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ORIGINAL ARTICLE

Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses

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Abstract Tissues used for clinical diagnostics are mostly formalin-fixed and paraffin-embedded (FFPE) which provides many advantages. However, the quality of the obtained nucleic acids (NA) is reduced and this turns out to be a challenge for further molecular analyses. Although the spectrum of analyses of NA extracted from FFPE tissue has increased, the standard operating procedures for NA isolation from old tissue blocks still need to be improved. Here, we compared the efficiency of different NA extraction methods, using FFPE tissues of variable age and origin, with respect to downstream analyses. Our study showed that the phenol–chloroform isoamyl alcohol (PCI) and the commercial Qiagen protocol yielded samples with highest purity. The PCI protocol delivered the longest amplicons even from samples from the 1970s. We developed a short (1 h) tissue lysis procedure that turned out to be highly time- and cost-effective when DNA quality was tested using single and multiplex PCR. Compared to a 1-day lysis-protocol, the amplicons were only 100 bp shorter. In addition, single-copy genes used in daily routine were successfully amplified from long-term stored FFPE samples following 1-h tissue-lysis. The RNA integrity numbers (RIN) determined on RNA isolated from FFPE tissues indicated degraded RNA; however, all RINs were above the generally agreed threshold of 1.4. We showed that, depending on the purpose of the analysis, NA retrieved from FFPE tissues older than 40 years may be successfully used for molecular analysis.

Keywords Long-term preserved FFPE tissue · DNA · RNA · Rapid tissue lysis · PCR

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Introduction

In pathology archives, tissue samples are stored as formalin-fixed paraffin-embedded (FFPE) blocks, a common way to preserve specimens for a longer time period. FFPE tissue is relatively stable for several decades allowing applications for retrospective NA analyses, such as for prediction of therapy response and clinical outcome [1]. The advantages of FFPE tissue include easy handling, long-term cheap storage, suitability for immunohistochemical analyses, and low cost of large-scale application [2, 3]. However, formalin decreases the efficiency of PCR due to protein cross-linking. In addition, the degradation of nucleic acids increases during storage depending on the pH value of the fixative [4]. Nevertheless, due to the worldwide abundance and the associated great potential of diagnostic pathology tissue archives, many groups have used FFPE tissues for nucleic acid extraction and further analysis [5–9]. In recent years, using FFPE specimens of different origin and storage times, a variety of methods for DNA/RNA isolation and subsequent assay have

been developed. It was shown that PCR products between 100 and 300 bp could be generated from FFPE material [8]. Additionally, Gilbert and colleagues [10] reported a multiplex PCR procedure combined with minisequencing for high-throughput SNP typing applicable to 25-year-old FFPE tissue. Epigenetic studies performed with DNA isolated from 30-year-old FFPE tissue have been reported [11].

The scope of the present study was to establish standard operation procedures for DNA and RNA isolation from up to 40-year-old FFPE material in order to determine the quality and quantity of the NA samples obtained. We compared the properties of NA after extraction using commercially available kits from Qiagen (QG) and Norgen (NG) as well as the phenol–chloroform isoamyl alcohol (PCI) protocol. In addition, we developed a rapid lysis method to validate the quality of the FFPE tissue. The extraction protocols were ranked according to the suitability of the extracts for performing PCR and the obtained RIN value (RNA integrity number). For the amplification reaction, primers targeting three house-keeping genes (β-actin, GAPDH, and TBP) and targeting single-copy genes EGFR exon 19 and 21 were applied. The short (1 h) tissue lysis method turned out to be an efficient tool for testing the length of amplifiable fragments. Extractions using the PCI protocol and Qiagen kit resulted in the highest purity of NA. Furthermore, the longest PCR fragments and the highest RIN values from about 40-year-old FFPE materials were achieved by PCI extraction.

Materials and methods

Cellular material

NA were extracted from the following materials: (1) FFPE breast cancer specimens stored between 10 and 40 years (originating from three different pathology institutes); FFPE and frozen human colon tissue from the same patient from 2002; immortalized T47D breast cancer cells isolated from fresh cell culture; (2) T47D FFPE blocks fixed in formalin at pH 7 and pH 4.

For NA extraction, we used three 8-μm-thick sections of archival FFPE blocks, six 8-μm-thick sections of T47D cell blocks, 40 8-μm-thick sections of frozen colon tissue, or 5×10^6 freshly prepared T47D cells.

Preparation of FFPE blocks from T47D breast cancer cell line

T47D breast cancer cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum "Gold" (PAA Laboratories, Cölbe, Germany), 10 μg/ml bovine pancreas insulin (Sigma-Aldrich), and 1% penicillin–streptomycin (10,000 U/ml from Invitrogen, Darmstadt, Germany) at 37°C and 5% CO_2 in a humidified incubator. The FFPE T47D cell blocks were prepared as described previously with some modifications [12]. Briefly, 2×10^6 T47D cells were harvested and washed twice with D-PBS (Dulbecco's PBS, Gibco®, Invitrogen, Darmstadt, Germany). The pellet was resuspended in human plasma, before adding bovine thrombin (both from Sigma-Aldrich, Schnelldorf, Germany), and mixed immediately. The pellets were left for several minutes to agglomerate, brought into biopsy capsules (Leica, Nussloch, Germany), and subsequently fixed in 4% phosphate-buffered formaldehyde at pH 7 or at pH 4 for 16 h. Pellets were dehydrated and embedded in paraffin using a tissue processor (Sakura Tissue-Tek VIP from GMI Inc., Ramsey, USA).

Isolation of NA

The tissue section, which was used for extraction, was hematoxylin and eosin stained, scanned, and the area measured with the polygon tool (dotSlide 2.0, slide scanner; Olympus Deutschland GmbH, Hamburg, Germany). The area varied in size about 30-fold (between 0.26 cm^2 and 7.53 cm^2) due to different tissue or cell amounts, origins (breast or colon), and tumor size within the blocks. Deparaffinization of the FFPE sections was performed in Eppendorf tubes using xylene, followed by descending ethanol series. After final centrifugation and removing the supernatant, tubes were left open (10 min) for evaporation of residual ethanol. For NA extraction, two commercially available kits (Qiagen and Norgen) or the phenol–chloroform isoamyl alcohol extraction protocol were used. NA were isolated three times for DNA and two times for RNA using the different protocols. For comparison of the NA yields from three different extraction methods, a three-times-standard deviation was calculated [13]. All NA yields outside this range were considered significantly different and marked in Fig. 1 with an asterisk.

NA isolation from FFPE material using qiagen kit (QG)

DNA was extracted using the QIAamp DNA FFPE tissue kit (#56404; Qiagen, Hilden, Germany) according to manufacturer's instructions, with the following modifications: after 3 h of lysis with 40 μl proteinase K (20 mg/ml, Roche, Mannheim, Germany), 2 μl 100 mg/ml DNase-free RNase A (Qiagen) was added and the mixture was incubated for 2 min at room temperature before adding buffer AL. DNA was eluted with 30 μl TE buffer (10 mM Tris, 1 mM EDTA). RNA was extracted using the RNeasy FFPE kit (#74404; Qiagen) according to the manufacturer's instructions. RNA was eluted with 25 μl ultra-pure water and immediately supplemented with RNase Inhibitor (RiboLock™, Fermentas, St. Leon-Rot, Germany). For 1-h lysis–PCR,

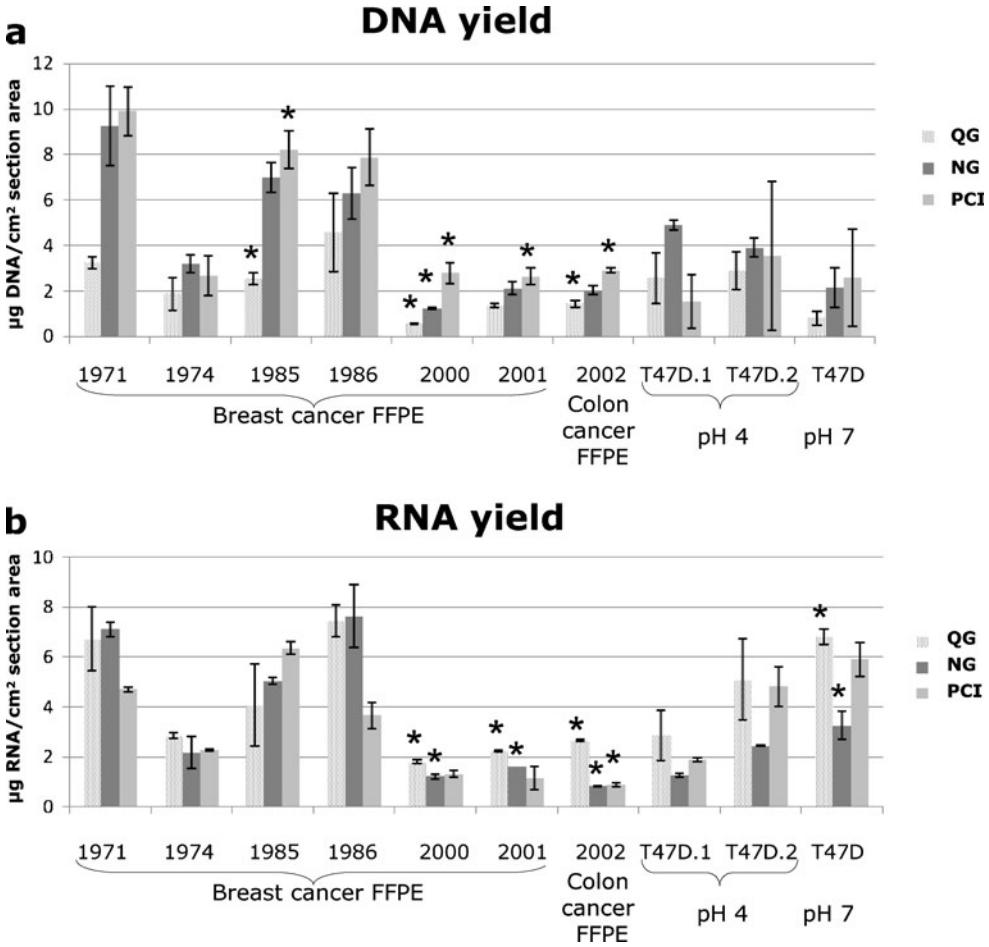

Fig. 1 Histograms showing total DNA (**a**) and RNA (**b**) amounts, respectively. Yield for each extraction method from all 10 FFPE blocks normalized by the actual tissue area measured in a subsequent section. Nucleic acids have been extracted with the Qiagen kit (*QG*), the Norgen Kit (*NG*), and a phenol–chloroform isoamyl alcohol protocol (*PCI*), and NA yields were determined spectrophotometrically. NA amounts, which are significantly different from the other extraction methods in terms of ±3-times-SD, are marked by *asterisks*

PCR-ready lysis solution (see PCI protocol) was used instead of the ATL buffer, followed by DNA purification via Qiagen silica columns.

NA isolation from FFPE material using norgen (NG)

The FFPE RNA/DNA purification kit (#25000; Norgen Biotek, Ontario, Canada) was used according to the manufacturer's instructions. After 1 h of lysis, 31 µl (10%) of the lysate was taken, heat-inactivated (10 min, 95°C), and used as PCR template. With the remaining lysate, incubation was continued as indicated in the manufacturer's protocol (1-day lysis). An additional RNA digestion step was introduced before adding binding solution (as described above). Lysis was carried out for 24 h for DNA and 3 h for RNA. DNA was eluted with 40 µl TE buffer and RNA with 25 µl ultra-pure water, and immediately supplemented with RNase Inhibitor (RiboLock™, Fermentas).

NA isolation from FFPE material using phenol–chloroform isoamyl alcohol (PCI)

The following PCR-ready lysis buffer was used for DNA and RNA extraction: 40 mM Tris, 1 mM EDTA (Sigma-Aldrich) and 0.5% Tween-20 (Carl Roth, Kalsruhe, Germany) in ultra-pure water. Deparaffinized and dried cell pellets were resuspended in 200 µl PCR-ready lysis solution, and 50 µl proteinase K was added. For extraction of DNA, samples were incubated under constant agitation (60°C, 1 h), vortexed, and 25 µl (10%) lysate was retained, heat-inactivated, and used as PCR template (1-h lysis). Lysis of the remaining sample was continued (1-day lysis). After heat inactivation of proteinase K (10 min, 90°C), 2 µl RNase A was added to each sample as described above. An equal volume of phenol–chloroform isoamyl alcohol solution (25:24:1) was added to the sample, vortexed, and centrifuged. Upper phases were transferred into new tubes and an equal volume of chloroform (EMSURE® by Merck, Darmstadt, Germany) was added, vortexed, and centrifuged. Upper phases were transferred into new tubes, 0.1 vol of 3 M sodium acetate pH 5.5 (Ambion, Austin, USA) was added, and tubes were vortexed before addition of 1 vol of isopropanol followed by precipitation of DNA (−20°C, overnight). The precipitated DNA was centrifuged, supernatants were removed, and pellets were washed twice with 70% ethanol. Then, pellets were dried at 35°C, 40 µl TE buffer was added, and tubes were heated (1 h, 45°C) for complete solubilization. For extraction of RNA, samples were incubated under constant agitation (55°C, 2 h), 0.1 vol of

3 M sodium acetate pH 4.0 was added, vortexed, and after addition of an equal volume of phenol–chloroform isoamyl alcohol solution, samples were incubated (4°C, 15 min). The following centrifugation led to separation of two phases, of which the upper was transferred into a new tube, mixed with an equal volume of chloroform, and again centrifuged. Then, the upper phase was transferred into a new tube, and 1 μl glycogen and 1 vol of isopropanol were added. RNA was precipitated (−20°C, overnight), then centrifuged and washed twice with 70% ethanol. Pellets were dried at 35°C, 25 μl ultra-pure water was added, and all tubes were heated (15 min, 55°C) for complete solubilization.

NA extraction from frozen tissue and T47D cells

Extraction of DNA and RNA from frozen colon tissue (25 mg of 8-μm tissue section) and from 5×10^6 fresh T47D cells was performed using the QIAamp DNA Mini Kit and the RNeasy Mini Kit (#51304 and #74104; Qiagen), respectively, according to the manufacturer's instructions. Purified DNA was eluted twice with 200 μl TE buffer and stored at −20°C. Purified RNA was eluted with 50 μl ultra-pure water and supplemented with RNase inhibitor.

Spectrophotometrical measurements

The total amounts of NA obtained with the three 1-day lysis extraction methods were measured using a spectrophotometer (NanoDrop ND-100; PEQLAB Biotechnologie, Erlangen, Germany). The amount of DNA obtained after 1-h lysis could not be spectrophotometrically determined due to sample consistency. For each sample, DNA and RNA concentrations, OD ratio 260/280 nm indicating protein contamination, and OD ratio 260/230 nm, indicating contamination with solvents, salts, and organic compounds, were determined in triplicate.

Microcapillary electrophoretic RNA separation

RNA degradation and RNA integrity numbers (RINs) for double RNA extractions with each method from each block were determined (Agilent 2100 Bioanalyzer; Agilent, Santa Clara, USA) according to the manufacturer's instructions after additional denaturation of the ladder (2 min, 70°C) before use. RNA (500 ng/μl) was diluted in RNase-free ultra-pure water. The software automatically generates the ratio of the 18S and 28S rRNA and a RNA integrity number (RIN) from 1 to 10, based on the entire electrophoretic trace of a sample. An RNA sample with RIN 1 is considered to be completely degraded, whereas RIN 10 indicates intact RNA [14]. A RIN above the minimum acceptable value of 1.4 is considered as still useful for analysis [15]. The average RNA quality is expressed as the average RIN ± standard deviation.

PCR-based assays

To assess the length of the maximum amplifiable PCR fragment, 10 primer pairs targeting three different human house-keeping genes [β-actin, GAPDH, and the TATA box binding protein (TBP), see Table 1] were designed for the amplification of fragments with an increasing length between 100 bp and 1,338 bp. The four primer pairs for the shortest fragments were used in a multiplex PCR, generating simultaneously 100, 150, 200, and 300 bp long fragments. For the amplification of EGFR exon 19 and exon 21, four primers (Table 1) and PCR conditions as published [16] were used. The PCR master mix contained 1.16× reaction buffer for BioThermAB™ Hot Start Taq DNA Polymerase (Ares Bioscience, Köln, Germany) and 232 μM of each dNTP (dNTP set, 4×100 μmol; Fermentas). For every PCR reaction after 1-day lysis, 1 μl of extract (100 ng/μl) and after 1-h lysis also 1 μl of lysate (concentration could not be determined) were used as templates. All other PCR reactions were performed under identical conditions: initial denaturation (4 min, 94°C), followed by 35 cycles of denaturation (1 min, 94°C), annealing (1 min, 64°C), elongation (1 min, 72°C), and a final elongation (7 min, 72°C). The PCR products were visualized by 1% agarose gel electrophoresis and stained with ethidium bromide.

Results and discussion

The NA amount depended on the isolation method and on FFPE material age

In spite of the many advantages formalin fixation of tissue samples for diagnostic purposes has to offer [2, 3], the use of FFPE material for molecular analysis remains problematic. While the tissue morphology is well preserved, the degradation of nucleic acids continues, in particular due to a time-dependent decrease of pH [4].

The correlation between the pH and NA quality was also observed in our study. The amount of DNA, after isolation with the PCI protocol, was higher than with the (commercially available) kits. However, the PCI extraction resulted in a higher variation in DNA yield between the three replicates (Fig. 1a). This outcome is associated with a DNA loss in silica columns and several processing steps, typical for conventional isolation methods, which is furthermore influenced by particularities of the used laboratory protocol. In Fig. 1a and b, the NA amounts, which are significantly different from the other extraction methods in terms of ±3-times-SD, are marked by asterisks.

We observed that the age and origin of the FFPE sample influenced the isolation efficiency. However, the DNA yield from 40-year-old FFPE material was relatively high in comparison to FFPE material, a 10-year-old block. Interestingly,

Table 1 Sequences and names of primers used for single and multiplex PCR, amplicon length (bp), and corresponding number. Sequences for the last four primers for the amplification of exon 19 and exon 21 of EGFR were used as published before [16] *F* forward, *R* reverse

Primer name	Primer sequence in 5′–3′ orientation	Amplicon length (bp)	Number/name
TBP-100 F TBP-100R	AACTTCGCTTCCGCTGGCCC AGTGCAGTGGTGGCCTTCGC	100	1
ACTB-142 F ACTB-142R	CCTGTGTTATCTTGGAGGTCCCCTGAAG ACCGTAGAGTGGTCACTCAATGAAG	142	2
GAPDH-200 F GAPDH-200R	GGGAAGTCAGGTGGAGCGAGGC TTGCGGTGGAAATGTCCTTTTCCAACTACC	200	3
ACTB-307/968 F ACTB-307R	CTTGAGGAGGTGGGAAGGGACTATTTGG CCCCTACCCCAACTTGACTTTGATTCCA	307	4
ACTB-411 F ACTB-411R	AAGTTCCCAAGCACAGAAGAGAACCTGT CTTCCTAGTGCTCTCCTATGCACCCTTC	411	5
GAPDH-500 F GAPDH-500R	GAAAAGGACATTTCCACCGCAAAATGGC GGTCAGAAATTAACTGGACAGGGCAAGC	500	6
GAPDH-687 F GAPDH-687R	ATTAGCCCAGTTTCATGCAGCAGAGAGA GGTGACTCAGCAGAGAAGACTTGAGGAG	687	7
GAPDH-781 F GAPDH-781R	AAAATCGGTAAAAATGCCCACCTCGCAT ATCAGCTAAAGATGTGCTTCCTCCGTGT	781	8
ACTB-968R	CTTCAGGGGACCTCCAAGATAACACAGG	968	9
ACTB-1338 F ACTB-1338R	CTGTGGCATCCACGAAACTACCTTCAAC CCAAATAGTCCCTTCCCACCTCCTCAAG	1338	10
EGFR-E19F EGFR-E19R	CGTCTTCCTTCTCTCTCTGT CCACACAGCAAAGCAGAAAC	148	Exon 19
EGFR-E21F EGFR-E21R	CCAGGAACGTACTGGTGAAA TGACCTAAAGCCACCTCCTT	129	Exon 21

the DNA amount derived from freshly prepared FFPE block fixed at pH 4, a pH value typical for long-term stored FFPE material, was reasonably good and higher than from samples from recent years (Fig. 1a). Furthermore, the composition of tissue samples differs in relation to tissue composition, such as inflammatory cells and parenchymal cells, resulting in differences in cell density and therefore different NA amounts in a given tissue surface area.

The RNA yield between the three isolation methods showed more consistency than for that for DNA; consequently, the determined standard deviation for respective replicates was lower than for DNA (Fig. 1b). The RNA amount isolated from older FFPE samples was slightly higher than that from tissues from recent years. The RNA yield from freshly prepared T47D cell blocks, fixed at pH 7, was much higher than the DNA amount obtained from the same sample, probably due to the isolation of RNA from exponentially growing cells cultivated at optimal conditions. In general, the NA yield depended on the origin of individual tissue blocks: breast cancer tissue fixed in 1971 or 1985 yielded higher NA amounts than breast cancer fixed in 2001 or fresh T47D cell blocks (fixed at pH 4) (Fig. 1a, b). As the exact fixation and storage conditions for archival samples are normally not known, we suppose that the variation of the NA content is due to variations in tissue condition and preservation between individual samples. This hypothesis is in accordance with the study by Bonin et al. [5] comparing different isolation protocols using colon, ovarian, and lung cancer tissues that showed variable NA yields.

The highest NA purity was obtained with PCI protocol and qiagen kit

The highest purity for DNA was obtained using PCI and Qiagen protocols (Fig. 2a). Only five out of all 30 DNA isolates using Norgen or Qiagen protocols did not reach the threshold of 1.8 (OD 260/280), indicating high protein contamination. Generally, using the Norgen protocol resulted in lysates highly contaminated with organic compounds and solvents (Fig. 2b, OD 260/230), and this method therefore should not be used for isolation of high purity NA using FFPE tissue.

Concerning the purity of RNA, all OD 260/280 values exceeded the threshold of 1.8, the lowest value being 1.83 and the highest 2.03, indicating low protein contamination (Fig. 2c). The OD 260/230 values of the Qiagen and PCI RNA lysates were above the threshold of 1.8, indicating that the RNA lysates were relatively pure (Fig. 2d). RNA isolated using the Norgen protocol showed low purity and was highly contaminated with salts, solvents, or organic compounds.

In summary, for both DNA and RNA, lysates obtained using PCI and Qiagen methods showed the highest purity. Our results confirmed previously published data showing satisfactory results for the Qiagen kits for extraction of DNA or RNA from FFPE tissue [11].

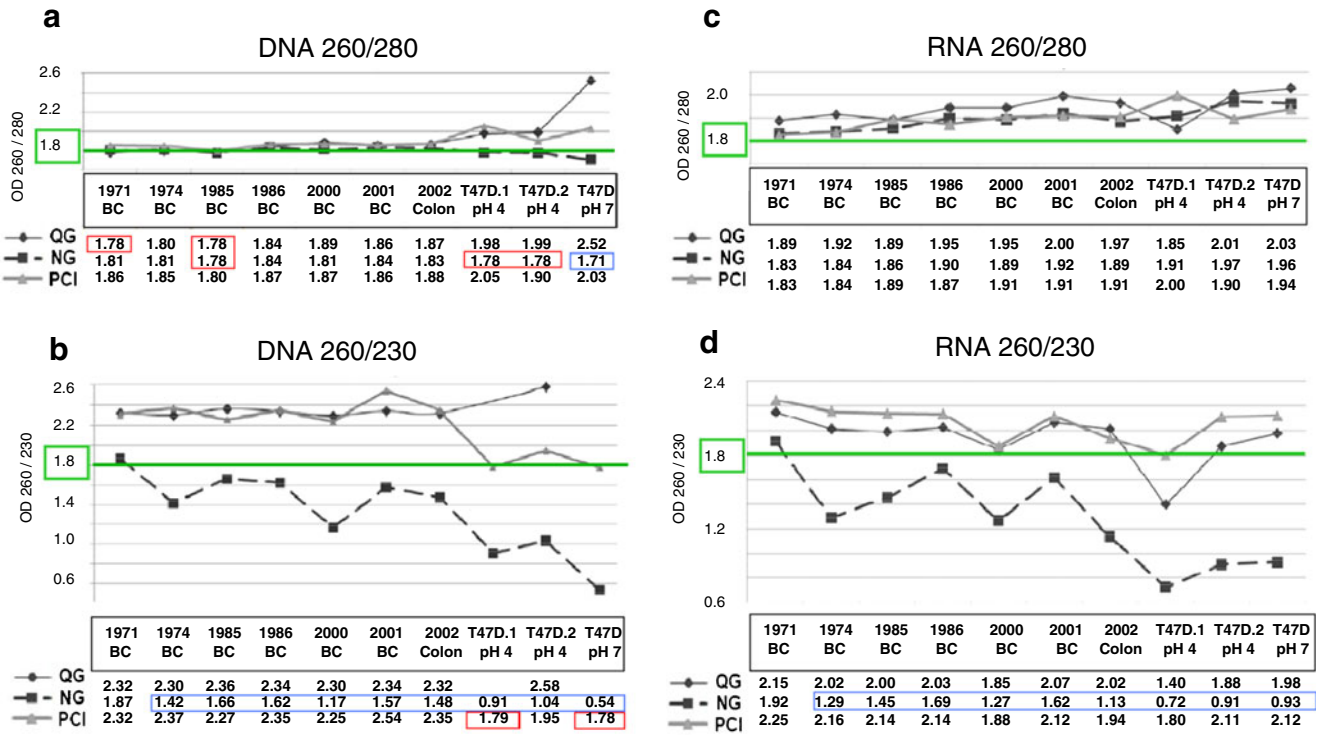

Fig. 2 Plots and tables of the OD 260/280 and OD 260/230 values for DNA (**a**, **b**) and RNA (**c**, **d**) for each extraction method from all 10 FFPE tissue blocks. Nucleic acids have been extracted with the Qiagen kit (*QG*), the Norgen kit (*NG*), or the phenol–chloroform isoamyl alcohol protocol (*PCI*), and OD values were determined spectrophotometrically. The threshold of 1.8 is marked; isolates which undermatch the threshold are framed in *red* (strongly—*blue frame*)

RNA isolated from FFPE tissue is highly degraded but still useful

For the assessment of RNA length, RIN (RNA integrity number) values for all isolates were established. RIN value of 1 indicates completely degraded RNA, whereas RIN value of 10 indicates intact RNA. A RIN value of 1.4 was considered as a minimum for successful execution of further analyses, such as RNA expression arrays [14, 15]. In all our samples, regardless of extraction methods and age of the sample, RIN values higher than 1.4 were achieved. No big differences were detected between the used extraction methods using the same breast cancer tissue (Fig. 3a). The Qiagen protocol was used for frozen colon tissue, the corresponding FFPE tissue as well as for fresh T47D cells (Fig. 3b). Here, the determined RIN for freshly prepared T47D cells was 10. RNA isolated from frozen colon tissue had a RIN value of 8.7, whereas the corresponding FFPE colon tissue had a value of 2.20. In Fig. 3c, an overview of all average RIN values per sample according to extraction method is shown. The average RIN values of FFPE tissue from 1971 to 2002 varied between 1.80 and 3.65, obtained after Qiagen extraction, between 2.3 and 2.5 after Norgen extraction and between 2.3 and 2.7 after PCI extraction. For frozen colon tissue from 2002 and fresh T47D cells, higher RIN values of 8.03 and 10, respectively, were obtained.

In general, independent from the extraction protocol, all FFPE samples showed a high level of degradation, confirming earlier studies [15, 17]. The high fragmentation of RNA from FFPE material is highly associated with the addition of methylol groups by formalin during fixation [9, 18, 19]. In comparison to DNA, RNA is more susceptible to degradation as it is a single strand. However, all samples showed RIN values higher than 1.4 and could be thus used for further analysis.

Fragmentation of genomic DNA increases with the age of FFPE material

As shown in Fig. 4a, DNA smear caused by fragmentation was detected in all three individually extracted samples using the same block. Similarly different FFPE samples, including the recently prepared blocks from the T47D cells fixed at pH 4, showed a different degree of DNA fragmentation. In contrast, isolates from the T47D cell block, fixed in formalin at pH 7, showed high molecular weight DNA (Fig. 4a). Therefore, the size of DNA fragments seemed not only to be influenced by the age or the pH value decrease during the storage but also by variations in the sample preparation methods. The latter is demonstrated in Fig. 4a in which samples from breast cancer (from year 2000) and colon cancer (from year 2002) showed a DNA smear and a

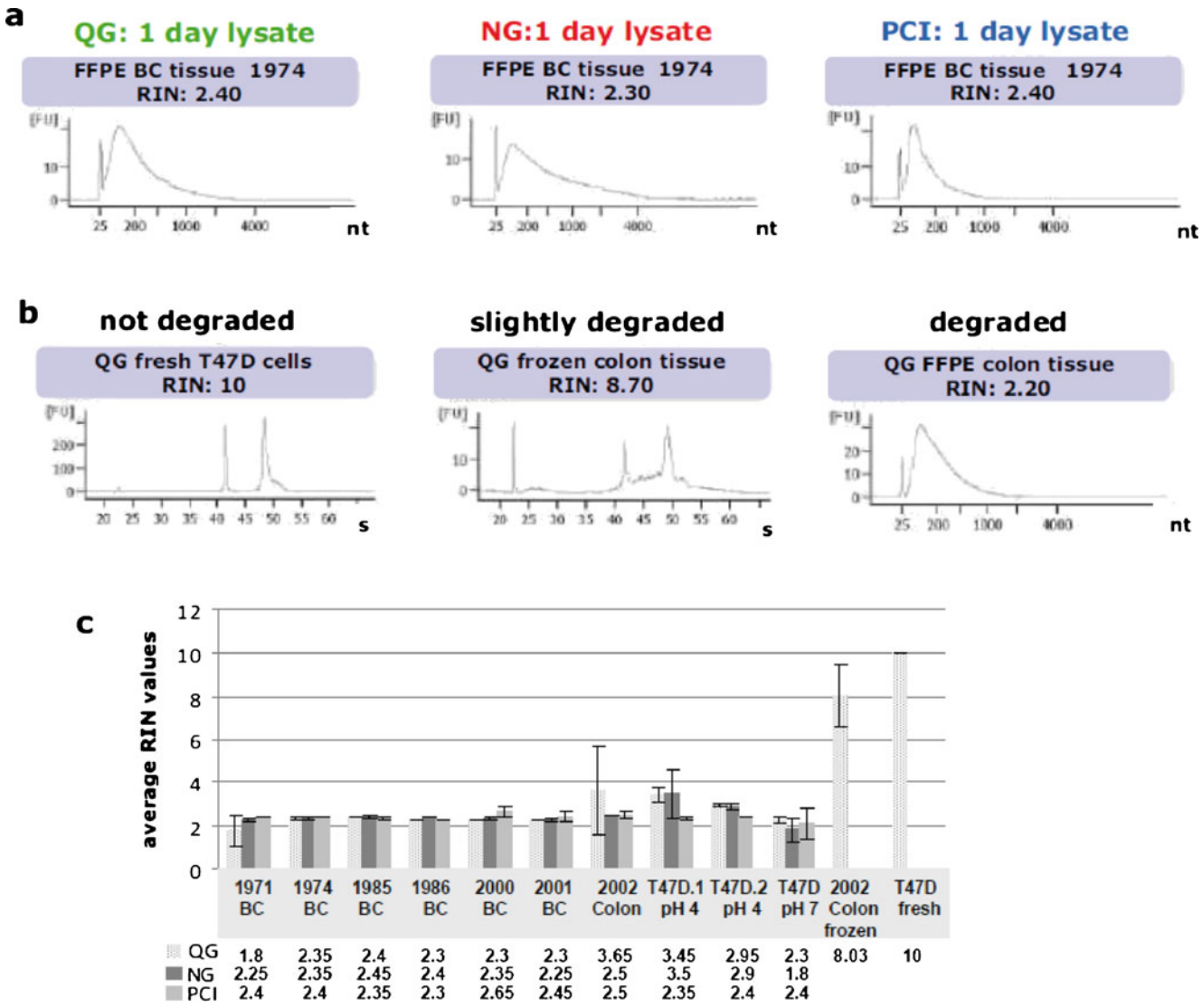

Fig. 3 Representative electropherogram curves from selected samples. **a** No difference between the different extraction methods, applied on a breast cancer (*BC*) FFPE tissue block from 1974. **b** RNA degradation shown by the electropherogram curve: *not degraded*: two distinct and sharp peaks at around 41 seconds (*s*) and 47 seconds correspond to the 18S and 28S rRNA, *slightly degraded*: a raised baseline along with less distinct 18S and 28S rRNA peaks and broad peaks between 25 and 200 nucleotides (*nt*) indicate small, degraded RNA products and tRNA in the frozen colon tissue, and *degraded*: the absence of 18S and 28S rRNA peaks shows a complete degradation of the RNA in the corresponding FFPE colon tissue sample, dependent on the degree of fragmentation the software shifts to a different scaling, e.g., from (s) to (nt). **c** Plot and table with the average RIN values per block or material for all 10 FFPE tissue blocks, frozen colon tissue, and fresh T47D cells. All extractions were carried out in duplicate, except from frozen colon tissue (*Colon frozen*) and fresh T47D cells, where extractions were carried out in triplicate. In the block "*1986 BC*", only one RIN value could be determined

band of high molecular DNA, whereas breast cancer sample of similar age (2001) only showed a degraded DNA smear.

In general, DNA fragmentation was strongly associated with the storage time: the older the FFPE sample the shorter the fragments, as shown in Fig. 4a. DNA fragmentation was compared between DNA isolated from freshly prepared T47D cells, recently prepared T47D FFPE blocks, and over 5-year-old T47D FFPE blocks. High molecular weight DNA was detected in fresh T47D cells and in recently prepared T47D FFPE cell blocks, while over 5-year-old T47D FFPE blocks yielded only a degraded DNA smear (Fig. 4b).

No significant differences were observed between the three different protocols for DNA isolation. Huang et al. [20] detected fragment sizes between 15 and 30 kb of extracted DNA from FFPE tissue. These results are comparable with freshly prepared FFPE material also tested by us as shown in Fig. 4b.

1-h lysis–PCR of FFPE material is a rapid novel method to validate DNA quality

To obtain information about the quality of DNA from long-term preserved FFPE tissue blocks using a rapid and low-

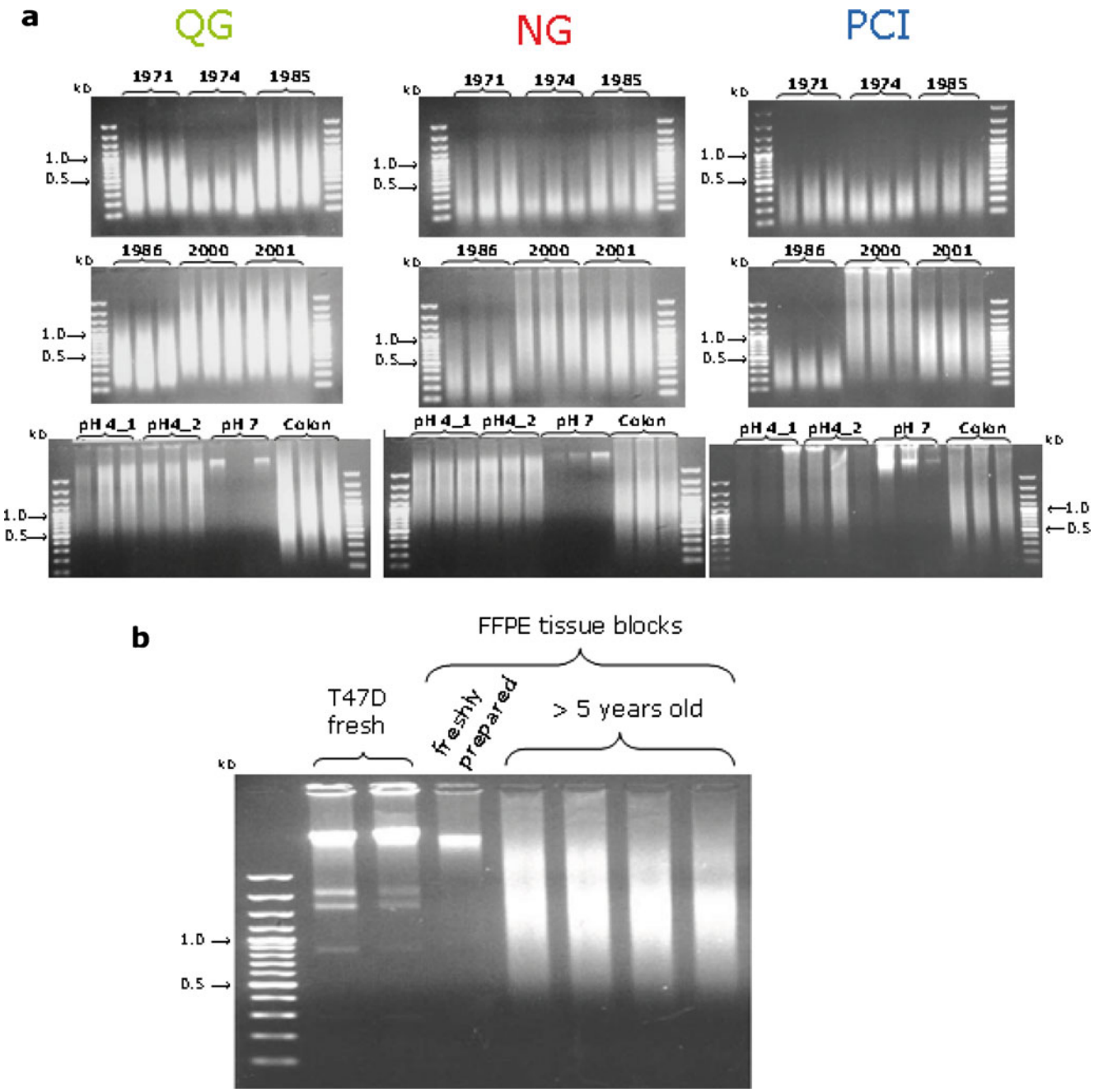

Fig. 4 Fragmentation of DNA extracted from different samples. **a** 1 μg genomic DNA from three individual extraction approaches per block were loaded on an agarose gel. From T47D cell line, two blocks were fixed in formalin pH 4; one was fixed in formalin pH 7. The used extraction protocols were Qiagen kit (*QG*), Norgen kit (*NG*), and phenol–chloroform isoamyl alcohol extraction (*PCI*). **b** 1 μg genomic DNA from fresh T47D cells, from recently prepared FFPE fixed, and from over 5-year-old FFPE T47D cell blocks (FFPE, >5 years old) were loaded

cost protocol, we tested a short (1 h) tissue lysis method using PCR as an endpoint (1-h lysis–PCR). Ten different primer pairs targeting three house-keeping genes (β-actin, GAPDH, and TBP) were used, resulting in amplified sequences varying in length between 100 bp and 1,338 bp. Amplification results were compared to PCR amplification data obtained after continued overnight lysis and subsequent purification steps (1-day lysis–PCR).

In Fig. 5a, representative examples are shown for PCR products obtained using the PCI method combined with 1-h lysis–PCR or 1-day lysis–PCR from breast cancer FFPE tissue from 1986 and from a FFPE T47D cell block, fixed at pH 7. The amplifications were performed in triplicate and the results indicate that the maximum amplifiable PCR fragment from the 1986 sample after 1-h lysis was 300 bp (lane 4). From the respective 1-day lysis–PCR, an additional

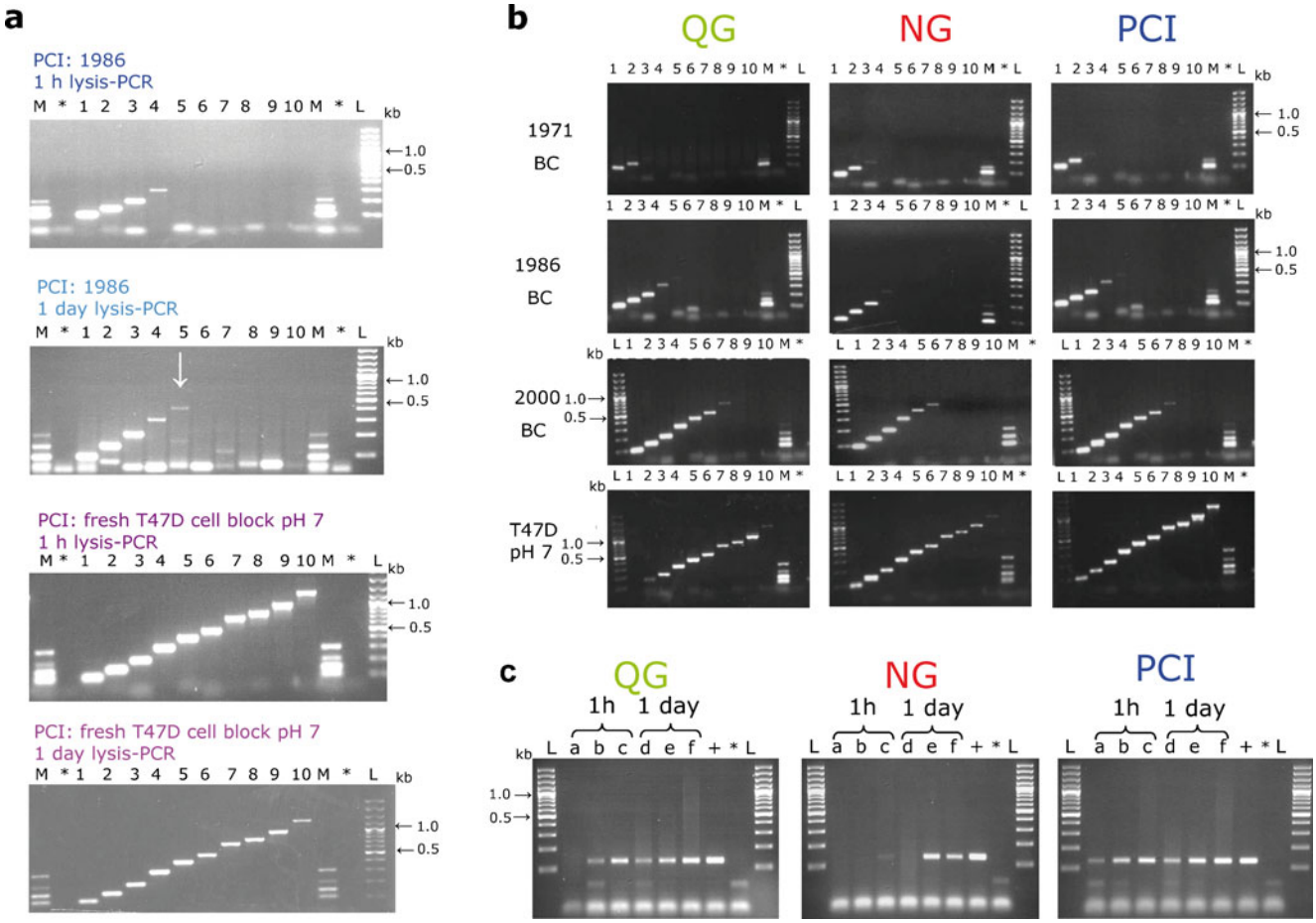

Fig. 5 Maximum amplifiable PCR products from DNA extracted from different FFPE samples. **a** Representative examples of PCR fragments obtained after 1-h lysis–PCR and after 1-day lysis–PCR with DNA isolated with PCI protocol. *M* multiplex PCR, *L* ladder, * negative control. Numbers *1–10* indicate fragment lengths shown in Table 1; *white arrow* indicates the 411 bp fragment. **b** Comparison of maximum amplifiable PCR products obtained after DNA extractions from different breast cancer (*BC*) FFPE tissues and T47D cells using the three different protocols and followed PCR (1-day lysis–PCR). **c** Examples of amplifications of 129 bp long EGFR exon 21 using three different protocols for 1-h and 1-day lysis, *a* lysate from 1971, *b* from 1986, *c* from 2000, *d* extract from 1971, *e* from 1986, *f* from 2000. +: positive control

weak 411 bp long PCR fragment was obtained (indicated by an arrow in Fig. 5a, lane 5). The 1-h lysis–PCR using PCI protocol generally yielded longer PCR fragments than the 1-h lysates from the Norgen or Qiagen kit, in particular with DNA derived from older FFPE samples (data not shown). The 1-day lysis–PCR results were highly comparable between the methods, although the maximum amplifiable DNA fragments were generated using the PCI protocol.

In Fig. 5b, examples of 1-day lysis–PCR assays using the three protocols are shown. The results confirm that the older the FFPE samples the shorter the DNA fragment size. DNA templates isolated with the PCI protocol resulted in longer PCR fragments than the silica column-based Norgen and Qiagen kits, probably due to the higher sensitivity and better DNA yield of the former method. Using the PCI protocol, weak bands of high molecular DNA were sometimes detected (Fig. 4a).

Importantly, our novel 1-h lysis–PCR method gave similar maximum fragment lengths amplified from long-term stored FFPE tissue as reported previously [6, 8]. Furthermore, in addition to the single PCR assays, multiplex PCR using four primer pairs for amplification of fragments between 100 bp and 300 bp was successfully performed using 1-h lysis–PCR method, although the 1-day lysis–multiplex PCR led to somewhat longer fragments (100 bp) with respective lysates (Fig. 5a, b). This is an efficient test to determine maximum amplifiable DNA after 1-h tissue lysis.

Furthermore, amplification of EGFR exon 19 and exon 21 from FFPE samples was performed using PCR published previously [16]. To demonstrate that 1-h lysates can be used for the amplification of single copy genes, these target regions were selected as representative targets in daily routine analysis. Representative examples of amplification of EGFR exon 21 from DNA obtained after 1-h and 1-day lysis using three protocols are illustrated in Fig. 5c. Here, 129 bp

long PCR fragments were generated even from 1-h lysates after DNA isolation from FFPE tissue from 1971 following PCI and QG protocol. The NG protocol was less effective in comparison to PCI and QG protocol. These PCR results are in agreement with our PCR results obtained by amplification of house-keeping genes, indicating that the PCI and QG protocols are most effective for DNA isolation and subsequent PCR. Furthermore, 1-h tissue lysis is as efficient as 1-day lysis for successful amplification of single copy genes even from over 40-year-old FFPE tissue samples.

In conclusion, fixation and storage conditions may make FFPE material unsuitable for further analysis. The development of tests for the prediction of therapy response makes it crucial to exploit available FFPE tissue banks using standard techniques. We established standard operation procedures for DNA/RNA isolation from long-term preserved FFPE tissue which can be routinely used in molecular diagnostic laboratories. We identify advantages and disadvantages of different methods. The Qiagen method and the PCI protocol resulted in highly pure nucleic acid samples. The maximum PCR amplicons were achieved after DNA extraction using the PCI protocol. However, this method, due to more preparatory steps, showed higher variation between the replicates. Importantly, an efficient novel method for tissue lysis and NA amplification (1-h lysis–PCR) and a multiplex PCR were established as a tool for rapid validation of the quality of DNA derived from long-term preserved FFPE tissue. As this approach delivered most relevant information on DNA quality, it might replace other more time-consuming and expensive isolation protocols.

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Conflict of interest The authors declare that they have no conflict of interest.

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