Optimal Fixation Conditions and DNA Extraction Methods for MLPA Analysis on FFPE Tissue-Derived DNA

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ABSTRACT

Objectives: *Molecular genetic analysis of formalin-fixed, paraffin-embedded (FFPE) tissues is of great importance both for research and diagnostics. Multiplex ligation-dependent probe amplification (MLPA) is a widely used technique for gene copy number determination, and it has been successfully used for FFPE tissue-extracted DNA analysis. However, there have been no studies addressing the effect of tissue fixation procedures and DNA extraction methods on MLPA. This study therefore focuses on selecting optimal preanalytic conditions such as FFPE tissue preparation conditions and DNA extraction methods.*

Methods: *Healthy tissues were fixed in buffered or nonbuffered formalin for 1 hour, 12 to 24 hours, or 48 to 60 hours at 4°C or at room temperature. DNA extracted from differently fixed and subsequently paraffin-embedded tissues was used for MLPA. Four commercial DNA extraction kits and one in-house method were compared.*

Results: *Tissues fixed for 12 to 24 hours in buffered formalin at room temperature produced DNA with the most optimal quality for MLPA. The in-house FFPE DNA extraction method was shown to perform as efficient as or even superior to other methods in terms of suitability for MLPA, time and cost-efficiency, and ease of performance.*

Conclusions: *FFPE-extracted DNA is well suitable for MLPA analysis, given that optimal tissue fixation and DNA extraction methods are chosen.*

Although fresh tissue freezing is considered one of the best methods of conserving nucleic acids and proteins, in a routine pathology setting, large amounts of tissue samples are fixed and stored in paraffin blocks primarily for further (immuno)histomorphologic examination. These formalin-fixed, paraffin-embedded (FFPE) tissues also represent a large and important source of biological materials for molecular diagnostics and research. However, FFPE tissue is considered a problematic starting material for most molecular genetic techniques due to the generally low quality of extracted DNA and RNA. Harsh conditions and treatment during FFPE tissue fixation and embedding contribute to the fact that DNA is often fragmented, partially denatured, and subjected to cross-linking (DNA-protein and/or DNA-DNA cross-linking).[1,2] During fixation, chemical modifications of DNA bases occur such as depurination and deamination of cytosines, leading to single-nucleotide exchange.[3] Multiple factors during the fixation process contribute to the quality of DNA extracted from FFPE tissue such as the time between the tissue acquisition and the start of fixation, the composition and pH of the fixative, the duration and temperature of fixation, and storage duration and conditions. There is no standard tissue fixation method, and therefore one needs to consider the conditions of the tissue handling before, during, and after fixation since these processes greatly affect the quality of DNA.[4,5] Furthermore, the quality of the FFPE tissue-extracted DNA sample might be influenced by the extraction method chosen. Depending on the requirements of the downstream molecular application in terms of DNA quality and quantity, as well as of the cost-effectiveness, one extraction method might be preferred among

various methods ranging from in-house protocols to commercial kits.

Despite the considerable damage introduced to DNA due to tissue fixation and paraffin embedding, multiple studies achieved reliable results using multiplex ligation-dependent probe amplification (MLPA) for the analysis of gene copy number and methylation status.[6-8] DNA fragmentation and denaturation induced by formalin treatment and paraffin embedding does not pose a big problem to MLPA because each MLPA probe hybridizes to a relatively short stretch (<100 base pairs) of single-stranded sample DNA.[9] Although MLPA has been successfully used on FFPE tissue-extracted DNA, no technical guidelines or recommendations are available concerning the optimal fixation procedure and the DNA extraction method to produce genomic DNA of suitable quality for MLPA.

In this study, the effect of the pH, duration, and temperature of tissue fixation on downstream MLPA analysis was evaluated. Furthermore, four commercial kits (QIAamp DNA FFPE Tissue Kit [Qiagen, Dusseldorf, Germany], RecoverAll Total Nucleic Acid Isolation Kit for FFPE [Ambion, Austin, TX], ZR FFPE DNA MiniPrep [Irvine, CA], and WaxFree DNA Extraction Kit [Sparks, MD] for paraffin samples) and one in-house method called, one-tube FFPE extraction, were compared to identify the best method to produce genomic DNA of sufficient quality for performing MLPA analysis.

Materials and Methods

FFPE Tissues and Fixation Conditions

To compare tissue fixation parameters, healthy fresh colon tissue was excised, fixed, and paraffin- embedded at the Symbiant Pathology Expert Centre (Alkmaar, the Netherlands). The 3- to 4-mm sections of fresh tissue were processed with a Leica TP1050 (Wetzlar, Germany) tissue processor using the following settings: 35°C (40 minutes in 70% ethanol; 35, 45, and 55 minutes in 96% ethanol; 45, 65, and 75 minutes in 100% ethanol; and 30 and 50 min in xylene) and 60°C (50, 90, and 110 minutes in paraplast). Subsequently, these sections were fixed with the following conditions:

1. 1 hour at room temperature (RT) in 10% buffered formalin
2. 12 to 24 hours at RT in 10% buffered formalin
3. 12 to 24 hours at 4 °C in 10% buffered formalin
4. 48 to 60 hours at RT in 10% buffered formalin
5. 12 to 24 hours at RT in 10% nonbuffered formalin (pH 3.0)

The 10% buffered formalin had a pH of 6.8 and ~4% formaldehyde content (cat. 4078; Klinipath, Duiven, the Netherlands). The 10% nonbuffered formalin had the following content: 10 mL 0.9% NaCl, 1.1 mL 36% formaldehyde, and 1.2 mL 100% acetic acid. Three 10-μm sections of 1-cm^2 paraffin blocks of all conditions were used for DNA extraction.

To compare DNA extraction methods, different FFPE tissue materials from eight healthy donors were made available by two institutions in the Netherlands: Symbiant Pathology Expert Centre, Alkmaar (colon tissue, further termed as *colonSymb*) and Department of Pathology, University Medical Centre Utrecht, Utrecht (breast, kidney, prostate, stomach, brain, bone marrow, lung tissue, and colon, further termed as *colonUMC*). Formalin fixation was performed for 12 to 24 hours at RT with 10% buffered formalin (pH 6.8) for all tissue blocks.

DNA Extraction Methods

To compare tissue fixation parameters, the QIAamp DNA FFPE Tissue Kit (cat. 56404; Qiagen, Germantown, MD) was used to extract DNA from FFPE tissues fixed with different conditions 1 to 5 described above following the manufacturer's instructions (QIAamp DNA FFPE Tissue Handbook June 2012). The protocol included an overnight lysis at 56°C (step 11) and incubation at 90°C for 10 minutes (step 12; deviation from the protocol to shorter incubation time was done to avoid extensive fragmentation). Elution of DNA (step 20) was performed two times from the same column, each with 30 μL TE buffer (T10E1 buffer: 10 mmol/L Tris-HCl [pH 8.5] and 0.1 mmol/L EDTA). Subsequently, this DNA was purified with the One Step PCR inhibitor Removal Kit (cat. D6030; Zymo Research, Irvine, CA) as it contained residual salt and further concentrated with DNA Clean & Concentrator-5 (cat. D4013; Zymo Research) to achieve a higher concentration of DNA. In total, 50 ng of extracted DNA was used for each MLPA reaction.

To compare DNA extraction methods, from each tissue block (kidney, prostate, stomach, colonUMC, colonSymb, brain, bone marrow, and lung), three 10-μm-thick sections were used per extraction; for breast tissue, 10 sections per reaction were used. Each extraction was performed in triplicate by the following methods:

1. QIAamp DNA FFPE Tissue Kit (cat. 56404; Qiagen) with overnight lysis at 56°C and total elution volume of 60 μL according to the manufacturer's instructions
2. RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion, cat. AM1975; Life Technologies, Carlsbad, CA) with overnight lysis at 50°C and total elution volume of 60 μL according to the manufacturer's instructions
3. ZR FFPE DNA MiniPrep (cat. D3065, version 1.0.1 protocol; Zymo Research), with total elution volume of 50 μL according to the manufacturer's instructions

4. WaxFree DNA extraction Kit for Paraffin Samples (cat. WF-20; TrimGen, Glencoe, MD) with total elution volume of 110 µL according to the manufacturer's instructions

5. In-house one-tube FFPE extraction method. The latter was performed as follows: to remove paraffin, three (or 10, for breast) 10-µm paraffin sections were transferred to 1.5-mL screw-cap tubes and heated at 90°C for 15 minutes in 200 µL one-tube FFPE extraction buffer (50 mmol/L Tris-HCl [pH 8.5], 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Tween 20, and 0.5% NP40). Then, 20 µL proteinase K solution (03115887001, 14- to 22-mg/mL solution; Roche, Welwyn Garden City, UK) was added, and the sample was incubated at 55°C overnight (~16 hours) for lysis of the tissues. Inactivation of proteinase K was achieved by heating the sample for 15 minutes at 80°C. The crude lysate was centrifuged for 10 minutes at 13,000 rpm, and 5 µL from the supernatant was used for each MLPA reaction.

For only one FFPE tissue (lung), 100 µL of the supernatant of the crude lysate was further purified using DNA Clean & Concentrator-5 Kit (cat. D4003; Zymo Research) and eluted in 20 µL TE.

The DNA yield was quantified by the Q-fragments ratio (available in Coffalyser software, MRC-Holland, Amsterdam, the Netherlands), which indicates the ratio of the quantity of the fragments' signals as opposed to the ligation fragments. First, a standard curve was made with Q-fragments ratios estimated for known DNA quantities of 1, 2.5, 5, 10, 20, 30, 40, and 50 ng. Based on the standard curve, the amount of DNA in a 5-µL volume used from each extraction in the MLPA reaction was quantified. Total yield was calculated by amplifying the DNA amount in 1 µL by the elution volume of each extraction. Nanodrop (Nanodrop-8000; Thermo Fisher Scientific, Waltham, MA) was used to measure DNA purity, indicated by the 260/280-nm ratio. The 260/280-nm ratio measurement was not possible for the DNA samples extracted by the WaxFree and one-tube FFPE extraction methods.

MLPA Procedure and Data Analysis

Two SALSA MLPA probe mixes were used: P027 (version C1, lot 0213; MRC-Holland) containing 50 probes and P105 (version D1, lot 0413) containing 55 probes. These two probe mixes were selected for the MLPA analysis as both contain probes targeting multiple genomic regions on chromosomes 1 to 20 and 22. For details about the probe mixes, see Supplementary Tables S1 and S2 (all supplemental materials can be found at *American Journal of Clinical Pathology* online). Commercially obtained blood-derived genomic DNA of multiple anonymous male or female donors
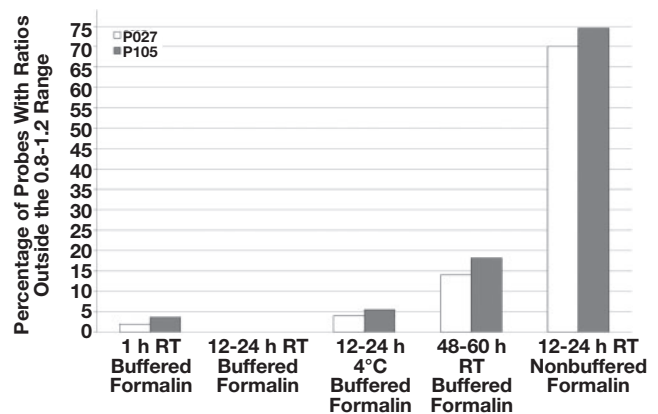

Figure 1 Multiplex ligation-dependent probe amplification (MLPA) probe copy number ratio deviation as a result of different formalin-fixed, paraffin-embedded tissue fixation conditions. Two MLPA probe mixes are presented in separate columns: P027 (white) and P105 (gray). Tissue fixation conditions are indicated on the x-axis. The percentage of probes with copy number ratios that fall outside the normal copy number ratio range (0.8-1.2) is indicated on the y-axis. RT, room temperature.

(Human Genomic DNA: Male [G1471] and Female [G1521]; Promega, Mannheim, Germany) was used as a normal diploid copy number reference sample DNA for normalization. All MLPA reactions were performed using a standard MLPA one-tube protocol (version MDP-005, available at www.mlpa.com) on a Biometra T1 Thermocycler (Biometra, Goettingen, Germany). Fragment separation was performed on an ABI PRISM 3100xl Genetic Analyser (4359571; Life Technologies). The generated raw data were analyzed with Coffalyser.Net v.140721.1958 software. Intrasample normalization and intersample normalization were done using a population analysis method. Copy number ratio values between 0.8 and 1.2 were considered normal.

Results

Optimal Fixation Conditions Enable Robust MLPA Analysis

Genomic DNA extracted from FFPE tissue sections, fixed under various conditions, was analyzed by MLPA using the P027 and P105 probe mixes and compared with the commercial reference sample DNA (with normal diploid copy number).

When comparing different FFPE tissue fixation conditions in terms of MLPA probe copy number ratio, the optimal fixation time was 12 to 24 hours with 10% neutral buffered formalin at RT. At these conditions, the copy

Table 1
Schematic Overview of the Main DNA Extraction Steps From FFPE Tissue Sections by Five Different Methods

DNA Extraction Method	Day 1	Overnight	Day 2
RecoverAll Total Nucleic Acid Isolation Kit	Deparaffinization with xylene Wash with 100% ethanol Sample drying	Protease digestion and sample lysis at 50 °C	Nucleic acid binding on glass-fiber filter Wash RNase digestion on filter Wash and DNA elution
QIAamp DNA FFPE Tissue Kit	Deparaffinization with deparaffinization solution	Protease digestion and sample lysis at 56 °C	Incubation at 90 °C RNase digestion DNA binding on silica-based membrane Wash and DNA elution
Zymo Research FFPE DNA miniprep	Deparaffinization with xylene Subsequent washes with 100%, 95%, and 75% ethanol, H_2O	Protease digestion and sample lysis at 55 °C	RNase digestion Incubation at 94 °C DNA binding on silica-based membrane Wash and DNA elution
WaxFree DNA Extraction Kit	Deparaffinization with Q solution Wash with wash buffer Resuspension in WaxFree Resin	Protease digestion and sample lysis at 55 °C	Incubation at 90 °C Sample cleanup through WR-filter
One-tube FFPE extraction	Paraffin melting in lysis buffer at 90 °C	Protease digestion and sample lysis at 55 °C	Protease inactivation at 80 °C Sample cleanup via centrifugation Optional column purification

FFPE, formalin fixed, paraffin embedded.

number ratios for all probes were within the 0.8 to 1.2 normal copy number ratio range **Figure 1**, and the standard deviation of copy number ratios was as low as 0.05 for the P027 probe mix and 0.06 for the P105 probe mix (Supplementary Tables S3 and S4). A dramatic increase to more than 70% of probes (35 of 50 probes and 41 of 55 probes in P027 and P105 probe mixes, respectively) showing copy number values outside the 0.8 to 1.2 range was observed when the 12- to 24-hour fixation was carried out in nonbuffered formalin (pH 3.0) at RT, making this the least suitable procedure for tissue fixation prior to MLPA (Figure 1 and Supplementary Tables S3 and S4). A very short fixation duration of 1 hour, an extended fixation time up to 48 to 60 hours, and a lower fixation temperature all increase the number of probes with copy number values outside the 0.8 to 1.2 range per MLPA analysis by more than 4%, more than 5%, and more than 18% of probes, respectively.

One-Tube FFPE Extraction Method Is the Simplest and the Least Laborious

All five tested FFPE tissue DNA extraction methods use different approaches for nucleic acid extraction. The four tested commercial kits require chemical deparaffinization in the initial step of FFPE tissue processing. After paraffin removal, the tissue is extensively washed to remove the solvents used. In contrast, the one-tube FFPE extraction method rehydrates the tissue and separates the paraffin by heating the FFPE section in a buffer; the paraffin floats on the surface of the solution during the subsequent tissue-processing steps. After rehydration, all methods use an overnight protease digestion step to

dissolve the tissue. The elevated temperature (>50°C) used results in reversal of a large part of the formaldehyde adducts and cross-links.[10] In the four commercial kits used, this is followed by different nucleic acid purification methods via glass-fiber filter, silica-membrane, or a resin/WR-filter. The one-tube FFPE extraction method results in a (often tissue-colored) crude lysate as only larger tissue remnants are removed by centrifugation. This crude lysate can be used directly but makes the spectrophotometric quantification of the DNA and sample storage more challenging. Therefore, an additional purification step (eg, by DNA Clean & Concentrator-5 Kit; Zymo Research) is recommended in case accurate DNA quantification and long-term storage are desired. **Table 1** provides an overview of critical steps in the workflow of selected DNA extraction methods from FFPE tissues.

The total duration of all five DNA extraction methods was comparable, except for the Zymo Research FFPE DNA miniprep kit, where several washes with ethanol contribute to a long handling time (approximately 3.5 hours) on day 1 of DNA extraction **Table 2**. The one-tube FFPE extraction method has fewer steps in the protocol and, as a result, shorter hands-on time in comparison to the other methods. This method skipped the paraffin removal and the lysate cleanup steps and was consequently the most time-efficient. The optional column purification would require approximately 20 more minutes. Storage of extracted DNA for most methods is not problematic (except for the one-tube FFPE extraction, as mentioned above) as they generally produce DNA of high purity (long-term storage, even at 4°C, is easily applied). Another important consideration for extracting

Table 2

Comparison of Selected DNA Extraction Methods for DNA Extraction From FFPE Tissue Sections

| FFPE DNA Extraction Method | Handling Time (4 Samples) | | Handling Procedure | Hazards | Automation System Available? | Relative Cost per Sample[a] | DNA Purity[b] | DNA Yield (ng) per Extraction[c] |
	Day 1	Day 2						
RecoverAll Total Nucleic Acid Isolation Kit	75 min	Overnight incubation 75 min	Simple	Xylene	Yes	€€€€	~1.8 (pure)	5-250
QIAamp DNA FFPE Tissue Kit	20 min	Overnight incubation 90 min	Simple	Xylene[d]	Yes	€€€	~1.8 (pure)	300-4,000
Zymo Research FFPE DNA miniprep	3.5 h	Overnight incubation 35 min	Laborious[e]	Xylene	No	€€€	~1.8 (pure)	40-3,600
WaxFree DNA Extraction Kit	60 min	Overnight incubation 20 min	Simple	None	No	€€	NA	200-9,000
One-tube FFPE extraction	25 min	Overnight incubation 30 min[f]	The simplest	None	No	€	NA	1,000-10,000[g]

FFPE, formalin fixed, paraffin embedded; NA, not available (the measurement with Nanodrop is unreliable).

[a]Price comparison is based on the cost of the kit sold in the Netherlands; the cost of labor is not calculated.

[b]Assessed by the 260/280 ratio; measurement was performed by a Nanodrop.

[c]Lowest to highest average amount obtained from 3 × 10-μm FFPE tissues, calculated by measuring the ratio of Q-fragments in the multiplex ligation-dependent probe amplification reaction (see Materials and Methods).

[d]In this protocol, the deparaffinization can be performed by xylene or a nontoxic alternative, such as a deparaffinization solution by Qiagen.

[e]Due to extensive ethanol washes.

[f]In case an additional cleanup column is used, the last step will increase total handling time with 20 minutes.

[g]The estimation of DNA quantity is not accurate for values higher than 10,000 ng.

DNA from a large number of samples is the possibility to use automation of the method. As illustrated in the Table 2, currently only two kits can perform automated DNA extraction. In terms of DNA yield, reagent costs, and hands-on time, the one-tube FFPE extraction method appears to be the most beneficial.

MLPA Results Were Influenced by FFPE Tissue DNA Extraction Method and Tissue Type

MLPA analysis of FFPE tissue-derived DNA extracted with five different methods demonstrated that the number of probes that fall outside the 0.8 to 1.2 normal copy number ratio range varied greatly depending on the extraction method and the tissue type. For most tissues, when analyzed by MLPA, the one-tube FFPE extraction method produced DNA that has the lowest number of probes (1%-27%) deviating from the normal copy number ratio range, followed by the Zymo Research FFPE DNA miniprep kit (1%-41%), WaxFree DNA extraction kit (1%-71%), and QIAamp DNA FFPE Tissue Kit (9%-71%) **Figure 2**. MLPA analysis on DNA extracted by the RecoverAll Total Nucleic Acid Isolation Kit presented the most (32%-62%) deviation of normal copy number ratios with around half of the probes showing copy number ratios outside the 0.8 to 1.2 range (Figure 2). For the different tissue types, MLPA analysis on DNA extracted from colon FFPE sections (colonSymb) with the QIAamp kit had the highest number of probes with copy number ratios outside the normal copy number range. A possible explanation is that salt, which is present in the tissue, could not be efficiently eliminated by this kit. The presence of excessive salt in the DNA sample impairs the

DNA denaturation process in the initial step of the MLPA reaction, mostly affecting the probe signals located in the CG-rich DNA regions.[11] Furthermore, the MLPA analysis of DNA extracted with the WaxFree kit and the one-tube FFPE extraction showed the highest number of deviating probe ratios from normal copy number ratios for lung tissue (Figure 2) **Figure 3**. This lung tissue–specific high variability of MLPA probes on DNA samples extracted with the one-tube FFPE extraction method was eliminated with an additional purification step (DNA Clean & Concentrator-5 Kit) of the crude lysate **Figure 3A**. The ability of the additional sample purification step to restore reliable MLPA analysis was clearly visible also on the electropherograms of MLPA fragments **Figure 3B,C**. The MLPA electropherogram of the nonpurified crude lysate shows a very high (off-scale) residual fluorescent polymerase chain reaction (PCR) primer peak **Figure 3B**. This large amount of unused primer is indicative of inefficient PCR. In contrast, the residual primer peak of the column-purified lysate is below the average peak height of the MLPA probes, indicating an efficient PCR reaction **Figure 3C**.

All extraction methods were highly reproducible, as indicated by the small error bars reflecting the standard error of the mean for most tissues samples (Figure 2 and Supplementary Table S6). DNA samples extracted with the one-tube FFPE extraction method had the highest reproducibility when analyzed by MLPA with SEM being zero for kidney and colonUMC FFPE tissue-extracted DNA samples (Figure 2 and Supplementary Table S6). The average amount of probes with copy number ratios outside the normal range in MLPA reactions was the lowest for kidney FFPE tissue
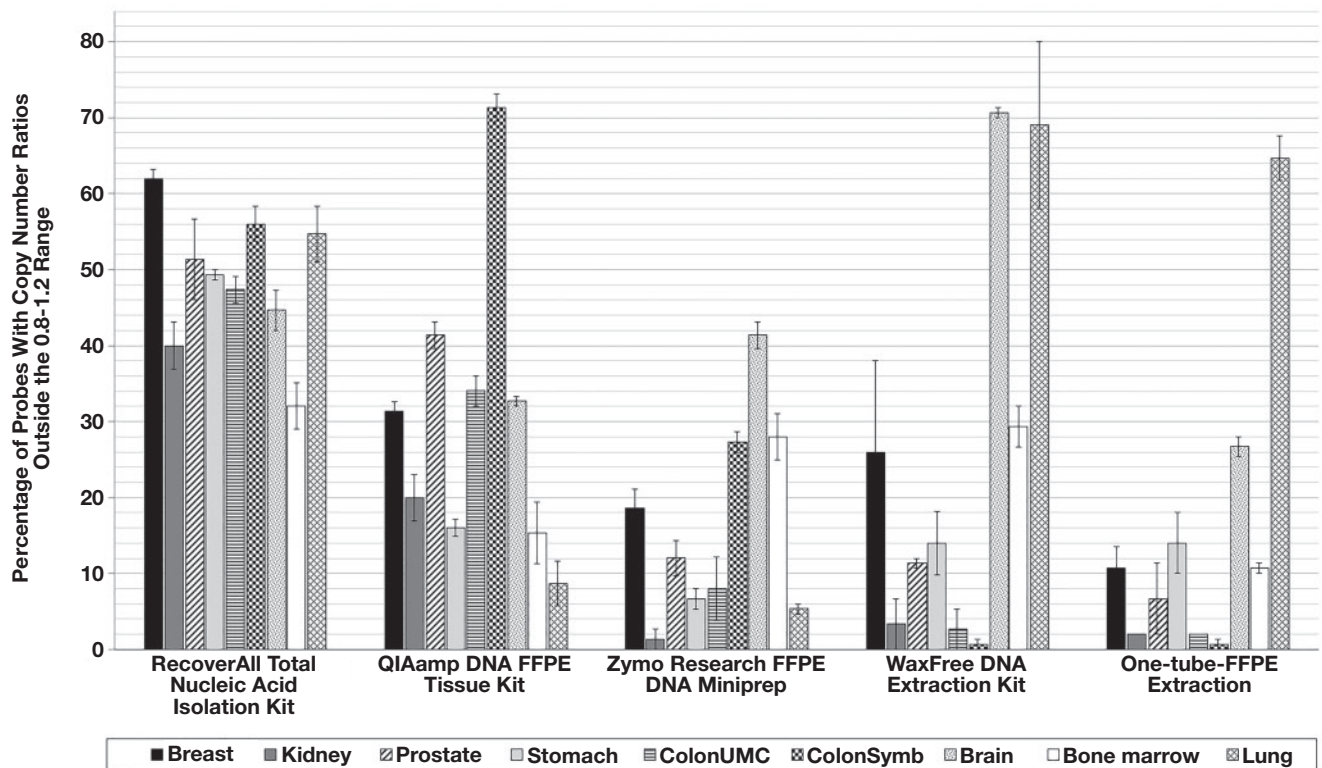
Figure 2 The influence of five different DNA extraction methods on the multiplex ligation-dependent probe amplification (MLPA) probe's copy number ratios. MLPA was performed on DNA extracted from eight various formalin-fixed, paraffin-embedded (FFPE) tissues with the P027 probe mix (50 probes). The y-axis represents the percentage of probes showing copy number ratios outside the 0.8 to 1.2 normal copy number range. The x-axis shows five different DNA extraction methods. Each bar represents a separate FFPE tissue-type block. For samples where DNA was extracted with the one-tube FFPE extraction method, results from crude (nonpurified) DNA lysate are presented for all tissues, except the lung. The crude lysate of the lung FFPE tissue was also purified. SEM (as indicated by error bars) was calculated by dividing the standard deviation of the mean of the number of probes with copy number ratios outside the normal range by the square root of the number of samples (triplicate) (Supplementary Table S6). colonSymb, tissue from Symbiant Pathology Expert Centre, Alkmaar; colonUMC, tissue from Department of Pathology, University Medical Centre Utrecht, Utrecht.

DNA samples (14%) and highest for brain FFPE tissue DNA samples (43%), regardless of extraction method (Figure S1 and Supplementary Tables S5 and S6). Among the tested DNA extraction methods, the Zymo Research FFPE DNA miniprep kit and the one-tube FFPE extraction method produced DNA with the lowest amount of variable copy number ratios averaged for all tissue types (17% and 10%, respectively) (Supplementary Figure S2 and Table S5).

Discussion and Conclusions

The recovery of DNA from FFPE tissues is a challenging task. Molecular techniques have an optimal performance on high-quality DNA samples extracted from blood or fresh-frozen tissues but may fail when FFPE-derived DNA is used. Therefore, multiple PCR-based and array technologies have

been optimized (either the method itself or data analysis) to overcome the suboptimal quality of FFPE-extracted DNA samples. MLPA, being a widely applied technique for gene copy number detection, has also been used on FFPE tissue-extracted DNA samples.[6-8,12,13] However, the preanalytic conditions (sample collection, fixation, embedding, and DNA extraction methods) of these studies are not always known for various reasons. The aim of this study was to investigate the effect of tissue fixation parameters as well as the best FFPE DNA extraction method for robust downstream MLPA application. For addressing tissue fixation conditions, the data presented are based on one tissue type (colon) and on two different MLPA probe mixes that contain probes targeting a wide variety of genomic regions. The nonbuffered formalin with a pH of 3.0 resulted in a very high variation in copy number ratios using DNA samples with known normal
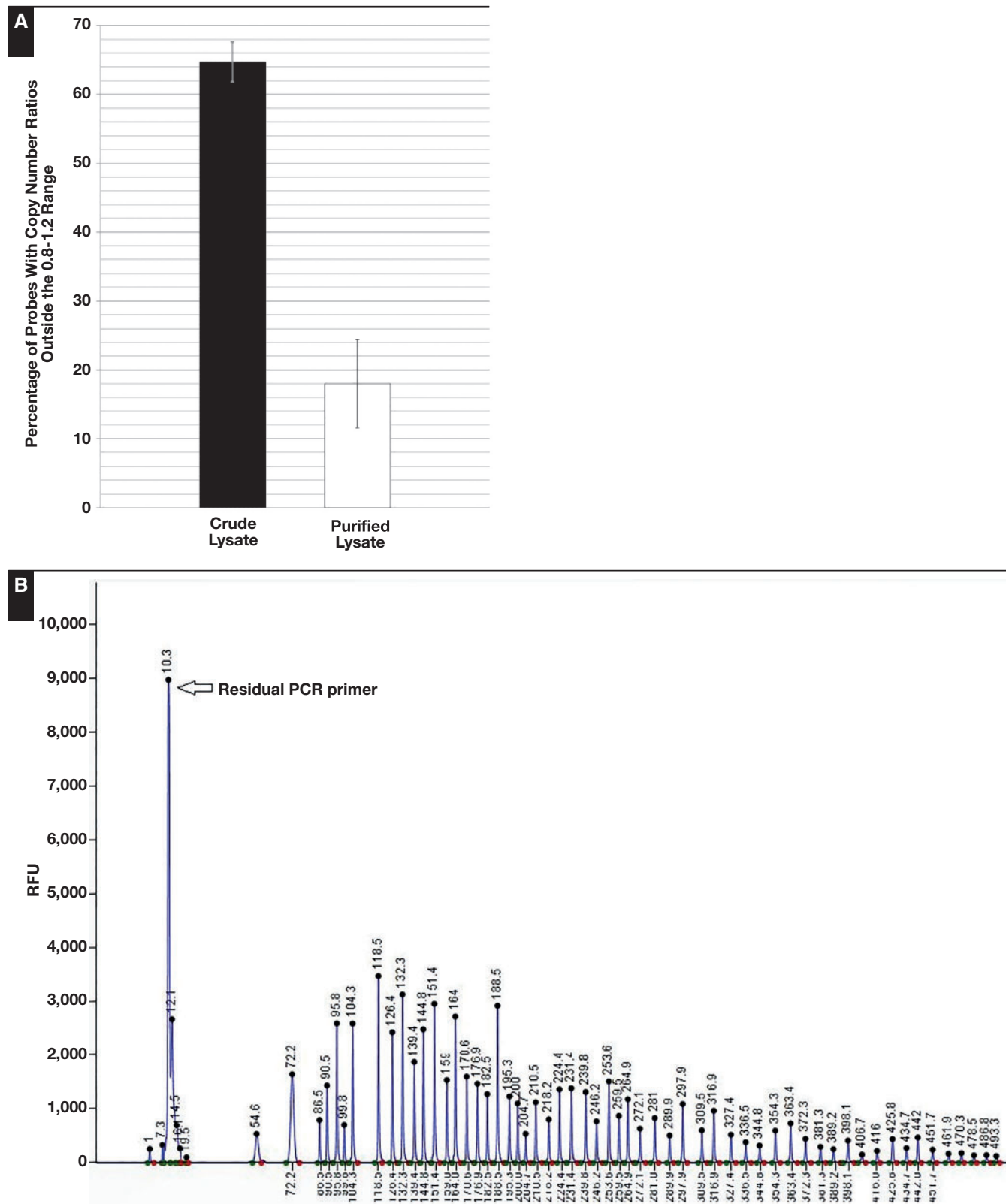
Figure 3 Multiplex ligation-dependent probe amplification (MLPA) on DNA in the crude lysate from formalin-fixed, paraffin-embedded (FFPE) lung tissue: before (**B**) and after (**C**) purification. **A**, Percentage of probes outside the range for normal copy number ratios (0.8-1.2) in the MLPA reaction performed on FFPE lung tissue crude lysate extracted with the one-tube FFPE extraction (black bar) and on the same crude lysate purified with the DNA Clean & Concentrator-5 kit (plain bar). **B**, MLPA electrophero-gram of FFPE lung tissue crude lysate; the arrow indicates the residual fluorescent MLPA primer peak. PCR, polymerase chain reaction; RFU, residual fluorescence unit.
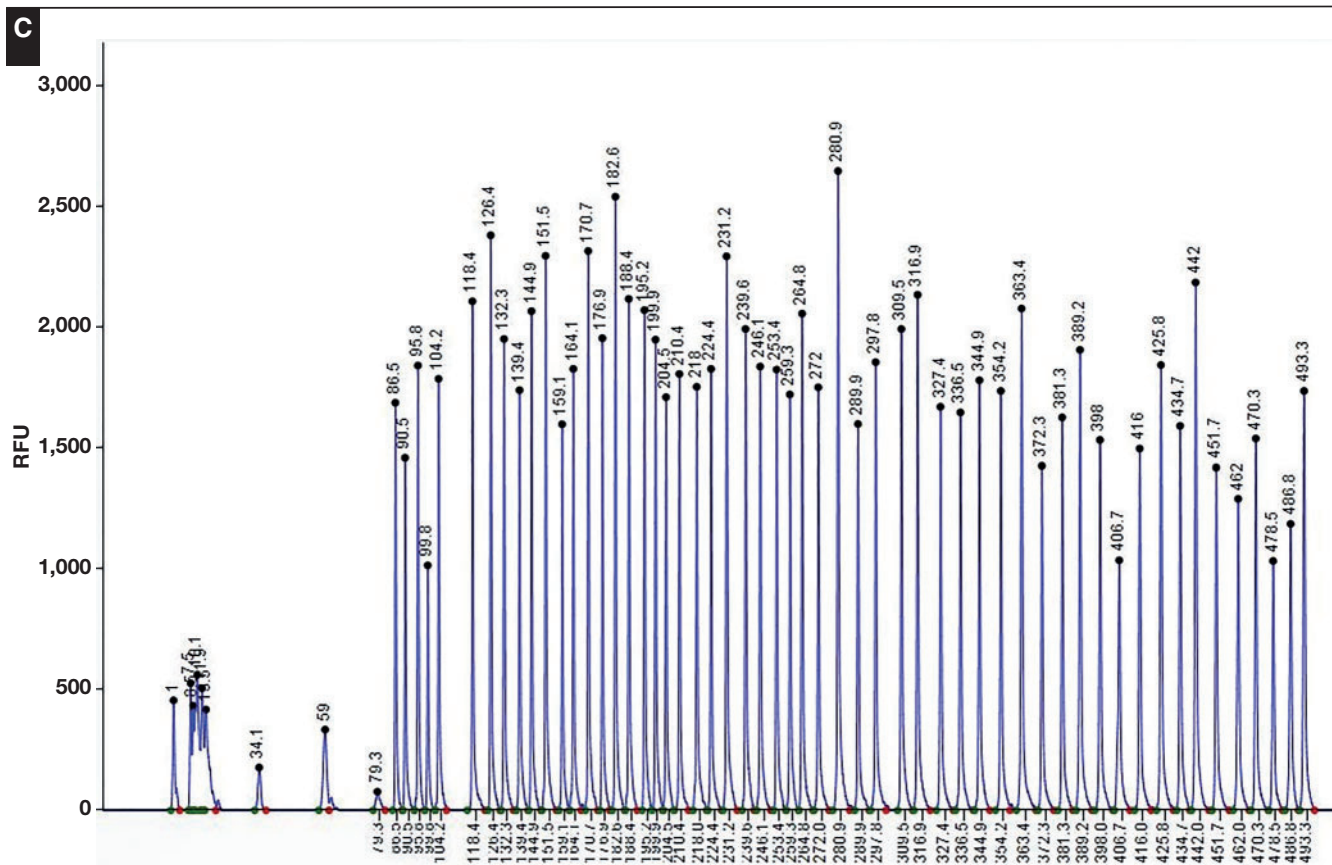
Figure 3 (cont) **C**, MLPA electropherogram of FFPE lung tissue crude lysate column purified with the DNA Clean & Concentrator-5 kit. RFU, residual fluorescence unit.

copy number ratios (most of the probes falsely indicating copy number changes). The extent of genomic DNA damage caused by nonbuffered formalin makes the MLPA analysis hardly possible. The best tissue preservation conditions tested were the 12- to 24-hour fixation with buffered formalin at RT. When DNA extracted from healthy colon tissue fixed with above-mentioned conditions was analyzed by MLPA, all probes showed a normal copy number compared with the commercial genomic DNA standard. Fortunately, most institutions already apply these optimal fixation conditions. As the fixation conditions affect copy number quantification to a certain extent, it is critical to use identical fixation conditions for all samples, including reference samples. Besides the fixation conditions addressed in this study, multiple other variables may play a role during FFPE tissue processing, including tissue type, thickness of the slices, and time from excision to fixation. These effects are not well studied in terms of suitability for MLPA.

Furthermore, this study demonstrates that FFPE tissue source and DNA extraction method influence MLPA analysis. All five tested DNA extraction methods provided genomic DNA suitable for downstream MLPA application. The RecoverAll and QIAamp kits demonstrated the highest

number of probes deviating from the normal copy number ratio range. One possible explanation for this can be "select-ive/incomplete" DNA extraction by these two kits (the other methods to a lesser extent) since some regions of genomic DNA (with a distance between MLPA probes up to 5 kb) seem to show high and some low copy number ratio values (Supplementary Tables S6 and S7).[14] Despite having this "selective/incomplete" extraction, the DNA with both methods is still suitable for MLPA if test samples are normalized against reference samples treated similarly. This will compensate for the bias introduced by the DNA extraction kit.

Of the five tested DNA extraction methods, the one-tube FFPE extraction method resulted in probe ratios most comparable with the commercial normal copy number DNA. In addition, this method allows skipping the chemical deparaffinization with xylene, omitting the use of a fume hood. Residual amounts of paraffin in the sample extracted with the one-tube FFPE extraction did not inhibit the MLPA assay. Paraffin remnants in the QIAamp FFPE extraction kit (by replacing the chemical deparaffinization step with heating at 95°C for 15 minutes) gave the same results as the original protocol (data not shown). Elimination of the deparaffinization step

with xylene reduces hands-on time and prevents handling of a hazardous substance. In addition, all steps of one-tube FFPE extraction are performed in one reaction tube, and no columns or filters are required. However, due to this feature, there are no purification steps, and any inhibitory factors present in the initial tissue will be carried over to the MLPA reaction and may possibly inhibit the latter, as shown for lung tissue.

Furthermore, storage of one-tube FFPE extraction-extracted DNA is challenging as the crude lysate is not an optimal environment for long-term stable DNA. Thus, an important recommendation for one-tube FFPE extraction-extracted DNA is to perform an additional purification step that, for example, uses silica-based columns (eg, DNA Clean & Concentrator Kit from Zymo Research used in this study). This additional purification step will also allow accurate determination of DNA concentration, which is not possible for the crude lysate.

In the current experimental design, high-quality commercial genomic DNA extracted from blood was used as standard for normalization of MLPA results. However, when performing MLPA analysis on clinical specimens, it is highly recommended to have the standard DNA sample isolated from the same tissue type and with the same fixation and extraction conditions. As this study has investigated only DNA samples from healthy donors with expected normal copy number ratios, further investigations of DNA extraction methods using tumor samples, for example, would be of a great importance for assessing the diagnostic accuracy of such samples.

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