Journal of Visualized Experiments

Optimized High Quality DNA Extraction from Formalin-Fixed Paraffin-Embedded Human Atherosclerotic Lesions

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61452R3
Full Title:	Optimized High Quality DNA Extraction from Formalin-Fixed Paraffin-Embedded Human Atherosclerotic Lesions
Corresponding Author:	Sabine Bauer Klinikum rechts der Isar der Technischen Universitat Munchen Klinik und Poliklinik fur Vaskulare und Endovaskulare Chirurgie München, GERMANY
Corresponding Author's Institution:	Klinikum rechts der Isar der Technischen Universitat Munchen Klinik und Poliklinik fur Vaskulare und Endovaskulare Chirurgie
Corresponding Author E-Mail:	s.bauer@tum.de
Order of Authors:	Sabine Bauer
	Jessica Pauli
	Jaroslav Pelisek
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Munich, bavaria, germany
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Genetics
Please provide any comments to the journal here.	

TITLE:

Optimized High Quality DNA Extraction from Formalin-Fixed Paraffin-Embedded Human Atherosclerotic Lesions

AUTHORS AND AFFILIATIONS:

Sabine Bauer[1,2,4], Jessica Pauli[1,2], Jaroslav Pelisek [1,3]

[1]Department for Vascular and Endovascular Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

[2]Deutsches Zentrum für Herz-Kreislaufforschung (DZHK), partner site Munich Heart Alliance, Berlin, Germany

[3]Department of Vascular Surgery, University Hospital Zurich, Zurich, Switzerland

[4]Department of Experimental Cardiology, German Heart Centre, Technical University Munich, Munich, Germany

Corresponding Author:

Sabine Bauer (s.bauer@tum.de)

Email Addresses of Co-Authors:

Jessica Pauli (jessica.pauli@tum.de)

Jaroslav Pelisek (jaroslav.pelisek@usz.ch)

KEYWORDS:

FFPE, DNA extraction, atherosclerotic lesions, automated system, atherosclerosis, calcified tissue, PCR, fragmentation, low cell amount, difficult tissue

SUMMARY:

Here, we present a protocol for semi-automated DNA extraction from formalin-fixed paraffin-embedded lesions of human carotid arteries. The tissue lysis is performed without toxic xylene, which is followed by an automated DNA extraction protocol, including a second lysis step, binding of DNA to paramagnetic particles for cellulose based binding, washing steps, and DNA elution.

ABSTRACT:

Formalin-fixed paraffin-embedded (FFPE) tissues represent a valuable source for molecular analyses and clinical genomic studies. These tissues are often poor in cells or difficult to process. Therefore, nucleic acids need to be carefully isolated. In recent years, various methods for DNA isolation have been established for tissues from many diseases, mostly cancer. Unfortunately, genomic DNA extracted from FFPE tissues is highly degraded due to the cross-linking between nucleic acid strands and proteins, as well as random breakings in sequence. Therefore, DNA quality from these samples is markedly reduced, making it a challenge for further molecular downstream analyses. Other problems with difficult tissues are, for example, the lack of cells in calcified human atherosclerotic lesions and fatty tissue, small skin biopsies, and consequently low availability of the desired nucleic acids as it is also the case in old or fixed tissues.

In our laboratories, we have established a method for DNA extraction from formalin-fixed atherosclerotic lesions, using a semi-automated isolation system. We compared this method to other commercially available extraction protocols and focused on further downstream analyses. Purity and concentration of the DNA were measured by spectrometry and fluorometry. The degree of fragmentation and overall quality were assessed.

The highest DNA quantity and quality was obtained with the modified blood DNA protocol for the automated extraction system, instead of the commercial FFPE protocol. With this step-by-step protocol, DNA yields from FFPE samples were in average four times higher and fewer specimens failed the extraction process, which is critical when dealing with small-vessel biopsies. Amplicon sizes from 200–800 bp could be detected by PCR. This study shows that although DNA obtained from our FFPE tissue is highly fragmented, it can still be used for successful amplification and sequencing of shorter products. In conclusion, in our hands, the automated technology appears to be the best system for DNA extraction, especially for small FFPE tissue specimen.

INTRODUCTION:

Formalin fixation followed by paraffin embedding (FFPE) is a standard procedure for long-term preservation of pathological specimen in biobanking[1]. These samples provide a valuable source for histological studies as well as molecular analyses, especially genetic studies[2]. Further advantages of FFPE tissues are better long-term storage, lower costs, and easier storage conditions. Our intention here is to provide a reliable and easy-to-use protocol for reproducible nucleic acid isolation from small amounts of FFPE sections, since high quality DNA extraction is the first crucial step in a wide range of molecular techniques and FFPE tissues are the most available source of samples.

New scientific approaches, such as next-generation sequencing (NGS) and “omics” research approaches require high quality of nucleic acids[3-5]. Extracting DNA from FFPE tissue samples remains a challenging endeavor. DNA from FFPE samples can vary widely in quality and quantity depending upon its age and fixation conditions. Formalin, the most frequently used compound, leads to DNA-protein crosslinking[6-8] and causes unspecific random breakage in the nucleotide sequence[9]. This may significantly impact downstream genomic analyses, since crosslinking can disable polymerase chain reaction (PCR) amplification[6,10]. Due to contaminants during the fixation process, purity of the DNA isolated from FFPE samples is often limited. In recent years, various methods for DNA isolation were established, mostly from cancer tissue specimens[2,11-13].

In general, protocols for the extraction of nucleic acids from FFPE tissue can be differentiated into three main groups. The first, most commonly used group of methods includes commercially available silica-based column systems[14]. The second group involves manual organic phase extraction methods with phenol and chloroform, first described by Joseph Sambrook and David W. Russell[15]. As a third group, automated systems were established over the last years such as liquid handling systems as well as paramagnetic particle-based systems[16]. Each of the three named systems holds different advantages and disadvantages such as dangerous chemicals (i.e., xylene, phenol, chloroform), high costs[17], manpower[18], and time consumption[19]. Especially, for the difficult tissue specimen as well as high throughput analyses standardization, reproducibility,

relatively low time-consumption, manpower, and costs are the most relevant features in finding a suitable method for nucleic acid isolation[20]. Automated extraction methods are known to show better reproducible results and are more sensitive for small biopsies. Moreover, less amount of tissue or blood is needed and the risk of clogging of the system due to high amounts of paraffin is reduced. Although machines for automated nucleic acid extraction and the needed kits are more expensive compared to manual methods, they are still convincing due to less problematic extraction processes. Literature search provides a lot of publications that illustrate a direct comparison between manual, column-based, and automated DNA and RNA extraction methods from different tissues and organisms, such as plants, animals, and humans as well as cells in culture[20-22]. There are also evidences present in literature to show that DNA and RNA isolated from 10-year old snap frozen tissue can be used for downstream analyses such as PCR, quantitative PCR, NGS, methylation analyses, and cloning[9,23-26].

The major problem with, for example, aged human vascular tissue, as well as small tissue biopsies, especially concerning FFPE samples, is the lack of cells in the highly calcified atherosclerotic lesions, which consequently leads to low concentrations of nucleic acids[1]. Although several methods for DNA extraction from FFPE tissue have already been established and are widely used, the manual sample preparation methods require long hands-on time[27] and toxic reagents such as xylene or phenol are necessary for deparaffinization[2]. As described, the deparaffinization process is a crucial time-consuming step (e.g., around 30 min) that markedly affects the quality and quantity of the extracted DNA (e.g., toxic effects on DNA, such as fragmentation and degradation of deparaffinization solution and high temperatures)[28]. Recently developed new DNA extraction protocols focus on using other non-toxic deparaffinization solutions, repair strategies and automated bead technologies. In particular, automated and semi-automated methods have been shown to be successful in DNA extraction with efficient recovery, lack of cross contamination, and easy performance[29]. We have established a protocol that overcomes these limitations. As a result, our technique allows a reduction of processing and hands-on time at highest quantitative and qualitative standards.

Especially for reproducible high-throughput analyses such as genotyping, epigenomic studies, and RNA sequencing the handling of FFPE specimen with column-based purification systems is often difficult and, time consuming (e.g., long deparaffinization steps, column clogging, and long hands-on times). Clogging of the silica membranes due to high amount of paraffin is the major issue. Other circumstances that can worsen the isolation of high-quality nucleic acids are small amounts of tissue such as micro-biopsies of skin, small mouse tissue, very fatty or calcified tissue as plaques, ossified tissue, and aged samples. Especially in diagnosis and forensics, automated and semi-automated systems such as liquid handling or paramagnetic particle-based extraction methods became more and more essential over the last few years[30,31], mainly due to relatively low hands-on times and the possibility of standardization. Most of the already published protocols work perfectly for smooth tissues with high or medium amounts of cells such as tumor biopsies or plant tissue[13,22,32]. Literature about methods for semi-automated particle-based methods used for isolating DNA from relatively hard-to-handle tissue such as fixed single cells, calcified vessels, collagen rich tissue, and fatty tissue with low cell numbers are only poorly described[33].

In this study, an optimized semi-automated method for DNA isolation from vascular paraffin embedded sections is described, comparing it to two manual column-based protocols. DNA quantity, purity, and the extent of fragmentation were used for validation. The commercially available blood DNA protocol, was used as a starting point and the manual steps of the semi-automated system were subsequently optimized for the use of FFPE as well as fresh frozen tissue samples from human and animal tissue, combining steps from the FFPE and the tissue protocol. The automated step of this protocol is pre-installed on the instrument and depends on the used kit (here, the blood DNA kit). With the described semi-automated cartridge-based system it is possible to isolate DNA from blood, fresh-frozen tissue, formalin-fixed tissue and even single cells with the same protocol, machine, kit and consumables, instead of using different protocols and kits for the instrument, as it is recommended by the company. There are only minor differences in the protocols, such as one buffer and some incubation times for the different applications, which makes this protocol very useful for extracting DNA from all kinds of tissues. Our protocol is primarily optimized for calcified, poor in cells and fibrous human vascular tissue, but can of course be used and further optimized for all kinds of difficult tissues mentioned above.

Summarized, for researchers in the cardiovascular field working on atherosclerosis (e.g., aorta, carotid arteries, coronary arteries) we provide an easy-to-use, point-by-point protocol for semi-automated DNA extraction from vascular FFPE samples.

PROTOCOL:

The permission to collect human carotid atherosclerotic specimens in our biobank was approved by the local Hospital Ethics Committee (2799/10, Ethikkommission der Fakultät für Medizin der Technischen Universität München, Munich, Germany). Written informed consent was obtained from all patients. Experiments were performed in accordance with the principles of the Declaration of Helsinki.

1. Tissue preparation

1.1 Prepare 5–8 tissue sections of 10 µm from the FFPE sample with the microtome and transfer it in a 1.5 mL tube. It is not necessary to reduce the excess of paraffin from the block.

NOTE: Thinner single sections instead of one large section accelerate the buffer reaction. Discard the first sections due to O_2 exposure. For bigger samples, it is also possible to use fewer sections.

1.2 Centrifuge these tubes in a bench top centrifuge, set to 5,000 x *g* for 1 min at room temperature to collect each sample on the bottom of the tube.

CAUTION: Too long centrifugation leads to clotting of the sample and complicates the lysis.

2. Lipid dissolution and deparaffinization

NOTE: This step is needed for deparaffinization and lipid digestion. The buffer used is less toxic

than commercial deparaffinization solutions.

2.1 Add 300 µL of the commercially available incubation buffer and 6 µL of 1-thioglycerol to each tube.

NOTE: Do not use more than 300 µL as this is the maximum volume of the automation system cartridge.

2.2 Vortex for 10 s and incubate the sample for 10 min at 80 °C and 500 rpm in a heating block to solubilize paraffin.

CAUTION: The tissue should be completely dissolved in the end. If necessary, vortex several times during incubation.

3. Sample and protein digestion

NOTE: Native digestion with protease K is crucial to have clean DNA extracts without proteins. It also reduces any contaminating proteins present. Furthermore, nucleases are also destroyed to save the DNA[34]. This overnight step is also needed for complete sample digestion.

3.1 Let the sample cool down to 60 °C and then add 30 µL of the provided Proteinase K solution.

3.2 Vortex again and incubate the mixture at 65 °C and 500 rpm overnight (4–20 h) in a heating block. Vortex the samples during incubation from time to time for complete sample digestion.

NOTE: Overnight incubation leads to better results. Mixing steps are recommended every 30–60 min. In the end, there should not be any visual tissue piece inside the tube.

4. Cell lysis

4.1 Add 400 µL of the lysis buffer, provided in the blood kit and vortex shortly.

4.2 Incubate the sample again at 65 °C for 30 min with 500 rpm.

4.3 Let the sample cool down to room temperature. The paraffin will harden on top.

CAUTION: Do not vortex again, to keep the paraffin separated from the sample. Otherwise, the paraffin is mixed with the tissue, which destroys the sample. The sample will be collected in step 6.1.

5. Preparation of the pre-dispensed cartridges

5.1 Turn on the machine, as well as the associated tablet computer.

5.2 Start the software app and click on the **Door** button to open the instrument.

5.3 Remove the rack from the instrument and insert the pre-filled cartridge into the probe rack. Ensure that the cartridge clicks twice when into place and remove the sealing foil.

5.4 Add the plunger in the last (8) well of the cartridge. It serves as pipette tip in the instrument.

5.5 Fill the provided 0.5 mL elution tubes with 65 µL of elution buffer, provided with the kit. Leave the tubes open and insert them into the dedicated position in the front part of the rack, after the cartridge.

NOTE: The minimum volume for elution is 60 µL. The system will lose 5–10 µL of the added elution volume.

6. Automated DNA extraction

6.1 Carefully puncture the paraffin on top of the 1.5 mL tube from step 4.3 to reach to the clean sample at the bottom of the tube without mixing it with paraffin again.

6.2 Transfer the whole mixture (730 µL) of the prepared sample in the first well of the cartridge.

6.3 Insert the rack into the automated DNA extraction machine. Ensure that the rack locks in the back of the machine first and in the front afterwards.

6.4 Start the run by clicking the upper left orange **Start** button in the software. A window with different pre-installed protocols will open. Select **Blood DNA protocol** on the instrument. Confirm that plunger, elution tube, and sample were added by clicking yes in the software. The door of the instrument will close automatically, and the run starts (the light will turn green). The run will take approximately 38 min. No further calibration is needed.

NOTE: Watch until the system has picked up the plungers for all samples in the rack. If this is not happening the system stops automatically and the machine protocol must be restarted.

6.5 Ensure that the system performs the automated lysis step in the first well of the cartridge, followed by washing steps in wells 3 to 7. There is no further programming step needed. The complete program is pre-installed by the company.

6.6 Once done, ensure that the system elutes the DNA in the prepared elution tubes via the added plunger. The magnetic particles stay in the plunger. The plunger in the end goes back to the last well of the cartridge.

7. Finish the run

7.1 When the run is completed (the machine shows blue light), open the instrument by clicking the button for opening (door-sign) and remove the rack from the system.

7.2 Discard the cartridges.

7.3 Reinsert the empty rack to the instrument and close the door via the door button in the upper-right corner. Close the software app and turn off the machine, as well as the tablet computer.

7.4 Store eluates at -20 °C for long-term storage or at 4 °C for short-term storage or use it directly for downstream analysis or concentration measurements.

REPRESENTATIVE RESULTS:

For the establishment of the protocol, 5 FFPE tissue blocks from patients with atherosclerosis of the carotid artery were used. DNA was isolated with an optimized semi-automated protocol (kit C) as well as with two commercially available manual column-based protocols (kit A and kit B, see **Table of Materials**). DNA extraction with kit A and B was performed according to the manufacturer´s protocol. The only change that was made in the protocol of the two commercially available kits (kit A and kit B): deparaffinization in the beginning was performed twice due to high amounts of paraffin surrounding the sections. After deparaffinization with xylene, DNA was bound to the column-membrane and washed three times. After drying the membrane by centrifugation, DNA was eluted with 60 µL of the provided elution buffer.

To determine the optimal quantity of samples needed for reproducible results from every patient, different amounts of material were taken. Consecutive tissue sections of 2 x 10 µm (20 µm in total), 5 x 10 µm (50 µm in total), and 8 x 10 µm (80 µm in total) were used (**Figure 1**). The quality and quantity of total genomic DNA isolated with the automated system and the two-manual column-based systems were assessed both with spectrometry, a microplate reader spectrometric system as well as fluorometry. Fragmentation analysis was performed using automated screen-tape technology and polymerase chain reaction (PCR). Statistical analysis was done using commercially available software (see **Table of Materials**).

The semi-automated protocol leads to higher quantity of the extracted DNA

Using micro-volume spectrometry and plate reader spectrometry as well as fluorometry as shown in **Figure 2**, the total amount of genomic DNA was compared for the different amounts of tissue sections (20 µm, 50 µm, 80 µm in total). A linear increase of DNA concentration was obtained with the cartridge-based semi-automated kit C, with all three quantification methods. For the manual kits A and B, no significant differences were observed between 20 µm, 50 µm, and 80 µm sections. The mean concentration of all five tissue samples measured with micro-volume spectrometry and microplate reader technology for DNA extracted with the semi-automated protocol (**Figure 2A,B**, **Table 1**) showed a significant, a four times higher,

concentration (45 ng/µL) compared to the column-based protocols A and B ($p = 0.016$). Kit A attained mean concentrations of 12.5 ng/µl, while Kit B attained only 8.5 ng/µL. **Figure 3** shows the quality of the extracted DNA with the three named methods. Summing up that there is no big difference in quality. The semi-automated kit shows more stable quality, the measured quantities are comparable. Similar results were found by using fluorimetric measurements of the DNA concentration (**Figure 2C**). Mean concentrations of DNA using kit A were further reduced to 3 ng/µl compared to the spectrometric analysis. Summarizing the quantity of DNA, the automated protocol turned out to be the most efficient method for low cells-tissue samples.

Automated extraction protocol leads to a better quality of the extracted DNA

Furthermore, the quality of DNA isolated with the three different protocols was assessed spectrophotometrically, both using spectrophotometer and microplate reader, calculating the A260/280 ratio (**Figure 3A,B and Table 2**). Nucleic acids have their absorbance maximum at 260 nm. Purity of high quality DNA is generally in the range from A260/280 values of 1.6–1.8, as evidenced by the two dashed lines in **Figure 3A,B**[35]. The mean ratio of the manual column-based kit A was 1.92 ± 0.6 in spectrometry and 2.58 ± 1.2 measured with microplate-reader technology. Kit B showed smaller ratios (1.45 ± 0.3 and 1.85 ± 0.7). DNA isolated with the semi-automated kit C showed purity ratios of 1.4 ± 0.07 in spectrometry and 1.68 ± 0.05 calculated with the microplate reader. In both the measurements, the automated isolation method showed less variation in purity ratio than the manual kits but only kit A showed significant differences in paired t-test analysis because of the huge scattering ($p = 0.0025$ and $p < 0.001$). Regarding the microplate measurement, all samples isolated with the automate system passed the purity criteria (ratios between 1.6 and 1.8), whereas kit A and kit B in many cases failed these criteria ($p < 0.001$). Spectrometry (**Figure 3A**) evidenced that not all samples isolated with the optimized cartridge system (minimum ratio = 1.36) reached the defined purity threshold (dashed lines, indicating the A260/280 ratio of 1.6 and 1.8), indicating buffer contamination. In addition, huge variation of the calculated ratio for kits A and B was observed. Consequently, semi-automated DNA isolation provides samples with comparable and reproducible quality, whereas the DNA quality is widely fluctuating with column-based isolation methods. Another quality check was performed using DV200 calculation (**Figure 3C, Table 2**). Here, the manual column-based extraction system A showed a value of 90% ± 3.8 for fragments bigger than 200 bp, whereas kit B only showed 85% ± 7.1 ($p = 0.01$). Also, in this analysis, DNA extracted with the semi-automated protocol showed the highest percentage of fragments bigger than 200 bp (92% ± 3.6).

Summing it up, quality of the DNA extracted with the semi-automated system was more stable and reproducible, whereas DNA isolated with the column-based systems showed more variation in quality ratios. DV 200 indicated similar quality for kits A and C, whereas kit B had less fragments bigger than 200 bp.

DNA fragmentation is independent of the used protocol

Fragmentation of the DNA was assessed with semi-automated screen tape technology. In **Figure 4A**, representative gels are shown to display the extent of fragmentation of all samples used in this study. Dark color indicates a higher density whereas grey shaded images indicate lower densities. Here, similar patterns were observed for all samples and in all three kits. The first three

samples show the darkest region (which equals the highest accumulation of the fragmented DNA), in the lower part of the gel representing the smaller DNA fragments, samples from patient 4 and 5 show more (denser) fragments in the upper part of the gel, indicating less fragmented DNA. In **Figure 4B**, the difference in DNA fragmentation using various amounts of material (20 µm, 50 µm, and 80 µm) is shown for one representative sample using all three isolation strategies. Electropherograms show the corresponding fragment distribution. The data is summarized in **Table 3**. Here, the only visual difference between the three kits was found in the graphs of the 20 µm sections, with the automate kit showing a clearer electropherogram with more DNA detected. For 50 µm and 80 µm sections, no visible difference in the electropherogram was found between the different kits used in the study.

Figure 5A shows the mean distribution of the fragment sizes for the semi-automated cartridge system kit C as well as for the two manual kits A and B. Mean distribution of the fragment sizes for the automated system shows the following results: 5% of the DNA fragments were smaller than 200 bp, 62% sized between 200 bp and 1500 bp and 29% of the DNA fragments were longer than 1,500 bp. Higher percentage of augmented DNA fragmentation was measured with Kit B (12%) and Kit A (8%). Medium size fragments were similar for kit A (62%) and kit B (57%). Regarding the longer DNA fragments, no significant difference (kit A 26%, kit B 27%) were observed. This leads to the conclusion that DNA isolated with the optimized semi-automated kit (kit C) seems to be less fragmented than DNA isolated with column-based kits.

The fragmentation peak (highest amount of DNA for a certain fragment size) in all three kits was analyzed for each of the five tissue samples (**Figure 5B**). No differences between the three isolation methods could be detected concerning the overall DNA fragmentation. Of note, regardless of the kit utilized, samples 1 to 3 show an average maximum peak between 280 bp and 620 bp whereas samples 4 and 5 are less fragmented with an average fragmentation peak between 1,200 bp and 1,800 bp. These results indicate that DNA fragmentation is affected by the quality of the specimens itself.

DNA extracted with the semi-automated kit is suitable for PCR analysis

Ultimately, eight DNA samples isolated with the optimized protocol were used for downstream analysis performing PCR of different fragment lengths. Appropriate primers for the beta-actin housekeeping gene were chosen. A primary smooth muscle cell line served as a control (**Figure 6**). By displaying the detectable fragments in agarose gel electrophoresis, both samples with large maximum fragments (500 bp–800 bp for samples 1, 2, and 8) as well as samples with only 200 bp of maximum fragments were detected (samples 6 and 7), independent of the extraction method used.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental workflow. The main steps of sample preparation and semi-automated DNA extraction are shown in the context of the used specimens and in comparison, with the manual DNA extraction kits A and B.

Figure 2: Concentration measurement. The upper part (**A-C**) shows the DNA concentrations measured with spectrometry, microplate reader technology and fluorometry using 20 µm, 50 µm, and 80 µm sections (each, n = 5) for all three different kits applied. The lower part shows the distribution of the DNA concentrations of five samples (80 µm) measured with spectrometry (**D**), microplate reader technology (**E**) and fluorometry (**F**). p-values are calculated with the paired t-test, $p < 0.05$ was considered significant.

Figure 3: Purity of the isolated DNA. The purity of DNA was assessed by calculating the A260/280 ratio in two different spectrometers (**A,B**) for all three different kits (n = 15). Dashed lines demonstrate the optimal ratio (1.6–1.8). (**C**) shows the DV200 (fragments >200 bp) in % calculated via fragmentation analysis. P-values are calculated with Mann-Whitney test and shown in the graph accordingly, $p < 0.05$ was considered significant.

Figure 4: Representative images from the fragmentation analysis. The fragmentation of each individual sample 1–15 is shown in the screen-tape traces. For every sample (n = 5) different amount of DNA from 20 µm, 50 µm, and 80 µm sections were analyzed separately (n = 3 for each sample) (**A**). (**B**) shows the DNA fragmentation of one representative specimen for 20 µm, 50 µm, and 80 µm sections for all three kits.

Figure 5: Fragment distribution by screen-tape analysis. (**A**) shows the distribution of short, medium, and long fragments within the samples isolated with different kits. (**B**) shows the maximum fragmentation peak of the DNA isolated from samples 1–5 plotted for the individual kits. For every kit, the 20 µm, 50 µm and 80 µm sections of each samples are pooled. p-values are calculated with paired multiple t-test and shown in the graph, $p < 0.05$ was considered significant.

Figure 6: Fragmentation analysis with PCR. This gel electrophoresis shows the PCR products with different amplicon lengths of the beta-actin housekeeping gene for eight different samples and one control cell line. Lanes are named as follows: 1 = 100 bp, 2 = 200 bp, 3 = 300 bp, 4 = 400 bp, 5 = 500 bp, 6 = 600 bp, 7 = 700 bp, 8 = 800 bp, 9 = 1,000 bp, and 10 = 1,300 bp.

Table 1: Results of quantitative analysis. This table shows the minimum, maximum, and mean concentration, of the DNA isolated from the five patients with the three different kits. Concentrations were measured spectrometrically using micro spectrometer and microplate reader, as well as fluorometrically.

Table 2: Results of qualitative analysis. A260/280 ratios calculated with micro-spectrometer as well as microplate-reader are shown for every sample (n = 15) and the three different extraction methods. DV200 (fragments bigger than 200 bp) calculated via fragmentation analysis is also shown for kits A, B, and C.

Table 3: Results of fragmentation analysis. Here, the percentage of the fragments smaller than 200 bp, bigger than 1,500 bp and between 200 bp and 1,500 bp is shown for all five patients, the

different amounts of starting material (20 µm, 50 µm, and 80 µm) and the three different isolation methods. Percentages smaller than 2% were named as not detectable.

DISCUSSION:

DNA extraction methods for FFPE tissue vary in quality and quantity of isolated DNA, which inevitably affects the performance of further downstream analyses. Thus, automation is becoming imperative to improve workflow and standardization, as well as quality management. Therefore, in the present study, a semi-automated method for DNA extraction from FFPE samples was evaluated demonstrating better results than the other tested manual column-based protocols.

To optimize the described semi-automated method, our aim was to have one general protocol for every kind of tissue and body liquid, as well as fixed specimen. For this, we used the commercially available DNA Blood kit from the company (**Table of Materials**) for optimization. First of all, we included an incubation step for deparaffinization, using the commercially available incubation buffer and 1-thioglycerol of the same company. In different tests regarding the incubation time and optimum melting temperature of the paraffin, we chose a 10 min incubation step at 80 °C as intended in other commercially available FFPE kits. Concerning the amount of FFPE slices, most companies recommend 2 µm x 10 µm slices for DNA extraction. As known in this magnitude, it is extremely difficult to isolate DNA from specimen containing little cell amount. For this, we tried different amounts of fixed tissue slices and found that using 50 µm of tissue in total (best in single sections of 5 µm–10 µm) showed the best results. This is at any rate suitable for tissue with low cell amounts, but tissue with higher amounts of cells can also be treated like this. Especially column-based systems here show a high limitation with clogging of the silica membrane. The Proteinase K digestion was suggested to last between 4–20 h. Here, we found out that an incubation time of 4–6 h is indeed fine, but overnight incubation leads to more sufficient quality and quantity of DNA. For better cell lysis, it also appeared helpful to add another 10 µL of Proteinase K on the next day and incubate again for 1 h. As a shaking/mixing speed we chose more than the suggested 300 rpm since we saw that stronger mixing (500 rpm–600 rpm) leads to better proteinase K digestion, and therefore, higher amounts of DNA. Also, additional mixing steps (such as vortexing or full speed shaking) during the incubation leads to better DNA extraction. Contrary to the original FFPE protocol, we chose further incubation time after the addition of lysis buffer to the sample, with the lysis being more sufficient at 65°C. The last optimization step of the protocol was the addition of another incubation buffer. The system requires at least 60 µL of elution buffer. With the automated system being known to loose 5 µL –10 µL, we choose to have an elution volume of 65 µL.

This protocol has some crucial steps to note. The first one is to set the right temperature to solubilize the paraffin. 80 °C seems to be the best temperature for optimal melting. It is also crucial to cool the sample to 60 °C before adding proteinase K to prevent this enzyme from degradation. Next, it is important to not vortex the sample again after the 30 min incubation time with lysis buffer since the paraffin has to harden on top. Otherwise, the sample will be contaminated with paraffin. As a last step, it is also important to add some extra elution buffer volume since the system loses 5 µL–10 µL. We think that 65 µl of elution buffer is a good amount.

If higher amounts of DNA are expected, the volume can of course be increased.

The optimized semi-automated isolation method provided the highest concentrations of total genomic DNA in all FFPE samples. Using the same amount of elution volume, also the total yield of DNA was four times higher. Additionally, the column-based kits showed no increase in DNA yield using thicker tissue sections, whereas the automate system demonstrated a linear increase of the DNA concentration associated with the augmented amount of tissue used. These differences can be explained mainly due to the paraffin clogging in the columns and the consequent lowering of the DNA binding capacity. Our data also suggests that larger amount of FFPE tissue, in our case 80 µm sections, are not necessary when isolating DNA with the semi-automated system. We could show that already a smaller amount (20 µm sections) leads to a sufficient yield of DNA. Thus, valuable tissue material can be saved. Moreover, DNA extracted with the semi-automated protocol, fulfilled, in most cases, the required purity criterion for high quality DNA (A260/280 ratio between 1.6 and 1.8). In contrast, DNA obtained from column-based isolation systems showed wide variation in a DNA purity and fewer samples fit the mentioned optimum ratio.

Sarnecka et al. described recently that a magnetic particle-based system is more sufficient than column-based systems[12]. Khokhar and colleagues could demonstrate that the automated system based on another protocols is more sufficient for smaller amplicons and widely robust in terms of quantity and purity of the extracts compared with other methods[36]. Our samples confirmed these findings showing a high distribution in the A260/280 ratio of the DNA isolated by the other kits.

Regarding the fragmentation analysis, eight DNA extracts obtained from these three commercial methods were used for PCR amplification. Different fragment lengths of one housekeeping gene (beta actin) were amplified and visualized using gel electrophoresis. There was only mild variation between the individual samples that may be attributed to the fact that all tissues have similar stage of atherosclerosis. This assumption was confirmed by histology (data not shown). Furthermore, this data shows that all samples were treated with formalin in the same standard manner. No significant differences were observed with regards to the fragment length obtained from the different samples independent of the method used for DNA extraction. Hence, fragmentation of FFPE tissue does not depend on the method used for DNA isolation but is rather affected by the variability of the human specimens and formalin treatment.

Since the used semi-automated system is described as a magnetic particle mover, not a liquid handler, it also offers some advantages over other semi-automated systems. The main advantage is the minimal risk of cross-contamination, since no liquid handling or splashing happens during sample processing. Another point we want to mention is the high amount of samples, which can be processed in one run of the system, without having much more hands-on time. Using a semi-automated system also leads to a lower probability of user mistakes than with the column-based or phase-separation methods. Especially for biobanking analyses and diagnostic work as well as for handling difficult tissues the semi-automated method described above is highly recommended. In these areas, it is very important that different workers and sometimes even

unexperienced people all come to fast, easy, and reproducible results. Other advantages of the system are the generally applicable protocol, which can be used for every kind of tissue as well as blood, other body liquids, and also cells with only one minor change in the first step of the protocol, where no incubation buffer is needed.

Even if our results clearly demonstrated the superiority of the automated system for DNA extraction from FFPE over the manual protocols, it is to mention that we have compared only three commercially available kits. Consequently, our conclusion concerns only the methods tested in our study. In addition, the number of samples was low, and we used only atherosclerotic plaques for comparison. In order to generalize the results of our study, other tissue samples in a greater number would be necessary.

Nevertheless, the automated system seems to have considerable advantage over the manual kits, particularly for specimens with low amount of expected nucleic acids. Also, the system itself has certain limitations. For example, as mentioned above, it is necessary to use in minimum 60 µl for elution, because the plunger needs this amount to elute the DNA. Also, the loss of elution volume is higher for the semi-automated system than for the column-based systems. The automated method has a loss of around 10 µl of elution volume, whereas the manual methods only have a loss of 5 µl. Also, the higher costs have to be mentioned. But for high throughput analyses, the semi-automated system still offers the best benefits compared to costs. As a last limitation of the system, it is important to note that automated and semi-automated extraction is a self-contained closed system. It is, of course, possible to isolate, for example, DNA and protein, or also RNA and DNA from one sample, but it is not that easy and often used with manual phase separation methods. All buffers are customized to the kit and are not usable for other methods in contrast to Trizol reagents. At the end, it is also important to mention that, in general, DNA from FFPE samples is not suitable for whole genome sequencing because the fragmentation of the nucleic acids is too strong.

In conclusion, in this study, we demonstrate that a fully automated DNA isolation method might be an excellent tool to reduce the hands-on time and the amount of starting material. Also, it seems to be the best choice to reduce the contamination risks between samples. In addition, the flexibility of the automated system allows processing of not only one but up to 48 samples per run, which makes this method a useful tool for future high throughput analyses of samples with low number of cells and thus minor amount of nucleic acids. The quality and quantity of the isolated DNA using the automated system appears superior over other manual kits and also usage in further analyses as Southern blot, single nucleotide genotyping, cloning analyses, methylation assays as well as PCR amplification is possible.

ACKNOWLEDGMENTS:

The establishment of the protocol for automated DNA extraction was supported by Dr. Paul Muschler from the Promega company. We thank Paul Muschler for his support and scientific contribution. We also thank our colleague Dr. Moritz von Scheidt (German Heart Center Munich) for providing us with the Maxwell instrument and for supporting the experimental part. All experiments were performed in the laboratories of German Heart Center (Munich, Germany) and

Klinikum rechts der Isar (Munich, Germany). The research was funded by DFG (PE 900/6-1).

DISCLOSURES:

The authors declare that there is no conflict of interest.

REFERENCES:

1. Pelisek, J. et al. Biobanking: objectives, requirements, and future challenges—experiences from the munich vascular biobank. *Journal of Clinical Medicine*. **8** (2), 251 (2019).
2. Goelz, S. E., Hamilton, S. R., Vogelstein, B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochemical and Biophysical Research Communications*. **130** (1), 118–126 (1985).
3. Busch, A., Eken, S. M., Maegdefessel, L. Prospective and therapeutic screening value of non-coding RNA as biomarkers in cardiovascular disease. *Annals of Translational Medicine*. **4** (12) (2016).
4. Kandpal, R. P., Saviola, B., Felton, J. The era of 'omics unlimited. *BioTechniques*. **46** (5), 351–355 (2009).
5. McDonough, S. J. et al. Use of FFPE-derived DNA in next generation sequencing: DNA extraction methods. *PloS One*. **14** (4) (2019).
6. Gilbert, M. T. P. et al. The isolation of nucleic acids from fixed, paraffin-embedded tissues–which methods are useful when? *PloS One*. **2** (6) (2007).
7. Williams, C. et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. *The American Journal of Pathology*. **155** (5), 1467–1471 (1999).
8. Solomon, M. J., Varshavsky, A. Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. *Proceedings of the National Academy of Sciences*. **82** (19), 6470–6474 (1985).
9. Gillio-Tos, A. et al. Efficient DNA extraction from 25-year-old paraffin-embedded tissues: study of 365 samples. *Pathology*. **39** (3), 345–348 (2007).
10. Srinivasan, M., Sedmak, D., Jewell, S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology*. **161** (6), 1961–1971 (2002).
11. van Eijk, R., Stevens, L., Morreau, H., van Wezel, T. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. *Experimental and Molecular Pathology*. **94** (1), 121–125 (2013).
12. Sarnecka, A. K. et al. DNA extraction from FFPE tissue samples–a comparison of three procedures. *Contemporary Oncology*. **23** (1), 52 (2019).
13. Kalmár, A. et al. Comparison of automated and manual DNA isolation methods for DNA methylation analysis of biopsy, fresh frozen, and formalin-fixed, paraffin-embedded colorectal cancer samples. *Journal of Laboratory Automation*. **20** (6), 642–651 (2015).
14. McCormick, S. F. *Multilevel adaptive methods for partial differential equations*. (SIAM) (1989).
15. Sambrook, J., Russell, D. W. Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harbor Protocols*. **2006** (1), pdb. prot4455 (2006).
16. Berensmeier, S. Magnetic particles for the separation and purification of nucleic acids. *Applied Microbiology and Biotechnology*. **73** (3), 495–504 (2006).

17. Petrigh, R. S., Fugassa, M. H. DNA extraction and a cost-effective detection method for Echinococcus granulosus protoscoleces. *Veterinary Parasitology*. **198** (3–4), 410–413 (2013).
18. Lee, J.-H., Park, Y., Choi, J. R., Lee, E. K., Kim, H.-S. Comparisons of three automated systems for genomic DNA extraction in a clinical diagnostic laboratory. *Yonsei Medical Journal*. **51** (1), 104–110 (2010).
19. Rohland, N., Siedel, H., Hofreiter, M. A rapid column-based ancient DNA extraction method for increased sample throughput. *Molecular Ecology Resources*. **10** (4), 677–683 (2010).
20. Gutiérrez-López, R., Martínez-de la Puente, J., Gangoso, L., Soriguer, R. C., Figuerola, J. Comparison of manual and semi-automatic DNA extraction protocols for the barcoding characterization of hematophagous louse flies (Diptera: Hippoboscidae). *Journal of Vector Ecology: Journal of the Society for Vector Ecology*. **40** (1), 11–15 (2015).
21. Seiler, C. et al. Nucleic acid extraction from formalin-fixed paraffin-embedded cancer cell line samples: a trade off between quantity and quality? *BMC Clinical Pathology*. **16** (1), 17 (2016).
22. Harada, S. *Sample Preparation Techniques for Soil, Plant, and Animal Samples*. Springer. 125–138 (2016).
23. Haile, S. et al. Automated high throughput nucleic acid purification from formalin-fixed paraffin-embedded tissue samples for next generation sequence analysis. *PloS One*. **12** (6), e0178706 (2017).
24. Fujii, T. et al. Evaluation of DNA and RNA quality from archival formalin-fixed paraffin-embedded tissue for next-generation sequencing–Retrospective study in Japanese single institution. *Pathology International*. (2020).
25. Ludyga, N. et al. Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. *Virchows Archiv: An International Journal of Pathology*. **460** (2), 131–140 (2012).
26. Niland, E. E., McGuire, A., Cox, M. H., Sandusky, G. E. High quality DNA obtained with an automated DNA extraction method with 70+ year old formalin-fixed celloidin-embedded (FFCE) blocks from the indiana medical history museum. *American Journal of Translational Research.* **4** (2), 198 (2012).
27. Riemann, K. et al. Comparison of manual and automated nucleic acid extraction from whole-blood samples. *Journal of Clinical Laboratory Analysis*. **21** (4), 244–248 (2007).
28. Steinau, M., Patel, S. S., Unger, E. R. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. *The Journal of Molecular Diagnostics*. **13** (4), 377–381 (2011).
29. Dundas, N., Leos, N. K., Mitui, M., Revell, P., Rogers, B. B. Comparison of automated nucleic acid extraction methods with manual extraction. *The Journal of Molecular Diagnostics*. **10** (4), 311–316 (2008).
30. Okello, J. B. et al. Comparison of methods in the recovery of nucleic acids from archival formalin-fixed paraffin-embedded autopsy tissues. *Analytical Biochemistry*. **400** (1), 110–117 (2010).
31. Witt, S., Neumann, J., Zierdt, H., Gébel, G., Röscheisen, C. Establishing a novel automated magnetic bead-based method for the extraction of DNA from a variety of forensic samples. *Forensic Science International: Genetics*. **6** (5), 539–547 (2012).
32. Ivanova, N. V., Fazekas, A. J., Hebert, P. D. Semi-automated, membrane-based protocol for DNA isolation from plants. *Plant Molecular Biology Reporter*. **26** (3), 186 (2008).

33. Sengüven, B., Baris, E., Oygur, T., Berktas, M. Comparison of methods for the extraction of DNA from formalin-fixed, paraffin-embedded archival tissues. *International Journal of Medical Sciences*. **11** (5), 494 (2014).
34. Miller, S. A., Dykes, D. D., Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*. **16** (3), 1215 (1988).
35. Wilfinger, W. W., Mackey, K., Chomczynski, P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques*. **22** (3), 474–481 (1997).
36. Khokhar, S. K., Mitui, M., Leos, N. K., Rogers, B. B., Park, J. Y. Evaluation of Maxwell 16 for automated DNA extraction from whole blood and formalin-fixed paraffin embedded (FFPE) tissue. *Clinical Chemistry and Laboratory Medicine (CCLM)*. **50** (2), 267–272 (2012).

Figure1_Workflow

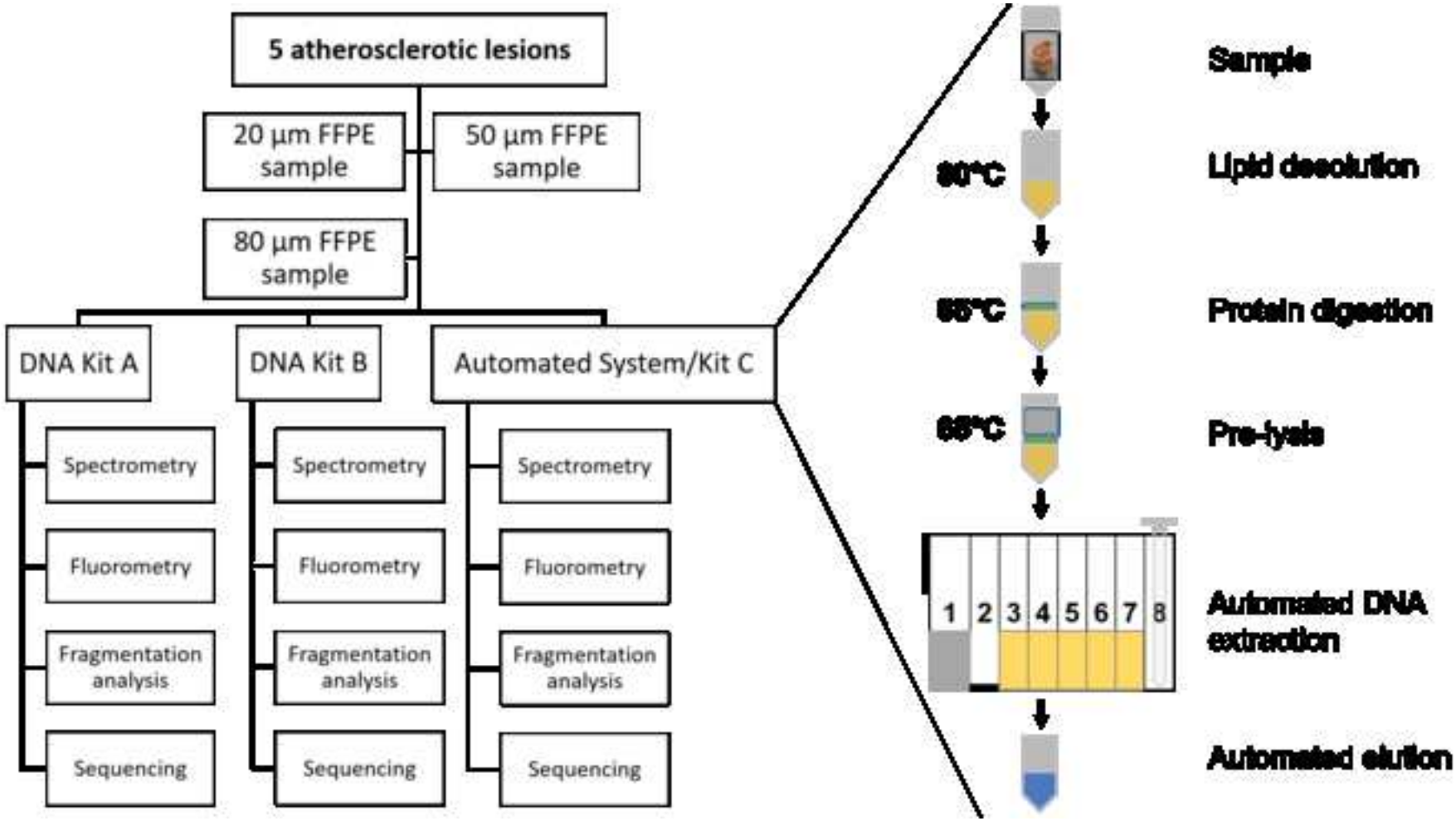


Figure3_Purity

Click here to access/download;Figure;Figure3_Purity_NEW.tif

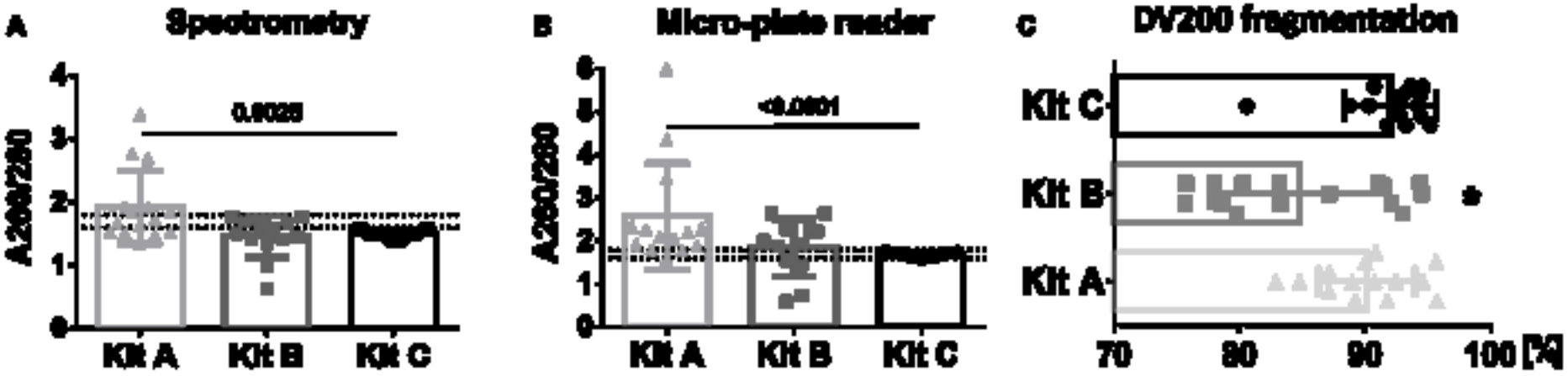


Figure4_Bioanalyzer

Click here to access/download;Figure;Figure4_Bioanalyzer_NEW.tif

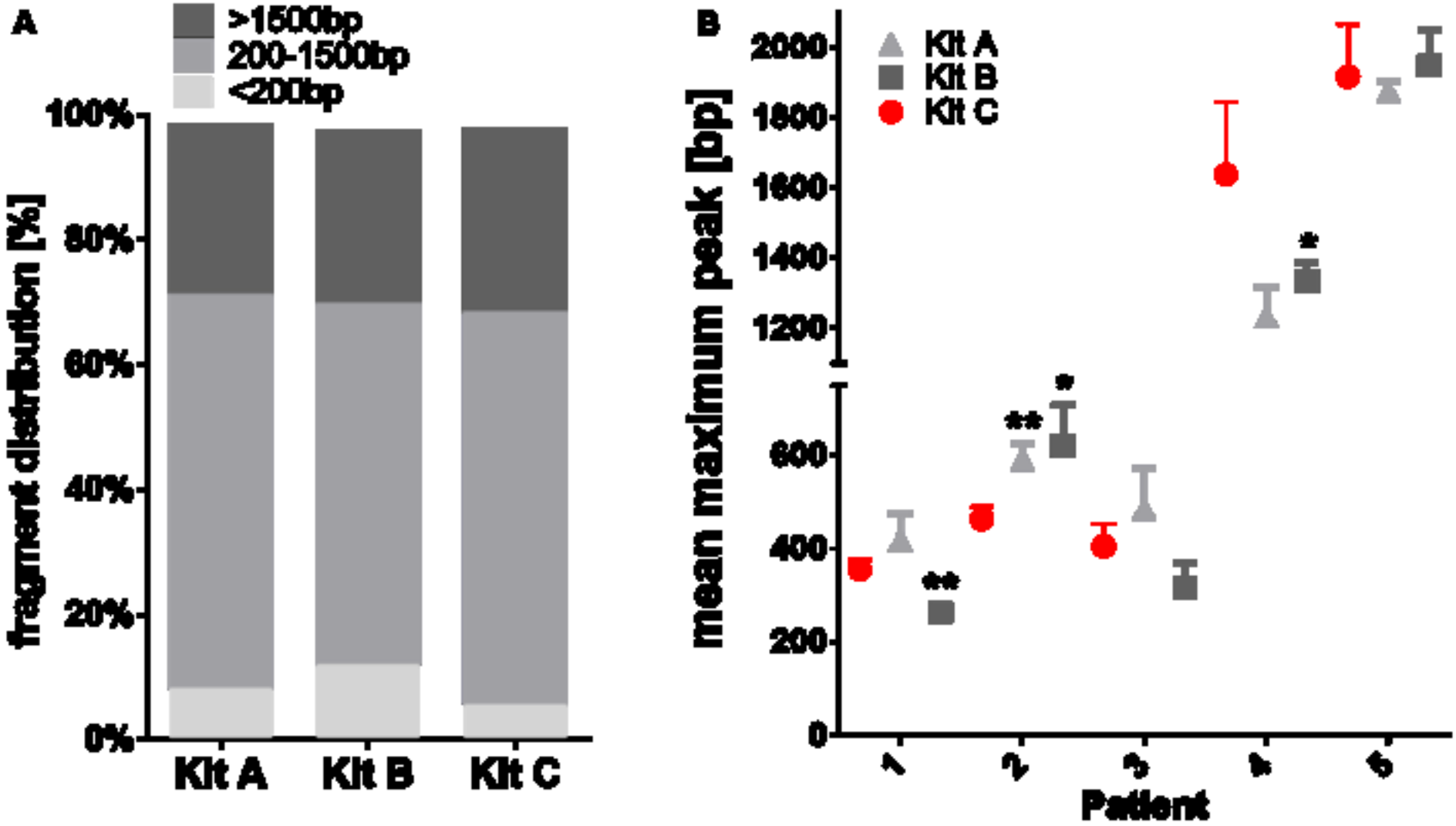
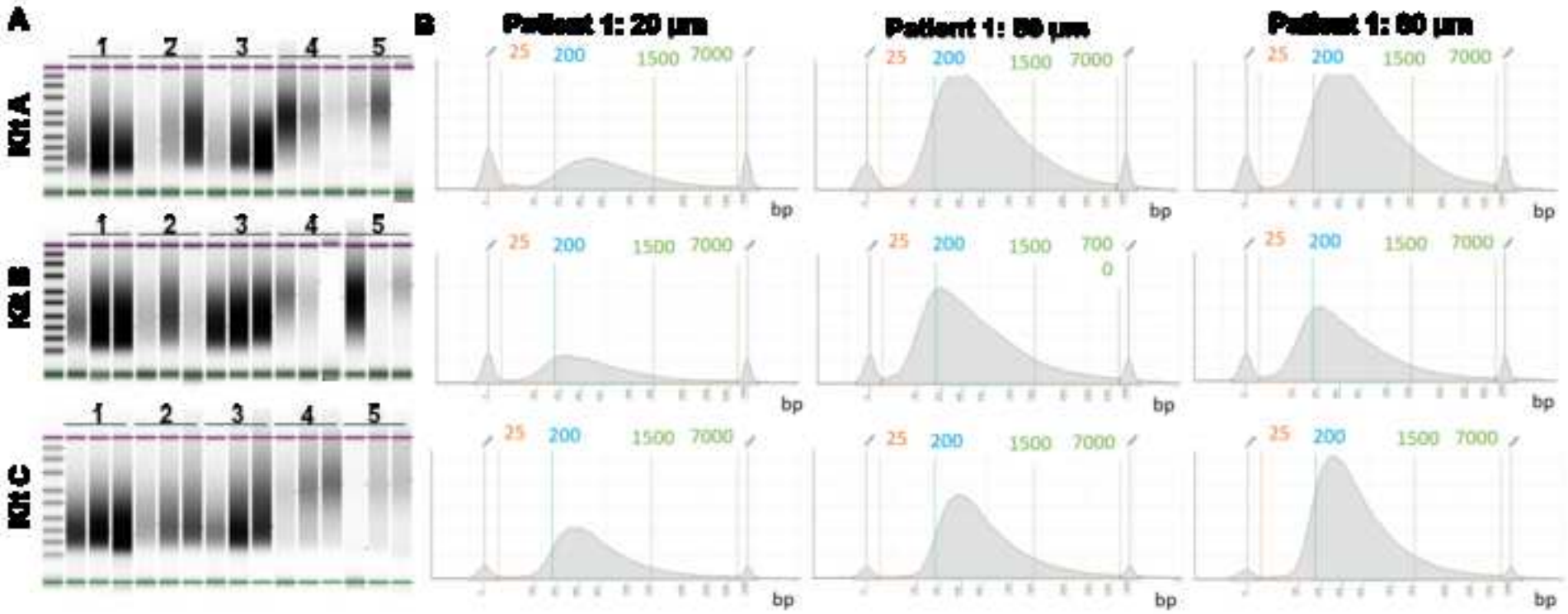


Figure5_Fragmentation

Click here to access/download;Figure;Figure5_Fragmentation_NEW.tif



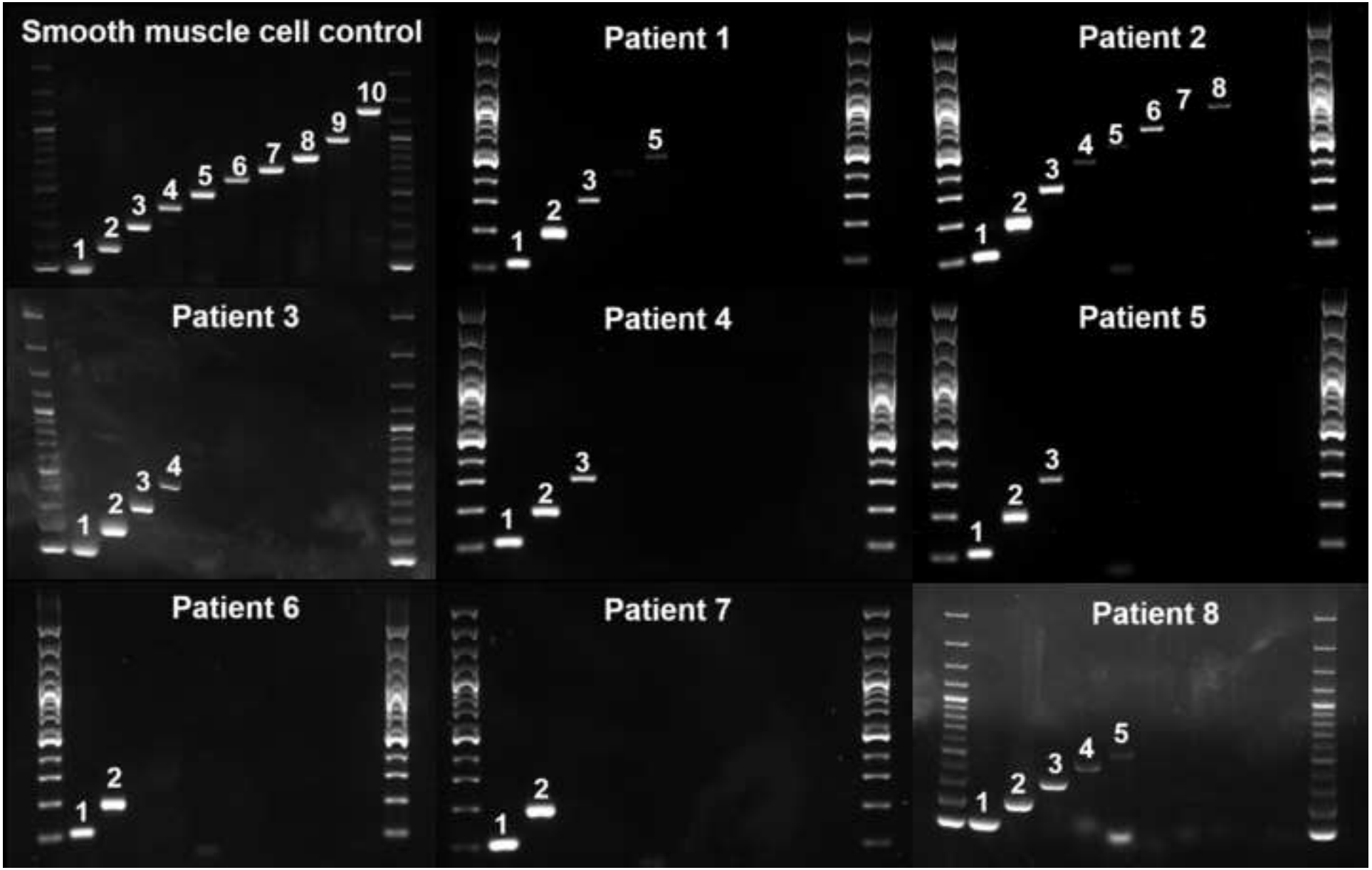


Figure1_meta

Click here to access/download

Video or Animated Figure

Figure1_Workflow_NEW_meta.emf

Figure2_meta

Figure3_meta

Click here to access/download

Video or Animated Figure

Figure3_Purity_NEW_meta.emf

Figure4_meta

Figure5_meta

Click here to access/download

Video or Animated Figure

Figure5_Fragmentation_NEW_meta.emf

Figure6_meta

Click here to access/download

Video or Animated Figure

Figure6_downstream-analysis_NEW_meta.emf

		Kit A
Spectrometer	max concentration [ng/µl]	26.2
	min concentration [ng/µl]	8.45
	mean concentration [ng/µl]	15.96
Microplate reader	max concentration [ng/µl]	24
	min concentration [ng/µl]	5.2
	mean concentration [ng/µl]	12.5
Fluorometer	max concentration [ng/µl]	10.3
	min concentration [ng/µl]	0.211
	mean concentration [ng/µl]	3.369

Kit B	Kit C
13.25	72.35
7.75	23.55
10.89	47.72
13.9	56.2
0	18.6
7.4	41.5
23.4	47.6
0.842	12.5
14.64	33.1

Table 2

Sample	µm	Kit	Microplate 260/280	Spectrometer 260/280	DV200 [%]
Patient 1	20	Kit A	6	1.65	90.72
Patient 1	50	Kit A	1.8	1.71	90
Patient 1	80	Kit A	2.09	1.345	88.98
Patient 2	20	Kit A	1.88	1.53	87.14
Patient 2	50	Kit A	2.29	1.465	92.46
Patient 2	80	Kit A	4.36	2.68	84.78
Patient 3	20	Kit A	1.91	1.85	89.24
Patient 3	50	Kit A	2.21	1.92	90.86
Patient 3	80	Kit A	2.06	1.675	94.59
Patient 4	20	Kit A	2.27	1.86	95.72
Patient 4	50	Kit A	not detectable	3.385	87.13
Patient 4	80	Kit A	2.33	1.905	95.64
Patient 5	20	Kit A	1.6	1.51	82.87
Patient 5	50	Kit A	1.79	1.525	91.76
Patient 5	80	Kit A	3.47	2.765	not detectable
Patient 1	20	Kit B	1.88	not detectable	80.23
Patient 1	50	Kit B	0.73	1.43	75.72
Patient 1	80	Kit B	1.44	1.585	75.64
Patient 2	20	Kit B	2.63	1.45	79.75
Patient 2	50	Kit B	1.92	1.765	87.14
Patient 2	80	Kit B	1.56	1.52	91.01
Patient 3	20	Kit B	not detectable	1.335	78.00
Patient 3	50	Kit B	2.28	1.63	83.21
Patient 3	80	Kit B	2	1.67	78.61
Patient 4	20	Kit B	0.59	0.625	94.37
Patient 4	50	Kit B	2.29	1.78	92.97
Patient 4	80	Kit B	not detectable	not detectable	not detectable
Patient 5	20	Kit B	2.63	1.41	83.15
Patient 5	50	Kit B	not detectable	1.005	92.06
Patient 5	80	Kit B	2.21	1.69	94.51
Patient 1	20	Kit C	1.69	1.495	90.73
Patient 1	50	Kit C	1.72	1.555	94.43
Patient 1	80	Kit C	1.72	1.565	88.71
Patient 2	20	Kit C	1.64	1.4	92.75
Patient 2	50	Kit C	1.63	1.455	93.26
Patient 2	80	Kit C	1.72	1.51	94.34
Patient 3	20	Kit C	1.63	1.4	91.78
Patient 3	50	Kit C	1.7	1.505	93.61
Patient 3	80	Kit C	1.74	1.595	94.61
Patient 4	20	Kit C	1.62	1.36	92.89
Patient 4	50	Kit C	1.71	1.495	94.2
Patient 4	80	Kit C	1.76	1.575	95.17
Patient 5	20	Kit C	1.57	1.36	80.55
Patient 5	50	Kit C	1.66	1.455	92.65
Patient 5	80	Ki t C	1.69	1.45	90.29

Table 3

Click here to access/download;Table;Table3_results of fragmentation analysis.xlsx

Sample	µm	Kit	Fragments % <200bp	Fragments % 200-1500 bp	Fragments % >1500bp	Fragments max peak (bp)
Patient 1	20	Kit A	9.04	77.08	13.64	484
Patient 1	50	Kit A	10.14	77.03	12.97	378
Patient 1	80	Kit A	10.91	76.56	12.42	393
Patient 2	20	Kit A	11.01	67.04	20.1	589
Patient 2	50	Kit A	6.09	68.67	23.79	628
Patient 2	80	Kit A	11.54	66.07	18.71	560
Patient 3	20	Kit A	10.22	77.29	11.95	412
Patient 3	50	Kit A	8.52	76.93	13.93	465
Patient 3	80	Kit A	4.79	77.5	17.09	580
Patient 4	20	Kit A	2.7	55.77	39.95	1331
Patient 4	50	Kit A	7.6	54.3	32.83	1183
Patient 4	80	Kit A	2.5	61.06	34.58	1185
Patient 5	20	Kit A	12.32	36.52	46.35	1895
Patient 5	50	Kit A	5.34	36.37	55.39	1856
Patient 5	80	Kit A	not detectable	not detectable	not detectable	not detectable
Patient 1	20	Kit B	18.51	69.28	10.95	283
Patient 1	50	Kit B	23.58	65.24	10.48	254
Patient 1	80	Kit B	22.42	67.14	8.5	257
Patient 2	20	Kit B	16.21	59.88	19.87	523
Patient 2	50	Kit B	9.39	63.7	23.44	678
Patient 2	80	Kit B	7.45	67.62	23.39	666
Patient 3	20	Kit B	18.53	65.15	12.85	370
Patient 3	50	Kit B	15.96	72.12	11.09	316
Patient 3	80	Kit B	20.7	71.49	7.12	266
Patient 4	20	Kit B	3.09	57.44	36.93	1296
Patient 4	50	Kit B	3.69	52.2	40.77	1373
Patient 4	80	Kit B	not detectable	not detectable	not detectable	not detectable
Patient 5	20	Kit B	9.86	34.96	48.19	1831
Patient 5	50	Kit B	3.76	31.42	60.64	2002
Patient 5	80	Kit B	1.93	33.24	61.27	2015
Patient 1	20	Kit C	8.88	81.47	9.26	358
Patient 1	50	Kit C	5.92	83.95	10.48	374
Patient 1	80	Kit C	11.02	80.18	8.53	334
Patient 2	20	Kit C	5.18	74.75	18	492
Patient 2	50	Kit C	4.96	75.67	17.59	458
Patient 2	80	Kit C	3.14	72.46	21.88	441
Patient 3	20	Kit C	7.15	79.05	12.73	393
Patient 3	50	Kit C	5.85	78.02	15.59	365
Patient 3	80	Kit C	2.59	68.54	26.07	458
Patient 4	20	Kit C	3.94	54.49	38.4	1433
Patient 4	50	Kit C	1.42	45.24	48.96	1634
Patient 4	80	Kit C	0.76	37.03	58.14	1849
Patient 5	20	Kit C	13.02	40.78	39.77	not detectable
Patient 5	50	Kit C	2.72	39.08	53.57	1813
Patient 5	80	Ki t C	5.25	31.87	58.42	2024

Table of Materials

Click here to access/download;Table of Materials;Material_table.xlsx

Material
1.5 ml tubes for sample incubation
1-Thioglycerol
Agilent tape station software 3.2
dsDNA HS Kit
FFPE DNA Purification Kits (Kit A)
FFPE tissue samples n=5
GeneRead DNA FFPE Kit (Kit B)
Heating blocks, set to 80°C and 65°C
High Sensitivity D5000 reagents
High Sensitivity D5000 ScreenTape
Incubation Buffer
Maxwell Blood Kit RSC including: Lysis Buffer, Elution Buffer, Proteinase K
Maxwell RSC 48 Instrument
Microcentrifuge
NanoDrop 2000c Spectrometer
Optical caps
Optical tube strips
Pipettors and pipette tips
Prism 6 for statistics, version 6.01
Qubit 3.0 Fluorometer
TapeStation 4200
Tecan Infinite M200 Pro

Company	Ref. Number
Eppendorf, Hamburg, Germany	30120086
Promega, Walldorf, Germany	A208
Agilent, Waldbronn, Germany	
ThermoFisher Scientific, Schwerte, Germany	Q32851
Norgene Biotek, Heidelberg, Germany	47400
Munich Vascular Biobank,Munich, Germany	
Qiagen, Hilden, Germany	180134
VWR,Darmstadt,Germany	460-0250
Agilent, Waldbronn, Germany	5067-5593
Agilent, Waldbronn, Germany	5067-5592
Promega, Walldorf, Germany	D920
Promega, Walldorf, Germany	AS1400
Promega, Walldorf, Germany	AS8500
Eppendorf, Hamburg, Germany	
ThermoFisher Scientific, Schwerte, Germany	ND-2000C
Agilent, Waldbronn, Germany	401425
Agilent, Waldbronn, Germany	401428
Eppendorf, Hamburg, Germany	
GraphPad Inc., San Diego, California	
ThermoFisher Scientific, Schwerte, Germany	Q33216
Agilent, Waldbronn, Germany	
Tecan, Männedorf, Swizerland	IN-MNANO

Klinik für Gefäßchirurgie
Klinikum rechts der Isar
der Technischen Universität München
Anstalt des öffentlichen Rechts

Direktor: Univ. Prof. Dr. H.H. Eckstein

Briefanschrift 81675 München
Telefon +49 (89) 4140 -2167
Telefax +49 (89) 4140 - 4861
E-Mail gefaesschirurgie@lrz.tum.de
Internet www.gchir.med.tum.de

Klinik für Gefäßchirurgie
Klinikum rechts der Isar, Ismaninger Straße 22, 81675 München

14.08.2020

Dear Editor,

enclosed please find our revised manuscript JoVE61452 “Optimized high quality DNA extraction from formalin fixed, paraffin embedded human atherosclerotic lesions”.

In accordance with the editor comments, we have proofread the manuscript modified the manuscript to address all objections and suggestions.

In particular, to the editor’s comments and our changes:

Title:
We have changed the title to “Optimized **high quality** DNA extraction …” Instead of “Optimized DNA extraction…” to clarify the aim of the manuscript

Authors and Affiliations:
We added an additional affiliation of Sabine Bauer to the list. We also added the email addresses of the co-authors Jessica Pauli and Jaroslav Pelisek.

Summary:
We proofread the summary after shortening and changed only one word.

Abstract:
We reword the sentence “Hence, nucleic acids need to be reliably isolated from small amounts of cells or difficultly to process specimen of FFPE sections“by building two separate sentence and reducing filler words.

So the blood DNA protocol was used to FFPE samples?
Yes, the blood DNA protocol provided from the company was used as a starting point. We changed some manual steps by adding some steps of the commercially available FFPE protocol (for FFPE kit) and changing some conditions.
Of course we also tried the FFPE kit with some troubleshooting steps but the changed blood protocol led to much better results.
Introduction:
Presently the introduction section is not organized. Please organize as:

a) A clear statement of the overall goal of this method → see Line 63-64
b) The rationale behind the development and/or use of this technique → see Line 110-112
c) The advantages over alternative techniques with applicable references to previous studies. → see Line 110-112
d) A description of the context of the technique in the wider body of literature → Line 67-76 sufficiently covers this comment.
e) Information to help readers to determine whether the method is appropriate for their application → see 145-147

In this section we added for each paragraph in the end one summary sentence recording to our step-by-step protocol to show more clearly the overall goal as well as the context of our method. For reasons of better understanding it was not possible to move the "literature-paragraph" to position d.

Please reword for clarity, including the significance of isolating nucleic acid from FFPE samples. "Hence, nucleic acids need to be reliably isolated from small amounts of FFPE sections, since high quality DNA extraction is the first crucial step in a wide range of molecular techniques. "
Here we added one sentence for clarify our intention of a step-by-step protocol and rewrote the sentence to be more clearly.
Thus, we have addressed all concerns of the editor. For your convenience, all changes to the revised manuscript are track-changed and highlighted in red ink.

In lines 84-89 we added two citations, where the methods were described first and added also the names of the authors.

"As already described, the deparaffinization process is also a crucial time consuming step that markedly affects the quality and quantity of the extracted DNA. "How is this time consuming and how it affects quality and quantity.
Here we added some examples to explain our point of view and show the effects on the tissue/DNA.

"Especially for reproducible high-throughput analyses such as genotyping, epigenomic studies and RNA sequencing the handling of FFPE specimen with column-based purification systems is often difficult and, therefore, time consuming. ". Please reword to bring out clarity and rationale.
Also here we added some examples to clarify the time consuming and problematic steps with column based systems.

"A blood DNA protocol was used as a starting point and subsequently optimized for the use of FFPE as well as fresh frozen tissue samples from human and animal tissue". Is this inbuilt in the automated system? Please bring out clarity.
Our method is a semi-automated method based on the Blood DNA kit, which provides some of the buffers and a protocol for DNA isolation from whole blood. In our manuscript we describe the semi-automated method for FFPE tissues. So we modified the protocol provided by the company by adding and changing the manual part. This is the part we are talking about in this paragraph. The automated part, done in the instrument is pre-installed and connected to the used cartridge. We changed some wording in this part of the manual to clarify, that we were talking about the manual step of the protocol.

“**The described protocol is based on a commercially available DNA Blood Kit protocol and a DNA FFPE Kit protocol and modified in some important steps”. This is confusing. Isnt this a part of the automated machine?**

Also here we talk about the manual part of the protocol, because the automated part is nearly not changeable, because the automated part depends on the used kit/cartridge. So we use blood kit we have to choose the blood protocol in the machine. The manual part on the other hand is nevertheless changeable. We changed the whole paragraph to clarify, that we are talking about the manual part of the protocol.

Please ensure that the steps are written in the order of it being performed. After tissue preparation shouldn’t it be tissue lysis and then lipid and protein removal. If there is some other reasons to place the steps in the order mentioned presently please bring out the clarity.

Of course you are right and we chose the headers not clearly. For this we changed the headers of the protocol as well as we added some notes to clarify the reason of this step.

For all steps please include how it is performed.

Here we added some steps with the buttons and exact handling of the probe.

How do you prepare the tissue sections from FFPE sample?

We used a microtome for cutting the sections. We added this to the manuscript

Step 1.2: we added the temperature (RT) and also some details about the sample and the tube. Yes, it is one sample/patient/tissue per tube without buffer. The step is only for collecting the sample on the bottom of the tube, so that the buffer in step 2 can reach all the tissue.

Step 2: we included the rationale and explanation of this step. Buffer concentration and composition is not known, because it is a commercial available buffer. (see Table of materials, here we already added). In step 2.2. we added the heating block to be more clear.

Where did you add the probe? Details about the probe?

Here there was a mistake in wording. Of course we talk about sample/tissue. We changed this in the whole manuscript.

Step 3: we clarified, that we talk about sample and protein digestion and added the rationale and explanation, as well as a citation to this step. Also in this step we added the heating block to clarify the handling.

How do you check for complete protein digestion visually?

We changed the statement protein digestion to sample digestion, which is checked visually. We also added an explanation how to check.

Step 4: Composition of the buffer used? If available commercially, please write it as commercially available lysis buffer and include in the table of materials.

The buffer is available commercial and provided with the blood kit. We added this comment in step 4 and also included the buffers of the kit in the table of materials.

Step 4.3 we added the reason for this comment. Vortexing of paraffin before hardening causes damage of the sample by remixing.

Step 5: we added some points about the instrument handling (5.1-5.3). we clarified step 5.5 by adding some information of the elution tubes and by rewording the sentences.

Step 6: We added an additional step (6.1) to clarify how to go through the hardened paraffin from step 4. We changed and reworded step 6.4 as well as the associated note.to clarify the machine handling and the single clicks.
In step 6.5 we added the comment, that there is no calibration for the instrument needed. The machine can be used directly after turning it on.

Step 7: Here we also added the steps of the machine handling and the clicks on the tablet computer.

How can you check the elution?
The elution of the DNA to the loaded elution tubes cannot be checked visually. You have to measure concentration to go sure there is DNA eluted.

Representative results:
Please ensure that the kit labeling A and B are marked in the table of materials as well for people to differentiate
We added kit A and B to the kit names in the table of materials

Please include the name in the table of materials.
The software (Prism) was already in the table of materials. We clarified this by adding the term software for statistics and the version of the software.

The semi-automated protocol leads to higher quantity of the extracted DNA. Please reword to bring out clarity. Also how do you ensure that the quality is same in each case to make such comparisons? e quality is same in each case to make such comparisons?
In this paragraph we are only talking about quantity of the extracted DNA. Quality assessment is done in the following step and explained in the paragraph “Automated extraction protocol leads to a better quality of the extracted DNA”. So, Figure 2 is only about quantity of DNA. Figure 3 therefore shows the quality of the samples. The automated system also shows similar to better quality. So the comparison of the quantity is reliable. We added one sentence in the paragraph relating to figure 3 to fix the comparison problem.

Please include agarose gel pictures of the eluted DNA as well with all three techniques and equal loading to see the quality.
Unfortunately, we had not enough DNA to perform an additional agarose gel with the same loading concentration (we only can provide a gel picture with different loading concentrations). Therefore, we added Figure 3C (DV200) as well as Table 3 to this paragraph. Table 3C shows the fragment distribution of all fragments bigger than 200 bp and the significant differences from Kit A and B to the semi-automated protocol C.
In Table 3 all ratios (260/280) calculated with spectrometer and microplate reader as well as the DV200 values are shown. Missing values are named as not detectable.

Significance or rationale behind reaching the dashed line? Also reasons of having two dashed line?

We added some explanations about absorbance ratios as well as a more detailed figure description. Additionally we included another figure for clarity.

Figure and table legends:

Please remove the commercial term from the figure and use generic term only.

We removed the commercial terms from figure 1 and replaced them with generic terms.

Please include the legends here as well. Please remove commercial terms from table 1, tapestation, Qubit, nanodrop etc . Table 2 is missing from the submission.

We included table legends for tables 1 and 2 as well as table 3 and the material table. This table was added for the qualitative analysis part to show fragmentation and A260/280 values. Additionally, we replaced all commercial terms by generic terms in table 1 and 2.

We hope, that our changes are according to your vision. We tried to address every comment to our best knowledge. If there are any open questions, feel free to contact the corresponding author directly. For your convenience, all changes to the revised manuscript are track-changed and highlighted in red ink.

We look forward to hear from you.

Yours sincerely,
Sabine Bauer