Received: May 2, 2023
Accepted: May 18, 2023

Acad Med J 2023;3(2):117-127
UDC:340.66:[604.2:577.213.7
https://www.doi.org/10.53582/AMJ2332117bs
Original article

DNA EXTRACTION FROM POST-MORTEM SAMPLES WITH DIFFERENT DEGREES OF DEGRADATION AND THEIR SUITABILITY FOR ION S5™ NEXT-GENERATION SEQUENCING SYSTEM

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Abstract

Molecular analyses occupy a significant part of laboratory tests in forensic practice. Pre-prepared commercial kits used for DNA extraction are a great choice for samples provided by living people, but in forensic practice most of the samples that undergo molecular processing are often degraded and may pose a challenge in the laboratory. A special challenge is to provide quality genetic material from postmortem samples that would be suitable for further analysis with massively parallel sequencing.

The aim of this study was to introduce and optimize a method for extracting DNA from postmortem specimens with varying degrees of degradation, such as blood, FFPE, and frozen tissue, suitable for Ion S5 ™ sequencing system.

Extraction protocols were modified to increase the quantities and the total yield of DNA. Thus, we doubled the quantity of the analyzed sample, the quantity of buffers and lytic material, and we also extended the incubation time with elution buffer. Quantification was made using Qubit 3.0 fluorimeter, followed by PCR quantification.

Blood produced the best yield of DNA, followed by formalin-fixed paraffin embedded tissue. The type of the sample, the degree of post-mortem damage, as well as the storage time of the sample significantly affect the amount of DNA material as well as its suitability for further analysis. Blood remains the first choice of sample that is suitable for further analysis with the Ion S5 ™ next-generation sequencing system.

Keywords: postmortem DNA extraction, DNA quantification, quality control, Ion S5 ™ next-generation sequencing system

Introduction

Molecular analyses occupy a significant part of laboratory tests in forensic medical practice. Modern research increasingly requires and includes genetic methods using massively parallel sequencing technology that offers ultra-high throughput, scalability and speed[1,2]. Worldwide, a huge number of gene panels for sequencing are in use, and they work well in clinical practice[3-5]. These samples are provided from living individuals, the biological sample is in optimal quantity and quality, and of course a new sample can be obtained if necessary. On the other hand, in forensic medical practice, only postmortem

samples are available, with ongoing autolytic processes. Autolytic processes sometimes begin in the period of agony, immediately before the onset of death and intensify proportionally with the increase of the postmortem interval. A special challenge is presented by tissues with advanced putrefactive changes, as well as samples of formalin-fixed paraffin tissue[6-8]. According to forensic storage and preservation protocols, formalin-fixed paraffin-embedded tissue is stored permanently and is always available for analysis, which in turn makes it attractive for further research[9-11].

The motive for a more serious inclusion of sophisticated genetic analyses with massive parallel sequencing in forensic-medical practice arose from cases of sudden death, which by indication belong to the forensic-medical expertise and which constitute a huge percentage of forensic-medical autopsies[12,4,13]. Forensic medical experts are tasked with determining the exact cause and mechanism of death[14-16].

The most common causes of sudden death worldwide are cardiovascular diseases[17-19]. Accurate determination of the underlying cause of sudden cardiac death is of particular importance because it allows prevention in asymptomatic family members who are at increased risk[20-22]. A special challenge in forensic medical expertise is the "negative autopsy", i.e., the absence of macroscopic and histopathological morphological changes and a negative toxicological finding. In these cases, post-mortem genetic testing, which is recommended in the latest "guidelines" from the European Associations for Cardiovascular Pathology and Forensic Medicine, is of particular importance and contributes to determining the underlying cause of death[8,12,4]. In such cases, the role of the forensic medicine expert is twofold: 1. He/she should quickly and efficiently determine the cause of death and the manner of death and 2. To initiate a process that would prevent sudden cardiac death in other family members[23-25]. Thus, advancing the post-mortem analysis of sudden cardiac death through molecular investigations with massive parallel sequencing will contribute to increasing the precision of forensic medical expertise and establishing preventive protocols for sudden cardiac death![13].

The specificity of the work in forensic medical practice, among other things, is characterized by a limited amount of biological sample, the impossibility of re-providing a sample for analysis, reduced quality of biological samples that always have a certain degree of degradation, as well as the need for long-term keeping the samples because they represent material evidence in court proceedings[10]. Hence, DNA analysis methods typically used in forensic medicine are based on short fragments that are easily amplified even from biological samples with a high degree of degradation[11]. Unlike typical forensic analysis, massively parallel sequencing requires biological samples of higher quantity and quality. Due to all the listed specificities of the forensic samples, sometimes there is a need to adapt and optimize the commercially available molecular methods and procedures in order to ensure the maximum quantity and quality of the sample. Also, the results in the forensic-medical operation must be reproducible in case there is a need for a second expertise for the needs of the court. Evidence material in the form of a biological sample, which is stored for a long time, must be properly stored in order to keep it in condition to provide adequate quantity and quality for analysis.

Aim of the study

The aim of this study was to evaluate and adapt a DNA extraction protocol with a commercial kit, used for forensic samples with different degrees of degradation, which will provide sufficient quantity and quality of DNA for further analysis.

Study objectives:

- to perform DNA extraction from different post-mortem samples (hemolyzed and/or degraded blood, putrefactive tissues and formalin-fixed paraffin tissues) using a modified protocol with QIAamp DNA Micro Kit;
- to evaluate the amount of DNA extracted from different post-mortem biological samples using the Qubit 3.0 fluorometer, as a quality control before further sequencing steps with the Ion S5 System;
- to evaluate the amount of amplifiable DNA extracted from different post-mortem biological samples using real-time PCR using the Quantifiler™ Trio DNA Quantification Kit (Thermofisher Scientific), as a quality control before further sequencing steps with the Ion S5 System;
- to determine the most suitable type of biological sample during a forensic-medical autopsy;
- to create an additional protocol for long-term storage of biological samples for sequencing with the Ion S5 System.

Materials and methods

Materials

The samples analyzed were taken from forensic-medical autopsies performed at the Institute for Forensic Medicine, Criminalistics and Medical Deontology, Faculty of Medicine in Skopje. All samples were secured according to the standard protocols for forensic autopsy, biological sample processing and storage prescribed by the European Council of Forensic Medicine and R99 recommendations. Three different types of samples were processed: 11 samples of peripheral hemolyzed whole blood stored at +4°C and with different storage periods, 3 samples of putrefied heart muscle tissue frozen at -20°C and 4 samples of cardiac muscle tissue embedded in paraffin blocks previously fixed in formalin.

Methods

DNA extraction

DNA extraction - peripheral hemolyzed whole blood

Blood used for DNA extraction was taken directly from a blood vessel of the deceased during autopsy and collected in sterile blood collection tubes. The blood was stored and kept at +4°C. All samples subject to analysis were brought to room temperature (15–25°C). The buffers used were also brought to room temperature. A genomic DNA extraction protocol from small volumes (1–100 µl) of blood was applied. Due to blood degradation, we doubled the starting blood volume and used 200 µl of blood. The amounts of buffers, proteinase K were also doubled and the incubation time of the elution step was prolonged to 5 minutes.

Procedure: Two hundred µl of whole blood was carefully pipetted in a 1.5 ml tube and DNA extraction procedure using silica membrane spin columns with the QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). Two hundred µl ATL buffer was added to the 200 µl blood. The procedure continued with the digestion of the sample by adding 20 µl proteinase K and 200 µl buffer AL during which we pulsed vortex for 15 s. We added carrier RNA to the AL buffer to improve DNA binding to the columns and achieve higher efficiency. We incubated the mixture at 56°C for 10 minutes to ensure a larger amount. The 1.5 ml tube was briefly centrifuged to remove the droplets from the inside of the lid. To the digested material, 200 µl of ethanol (96–100%) was added and the sample was vortexed for 15 s. followed by incubation at room temperature for 3 min. The entire lysate was then transferred to a QIAamp MinElute column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp MinElute column was placed in a new 2 ml collection tube, and 500 µL of buffer AW1 (Wash Buffer 1) was added and centrifuged at

6000 x g for 1 min. The QIAamp MinElute column was again transferred to a new 2 ml collection tube, 500 µl buffer AW2 (Wash Buffer 2) was added and centrifuged at 6000 x g for 1 min. The QIAamp MinElute column was placed in a new 1.5 ml collection tube and centrifuged at 20,000 x g; 14,000 rpm for 3 min. The QIAamp MinElute was placed in a new 1.5 ml microcentrifuge tube, and 100 µL of buffer AE was added, followed by incubation at room temperature for 5 min and centrifugation at 20,000 x g for 1 min.

DNA extraction – putrefied tissue

Tissue used for DNA extraction was taken during autopsy and collected in sterile tissue collection containers. The tissue was stored and kept in freezer at -20°C. A protocol based on magnetic particles - PrepFiler Express™ Forensic DNA Extraction Kit (Thermofisher Scientific) on the AutoMate Express Forensic DNA Extraction System (Thermofisher Scientific) was used.

Procedure: The tissue sample weighing 50-100 mg was placed in a 1.5 ml centrifuge tube. Five hundred µl of extraction buffer and 5 µl 1 M ditiothreitol was added. The mixture was incubated for 40 min. in thermoshaker set at 70°C and 1100 rpm, and processed by using the AutoMate Express Forensic DNA Extraction System. DNA was eluted in final volume of 50 µl.

DNA extraction – formalin-fixed paraffin-embedded tissue

Tissue used for DNA extraction was taken during autopsy and fixed in 10% formalin, then embedded in paraffin. The tissue was stored and kept at room temperature. A protocol was used to extract genomic DNA from tissue combining the QIAamp DNA Micro Kit and the QIAamp DNA Mini Kit. The sample incubation time and elution step incubation were prolonged.

Procedure: Extraction of DNA from paraffin blocks was performed with the QIAamp DNA Micro kit (QIAGEN, Hilden, Germany) by using a modified protocol designed to purify DNA from tissues. Twelve µm thick slices were cut from the paraffin blocks, which were then placed in a tube containing 1200 µl of xylene and vigorously vortexed for 10 s. The mixture was centrifuged at maximum speed for 5 min, the supernatant was removed, and 1200 µl of ethanol (96-100%) was added to the residue to extract the residual xylene from the sample. This was followed by centrifugation at maximum speed for 2 min, the supernatant was removed and the residue was incubated at room temperature for 10 min until the remaining ethanol had evaporated. The residue was resuspended in 180 µl of ATL buffer. For digestion, 20 µl of proteinase K was added to the suspension, then it was vortexed and incubated at 56°C overnight. Then, 200 µl buffer AL was added to the sample, followed by 200 µl ethanol (96-100%) and vortexed. The lysate was transferred to a QIAamp MinElute column and centrifuged at 6000 x g for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube, 500 µl buffer AW1 was added and centrifuged at 6000 x g for 1 min. The QIAamp MinElute column was transferred to another tube, 500 µl buffer AW2 was added and centrifuged at 6000 x g for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube and centrifuged at full speed for 3 min. to completely dry the membrane. The QIAamp MinElute column was placed in a clean 1.5 ml microcentrifuge tube, 50 µl buffer ATE was added to the center of the membrane, incubated at room temperature for 5 min and centrifuged for 1 minute at 20000 g.

DNA quantification by fluorimetric measurement

After DNA extraction, quantitation was initially done using a Qubit 3.0 fluorimeter by using Qubit™ dsDNA HS Assay Kit. Measurement with the Qubit Fluorimeter is an indirect method and is a dye that emits fluorescent signals only when bound to DNA. We used the

Qubit HS. The reagent was diluted using the buffer (both are part of the kit), 10 ul of the sample was added and the concentration was read using the Qubit 3.0 after 2 minutes of incubation. The Qubit measurement is precise, as only the concentration of bound DNA is measured, not the possible presence of RNA. The starting concentration for the procedures leading to last-generation sequencing is 20 ng/µl, so all samples should be normalized to that range. Poor quality or low concentration samples were reextracted using the same protocol. The extracted samples were then analyzed with real-time PCR in duplicates.

DNA quantification by real-time PCR

To evaluate the amount of amplifiable DNA extracted from different post-mortem biological samples by real-time PCR, we used the Quantifiler™ Trio DNA Quantification Kit (Thermofisher Scientific) on the 7500 Real-Time PCR System, according to manufacturer's instructions. The PCR reaction mix was prepared by mixing 10 ul PCR reaction mix, 8 ul primers and probes mix and 2 ul of DNA extract. The reactions were incubated at 95°C for 2 min., followed by 40 cycles consisting of 95°C for 9 s and 60°C for 30 s. The quantity of the DNA samples was measured by using the small autosomal target.

Results

The blood samples analyzed in this study were stored at +4ºC, completely hemolyzed with different storage periods. From all 11 blood samples that were analyzed, DNA was successfully extracted despite the high degree of sample degradation. The DNA concentration is expressed in ng/µl and the total yield is expressed in ng. The amounts of DNA obtained met the initial criteria for further analysis with the Ion S5 ™ System. Only one sample with a longer storage period provided a very low amount of DNA. Table 1 shows the individual values for each analyzed blood sample.

Table 1. Measured concentration for hemolyzed blood samples with different storage periods

Sample	Storage period (months)	DNA concentration (ng/µl) Qubit 3.0 HS	Total yield (ng)	DNA concentration (ng/µl) Real-time PCR
1	18	6,18	618 ng	7.87
2	12	10.8	1080 ng	27
3	7	10	1000	141
4	6	9.6	960	47
5	7	11	1100	41
6	110	1.27	127	1.35
7	2	10.2	1020	162
8	109	3.12	312	2.75
9	94	0.596	59.6	0.483
10	1	9.46	946	51
11	1	9.14	914	10.362

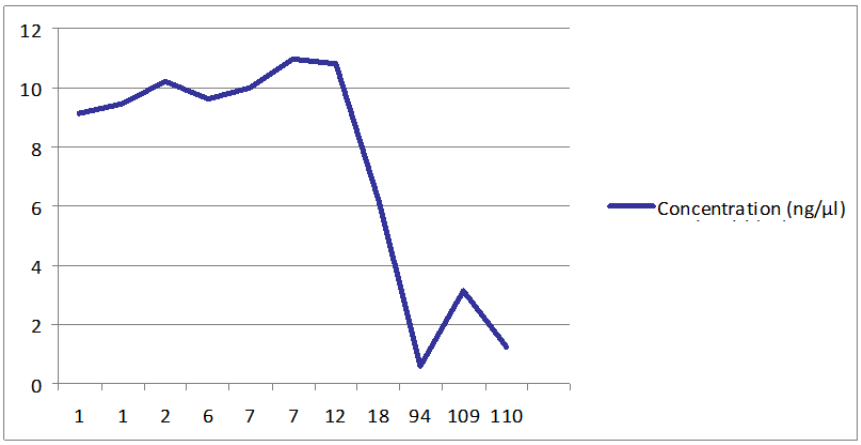

Fig. 1. Graphical representation of the dependence between blood samples storage time and DNA concentration measured with Qubit 3.0 fluorometer

Figure 1 shows the dependence of the storage time and the concentration of DNA that was measured with the Qubit 3.0 fluorometer and real-time PCR. It is clearly seen that there is a trend of decreasing concentration with increasing storage time of the sample and the degree of its degradation. Sample degradation significantly reduced the amount and total yield of DNA. However, despite the strong degradation of the sample, satisfactory quantities were provided for further analysis with the Ion S5 ™ System.

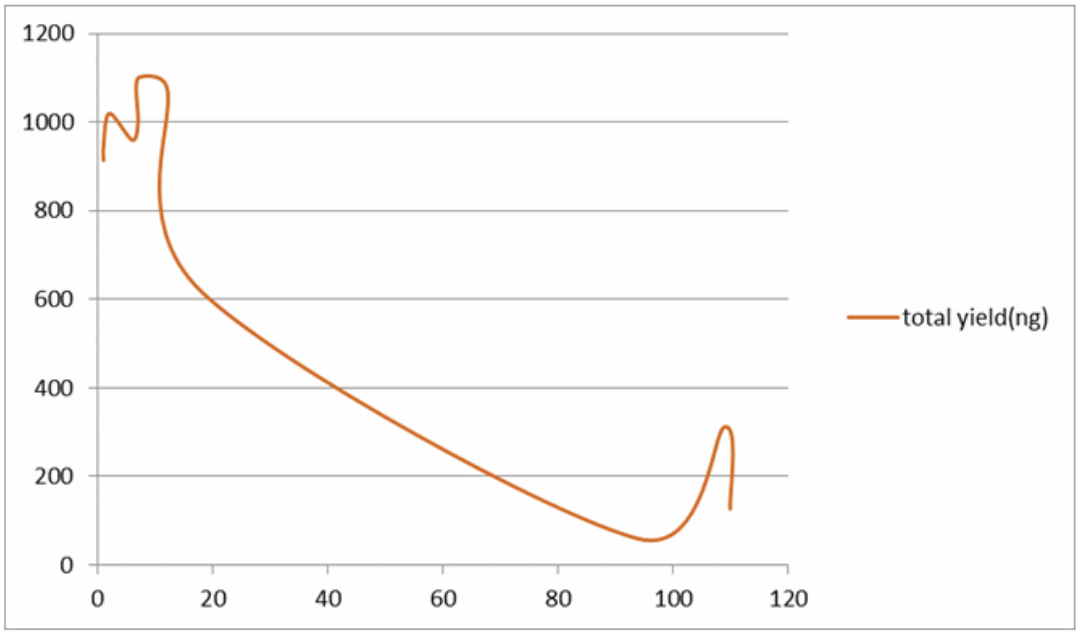

Fig. 2. Graphic representation of the dependence between blood samples storage time and total DNA yield

The results from the quantification of the DNA samples extracted from formalin-fixed paraffin embedded tissue samples are shown in Table 2.

Table 2. Measured concentration of tissue samples embedded in paraffin blocks after prior fixation with formalin

Sample	Storage period (months)	DNA concentration (ng/µl)	Total yield (ng)	DNA concentration (ng/µl) Real-time PCR
1	2	6.58	658 ng	0.809
2	2	4.36	436 ng	0.226
3	3	5.54	554	0.827
4	1	9.56	956	1.478

The samples of putrefied tissues frozen at - 20ºC represent material with an extremely high degree of degradation due to the advanced processes of tissue decomposition. From the three tissue samples that were analyzed, DNA was successfully extracted despite the high degree of sample degradation. The obtained amounts of DNA from putrefied tissues gave very small amounts of DNA and a low overall yield, for which the quality of the material must be assessed before its inclusion in further analyses.

Table 3. Measured concentration of frozen tissue samples with different storage periods

Sample	Storage period (months)	DNA concentration (ng/µl)	Total yield (ng)
1	3	3.86	386 ng
2	24	0.560	56 ng
3	19	7.50	750

Table 4 shows the typical yields of DNA during processing and analysis of a biological sample that was not degraded. The analyzed samples of whole blood, liver tissue and heart muscle tissue, in this case, provided a total yield that was up to ten times higher compared to samples with postmortem degradation.

Table 4. Typical yields of DNA during processing and analysis of a biological sample that was not degraded

Sample	Quantity at start	Total yield of nucleic acid (µg)	DNA yield (µg)
Blood	200 µl	4-12	4-12
Heart	25 mg	25-45	5-10

Discussion

DNA was successfully isolated from a total of 18 biological samples, of which 11 blood samples, 4 samples of formalin-fixed paraffin tissues and 3 frozen putrified tissues. All analyzed biological samples had a high degree of decomposition. In a typical forensic routine these results would be acceptable for further forensic DNA typing. However, given the relatively high cost of genetic testing by massively parallel sequencing, the specific quantitative and qualitative norms that are a prerequisite for analysis with the Ion S5 System, a selection must be made of samples that will be subject to further processing. In our practice, in the literature and in other research, it has been confirmed that DNA isolated using the kit that we used in the study, QIAamp DNA Micro Kit and PrepFiler Forensic DNA Extraction

Kit, can be used in a wide range of tests, including PCR and quantitative real-time PCR, Southern blotting, SNP and STR genotyping, pharmacogenomic research and massively parallel sequencing. However, these kits are commercially prepared and suitable for biological samples that have not gone through a process of decomposition. For these reasons, we have made adjustments to the protocols and method, so that it was applicable to our degraded biological samples, while providing a final product with improved quantitative properties. We chose to determine the amount and total yield of DNA using the Qubit 3.0 fluorimeter and real-time PCR. The Qubit dsDNA HS (high sensitivity) assay kit allows for precise measurement and was the choice for our study. By using this fluorimetric method, the concentration of the DNA was measured, without measuring the possible presence of RNA[26], which enabled us to accurately measure the DNA quantity and precisely select the samples that would enter further processing in the study. Apart from the decomposition of the tissues that occurred before securing the biological sample, we were also faced with additional degradation of the material due to the method of storage and additional processing of the post-mortem samples. The difference between the total expected yield of fresh samples (shown in Table 4) and the yield of the analyzed degraded biological samples was clearly visible. The amount of DNA extracted from the uncompromised samples was about ten times greater compared to the degraded samples.

The "molecular autopsy" in cases with sudden cardiac death can be only applied months after obtaining the sample. This is due to the need of complete negative finding from all other analyses, which makes the process lengthy and requires proper sample preservation protocols. Sometimes, these specific conditions result in poor starting sample quality because of degradation due to storage time of samples which in turn reduces the eligibility for analysis by massively parallel sequencing[4,20]. The results unequivocally show that as the sample storage time increases, the total DNA yield decreases. Thus, in the future in cases of sudden death, the protocols for the collection and storage of biological samples will need to be supplemented and in any case of sudden death where there is no macroscopically visible cause of death, an additional biological sample must be taken and stored immediately beside the standard ones. It is preferable to store blood and/or tissue samples, which will be frozen immediately at -20 and will continue to be stored at that temperature[27].

The analyzed FFPE had a short storage period, but the chemical and thermal damage to which the tissue was exposed in this process caused a significant degradation of the sample and represented a special challenge. From the four tissue samples that were analyzed, DNA was successfully extracted despite the high degree of sample degradation. Our results raised particular interest for further processing of formalin-fixed tissues that gave solid results as far as fluorimetric method was used, almost within the range of the results of the analyzed blood samples. However, the obtained real-time PCR quantification results, which measure the amplifiable DNA in the sample, showed presence of extremely low DNA amounts in the sample, which implies that despite the relatively high DNA concentration in the sample, it is most likely derived from short DNA fragments. Thus, when dealing with low quality samples, such as FFPE, additional quality assessment should be performed to determine the rate of sample degradation and/or the presence of co-extracted inhibiting substances. In other words, fluorimetric and spectrophotometric DNA quantification methods should be carefully taken in consideration, since they can overestimate the quantity of amplifiable DNA, leading to wrong decisions in the workflow. Danielle Mercatante Carrick *et al*. succeeded in whole-exome sequencing from formalin-fixed paraffin-embedded tissues, some of them stored for up to 30 years, under different conditions[1]. As this type of tissue is permanently stored at our institution but is also frequently available in clinical practice, it will be further processed in order to provide a high-quality sample for massively parallel sequencing. Certainly, formalin-

fixed paraffin tissues should be considered only as an alternative choice when other biological samples are unavailable, but not without quality preassessment.

Conclusion

In conclusion, biological samples that are available in forensic medical practice, despite the high degree of degradation, can still provide sufficient quantity and quality of DNA for further processing with massive parallel sequencing with the Ion S5™ next-generation sequencing system. Adaptation of standard commercial DNA extraction kits and protocols enabled successful extraction of material from all analyzed samples regardless of the degree of degradation and the manner in which that degradation occurred. Blood samples, although hemolyzed and degraded, still remain the first choice sample for analysis, but paraffin-embedded tissue after prior formalin fixation has shown solid potential for further use in practice, but only when no other sample is available. In the future, mandatory scalding and storage of tissue and blood at -20ºC should be introduced in the standard protocol for providing and storing tissue in cases of sudden death.

Conflict of interest statement. None declared.

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