatures for one week (Fig. 2). The storage temperature influences total RNA concentration and RNA fragmentation. Total RNA concentration as well as the amount of long RNA fragments (150–6000 nt) decreased over the tested temperatures (−80 °C > −20 °C > 4 °C > 24 °C) (Fig.3A and B; p = 0.0175; p = 0.0028). The RNA Quality Number (RQN) as an equivalent to RNA Integrity Number (RIN) also decreased significantly (p = 0.0045) (Fig.3: C). Contrarily, the relative ratio between the RNA fragments smaller than 150 nt and the RNA fragments between 150 and 6000 nt stayed stable over time (p = 0.25) (data not shown).

The different deparaffinization (Xylene; d-limonene and mineral oil) and purification methods (Promega Maxwell; Qiagen RNeasy) had no impact, neither on the purified amount of RNA, nor on the total RNA nor on the long fragment RNA quantity (Fig.3D–F). Regarding the tested purification methods, the total RNA quantity was significantly higher using the Qiagen protocol (Fig.3D: p = 0.0023; p = 0.0073 (d-Lim)). Furthermore, the quantity of long fragment RNA was significantly higher using the Qiagen protocol with xylene-based deparaffinization, compared to the Promega protocol (Fig.3E: p = 0.0087). The sections deparaffinized by d-limonene did not show a significant difference in RNA yield (Fig.3E). The relative ratio of the long fragment RNA to the total RNA was significantly higher using the Promega compared to the Qiagen protocol (Fig. 3F: p = 0.0005; p= < 0.0001 (d-Lim)).

To determine the effect of our results on molecular diagnostics, a reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were performed with the RNA and the cDNA samples respectively. The efficiency of the assays was 96.07% for *ACTB* and 79.80% for *GAPDH* (data not shown). For the negative controls *CCL18* and *PSMA5,* all Ct values were higher than 35 thus no efficiency can be calculated for these transcripts. The Ct values for ACTB and GAPDH (-26 Ct) were not significantly different when the various isolation methods were compared (data not shown).

4. Discussion

Formalin fixed, paraffin embedded tissue (FFPE) sample preparation and storage is a worldwide used biobanking method to preserve patients' tissues. However, longtime storage of FFPE tissue results in a severe negative effect on the RNA quality. Although formaldehyde denatures proteins including RNases [11], it has an overall negative effect on RNA integrity. Besides there are additional known RNA damaging mechanisms like oxidative deamination [12], UVlight and oxygen exposure [13].

The present data indicate a superior feasibility for routine diagnostic procedure of the Promega Maxwell platform compared to the Qiagen RNeasy FFPE kit. Nevertheless both protocols need approximately the same time for a small number of samples, for higher throughput, the semi-automated purification using the Maxwell platform gains advantage facing the Qiagen RNeasy protocol, particularly in overall isolation time as well as in steadily growing hands-on time for isolation. Besides, the Maxwell platform gave the higher RNA quality as most important indicator for sufficiency of subsequent molecular biological analysis [14]. We could verify the results of Seiler et al. showing a higher yield of total RNA is when using the Qiagen Kit in comparison to the Maxwell 16 platform as forerunner of the RSC. However, the Maxwell platform gave the higher RNA quality [14]. Despite the lower

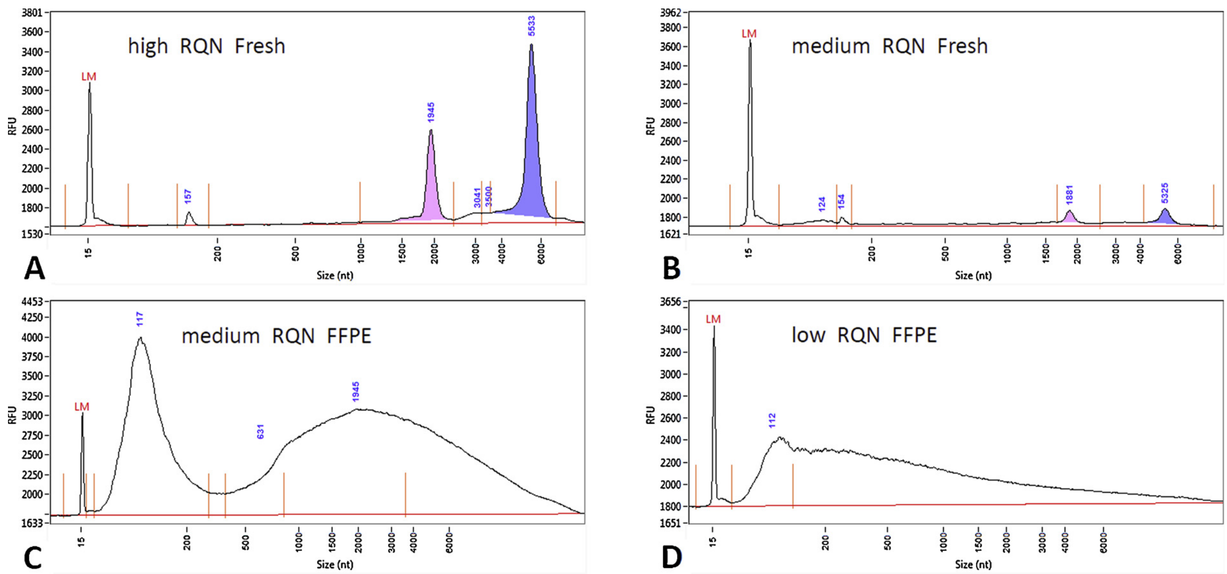

Fig. 2. Exemplary electropherograms of different RNA samples measured by AATI Fragment Analyzer. A) shows an ideal curve with a high RNA Quality Number RQN [10] while B) shows a medium RQN (6.4), both taken from freshly isolated RNA derived from human fibroblast MRC-5 cells. C) shows a moderate [5] while D) shows a low RQN (1.7) taken from human spontaneous pneumothorax samples, stored as formalin-fixed paraffin-embedded (FFPE) tissue for 3 years. The blue peaks indicate the 28 s rRNA while the pink peaks indicate the 18 s rRNA, respectively. The tissue stored as FFPE at room temperature (C and D) leads to massive degradation of the RNA compared to the fresh, directly processed samples (A and B). In the FFPE samples, no rRNA peaks are definable. The y-axis depicts the relative fluorescence units (RFU) and the x-axis the RNA fragment size in nucleotides (nt).

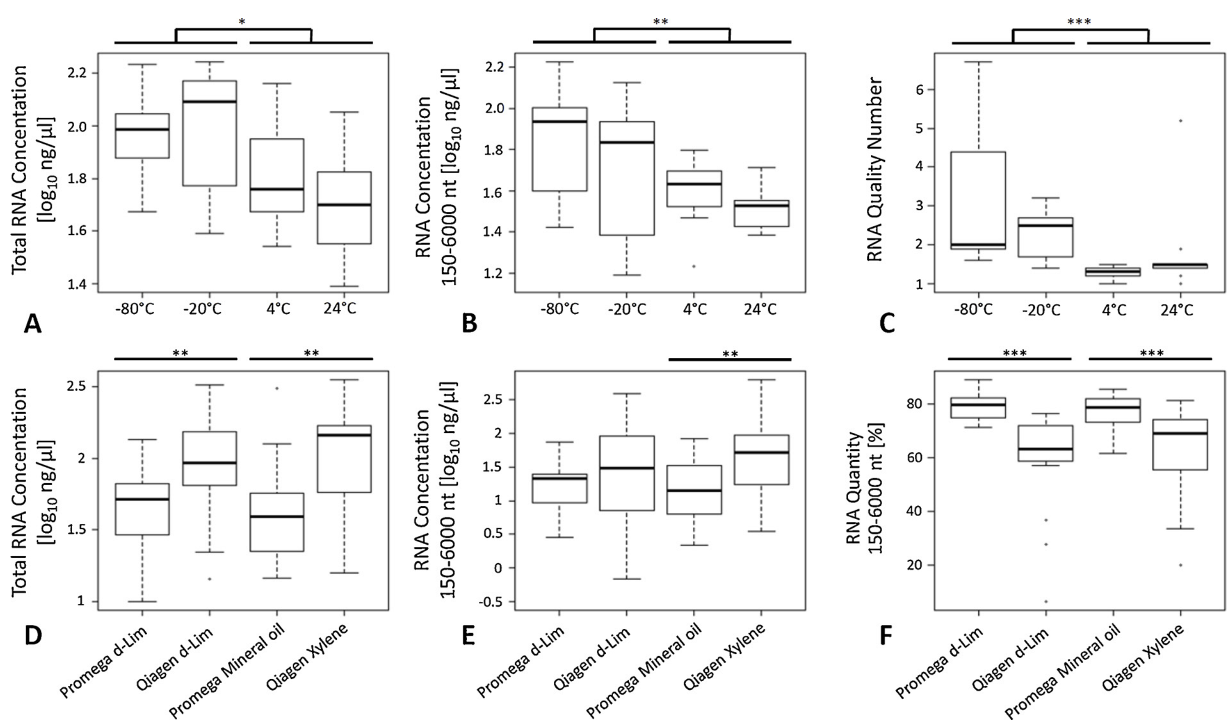

Fig. 3. Comparison of the RNA quantity and quality at different storage temperatures (A–C) and between the different deparaffinization and purification methods (D–F). The total RNA concentration was measured by Qubit 2.0 (A; D) while the rest (B; C; E and F) was measured by using AATI Fragment Analyzer. All formalin-fixed paraffin-embedded (FFPE) tissue specimens were stored for three years at ambient temperatures. A–C: The FFPE sections were stored for one week at different temperatures and the purification was performed using the Qiagen FFPE RNA Kit - Xylene protocol. Frozen samples (−80 °C; −20 °C) showed a higher total RNA concentration (A: p = 0.0175) and concentration of long fragments (150–6000 nt) (B: p = 0.0028) compared to non-frozen samples (4 °C; 24 °C). The same was observed for RNA quality (RQN, C: p = 0.0045). D–F: The sections were stored at −20 °C for one week, deparaffinized by different methods (xylene; dlimonene; mineral oil) and purified with either Promega Maxwell RSC RNA FFPE Kit or the Qiagen FFPE RNA Kit, respectively. D) The total RNA quantity was higher using the Qiagen Kit regardless of the deparaffinization agent, while (E) the long fragment RNA quantity (150 6000 nt) was only higher using the Qiagen – Xylene protocol (D; E: all $p < 0.009$). F) The relative ratio of the long fragment RNA to the total RNA was higher using the Promega Maxwell protocol (F: all $p < 0.0006$). *, **, *** indicate paired student's *t*-test or Wilcoxon signed-rank test p-values < 0.05, 0.01 and 0.001, respectively.

total yield, the Maxwell RSC protocol provides sufficient RNA even from small tumor biopsies for routine molecular diagnostic procedures. Of note, both methods have comparable costs regarding the RNA isolation for one sample when the purchase of the Promega Maxwell device is not taken into consideration.

The Promega kit takes advantage of the RNA electronegativity using paramagnetic particles to achieve purification. Therefore, the purification efficiency of short RNA fragments or miRNAs using the Maxwell platform is lower than the purification efficiency of longer nucleotides, while the Qiagen Kit as a commonly used silica bead-based ion-exchange extraction method is designed to isolate total RNA molecules longer than 70 nucleotides. This explains why there are no small fragments detectable when using the Promega kit (Fig. 3 F). This makes the Qiagen kit an important and reliable alternative for specific problems and study designs, focusing on the analysis of smaller RNA fragments.

Our data indicate that there is no significant difference regarding the deparaffinization agent on the RNA quality between d-limonene and the standard agents recommended by the different kits, as all used agents lead to efficient deparaffinization of the samples. As standard deparaffinization agent, the Qiagen RNeasy protocol uses Xylene (435 mg x m^{-3} MAK) and the Promega Maxwell protocol uses mineral oil. Both agents are considered carcinogenic, while deparaffinization with d-limonene (40 mg m^{-3} MAK) as alternative reagent is more environmentally friendly and less toxic to humans [15,16]. Therefore, we highly recommend replacing the common used agents by d-limonene, although d-limonene is about 005 € more expensive as e.g. xylene per each sample. Just to mention, another advantage of d-limonene worth mentioning is the rather pleasant scent compared to xylene. Nowadays, there are also several other substitutes for xylene described as well as chemical free methods based for instance on microwaves [16,17].

Although it is commonly acknowledged in the scientific community that sections should be either processed immediately or stored at low temperatures to avoid RNA degradation [18], published data supporting this empirically recordable finding is limited. Our data indicate that the storage temperature of the FFPE sections, a so far neglected factor, also influences the total RNA yield and RNA fragmentation processes. Besides, Maldegem et al. showed that tissue storage for RNA had no effect on the RNA quantity. There was no significant RNA degradation detectable up to 18 h, not even when the tissue was stored at room temperature [19]. The difference to our results might be explained by the storage duration (long-term vs. short-term).

Regarding RNA quality, it is important to reduce the time to a minimum between sample acquisition and fixation [4]. Moreover, if the fixation takes longer than 24 h, the samples are overfixed and this decreases significantly the RNA quantity [4]. Tissues fixed with formaldehyde at 4 °C reveal the least amount of RNA degradation [20], while our samples were fixed at room temperature. Unfortunately, for most of the FFPE samples no data are available concerning the exact fixation conditions, since at the time of sample acquisition the effect of the fixation conditions was of no relevance.

Isolated RNA is unstable and vulnerable to degradation. To overcome this obstacle the RNA has to be transcribed to cDNA [4]. For subsequent use by e.g. qPCR, the cDNA needs to have, depending on the used assay, a minimum length of at least 150 nt and a reasonable RIN. Both isolation methods are equally capable to extract even longer RNA fragments. For the RT and qPCR applications, our data indicate, assuming the concentration of the used RNA or cDNA input is the same to each reaction, the RNA isolation procedure from the FFPE sections has no influence on the Ct values of the PCR.

5. Conclusion

The feasibility and reliability of FFPE derived RNA for research studies and routine diagnostic procedures could be demonstrated in this study. Furthermore, we could prove the eligibility of d-limonene as an agent for deparaffinization with less environmentally toxic impact. Regarding RNA isolation, both isolation techniques are likewise useful with advantages and disadvantages depending on throughput and follow-up analysis, as both methods show only minor differences in RNA quantity and quality.

From the routine diagnostic point of view, the Promega Maxwell platform provides advantages such as higher level of standardization, less hands-on time and the quality of the RNA is higher. Analysis parameters such as amplicon-length have to be adapted and selected carefully when processing FFPE derived RNA in order to achieve robust and reliable analysis.

Compliance with ethical standards

This retrospective study was approved by the Ethics Committee of the Medical Faculty of the University Duisburg-Essen (Identifier: 14-5775-BO). The investigation conforms to the principles outlined in the declaration of Helsinki.

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Author's contributions

Sample acquisition was performed by JW and TM. Study design was outlined by KS, RW and FM. Cell culture experiments were performed by SB. Experimental execution was performed by JS. Data analysis was mainly performed by JS, MW and FM. Histomorphological assessment was performed by TH, TM and JW. Covariate acquisition was performed by MW, EM and SB. Manuscript preparation was mainly performed by JS, KS, TH and FM. All authors reviewed and revised the manuscript. RW and FM coordinated the project. Ethics committee's approval was obtained by JW, FM and RW.

Conflict of interest

All authors state that they have no conflicts of interest to declare.

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