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Original Article

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

Erin E Niland[1], Audrey McGuire[1], Mary H Cox[2], George E Sandusky[1]

[1]Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Van Nuys Medical Science Building, 635 Barnhill Drive, Room A128, Indianapolis, IN, 46202, USA; [2]Indiana Clinical Translational Science Institute Department of Medicine, 970 W. Walnut St. Indianapolis, IN 46202 USA

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Abstract: DNA and RNA have been used as markers of tissue quality and integrity throughout the last few decades. In this research study, genomic quality DNA of kidney, liver, heart, lung, spleen, and brain were analyzed in tissues from post-mortem patients and surgical cancer cases spanning the past century. DNA extraction was performed on over 180 samples from: 70+ year old formalin-fixed celloidin-embedded (FFCE) tissues, formalin-fixed paraffin-embedded (FFPE) tissue samples from surgical cases and post-mortem cases from the 1970's, 1980's, 1990's, and 2000's, tissues fixed in 10% neutral buffered formalin/stored in 70% ethanol from the 1990's, 70+ year old tissues fixed in unbuffered formalin of various concentrations, and fresh tissue as a control. To extract DNA from FFCE samples and ethanol-soaked samples, a modified standard operating procedure was used in which all tissues were homogenized, digested with a proteinase K solution for a long period of time (24-48 hours), and DNA was extracted using the Autogen Flexstar automated extraction machine. To extract DNA from FFPE, all tissues were soaked in xylene to remove the paraffin from the tissue prior to digestion, and FFPE tissues were not homogenized. The results were as follows: celloidin-embedded and paraffin-embedded tissues yielded the highest DNA concentration and greatest DNA quality, while the formalin in various concentrations, and long term formalin/ethanol-stored tissue yielded both the lowest DNA concentration and quality of the tissues tested. The average DNA yield for the various fixatives was: 367.77 µg/mL FFCE, 590.7 µg/mL FFPE, 53.74 µg/mL formalin-fixed/70% ethanol-stored and 33.2 µg/mL unbuffered formalin tissues. The average OD readings for FFCE, FFPE, formalin-fixed/70% ethanol-stored tissues, and tissues fixed in unbuffered formalin were 1.86, 1.87, 1.43, and 1.48 respectively. The results show that usable DNA can be extracted from tissue fixed in formalin and embedded in celloidin or paraffin from the early 1900's to present, and may be amplified through PCR and used for clinical and experimental studies.

Keywords: DNA, celloidin, museum specimens

Introduction

DNA and RNA have been used as markers of tissue quality and integrity throughout the last few decades. Numerous genomic tissue studies have been on post-mortem brain tissue. These studies on brain tissue have shown that both DNA and RNA quality is often good by most standards, despite long post-mortem intervals (PMI) and has led to genomic progress in the studies of neurological disorders [1]. Some reports have shown that age, sex, time from death to tissue removal, and the clinical agonal state and health conditions prior to death influence the DNA and RNA quality. Few genomic tissue studies have examined the tissue procurement process of other post-mortem human tissues [2-4].

The quality of the tissue largely depends on many external factors that may have effects on the quality of proteins, lipids, RNA, and DNA. Warm and cold ischemia times can affect both genomic and proteomic biomarkers [5]. It is common held theory that frozen tissue, even when stored for years, is suitable for DNA stud-

Table 1. A table depicting the types of cases (post mortem vs surgical), the years the samples were collected, and the fixation and storage methods used at the various time frames, and the source of the collection of the samples.

	Cases	Years Collected	Fixation method	Source
1	Post Mortem Autopsy	1970's	FFPE	IU Department of Pathology Archives
		1980's	FFPE	IU Department of Pathology Archives
		1990's	FFPE	IU Department of Pathology Archives
		2000's	FFPE	IU Department of Pathology Archives
2	Surgically Removed Cancer Sections	1998-2000	Formalin fixed, ethanol, paraffin embedded	NDRI; Philadelphia, PA
3	Organ Donor Tissue	1998-2000	UW solution then formalin fixed then placed into ethanol	NDRI; Philadelphia, PA
4	Celloidin Blocked Indiana Medical History Museum Cases	1911-1942	Unbuffered formalin fixative, Celloidin embedded	Medical History Museum formerly Central State Hospital for the Insane
5	Unbuffered Formalin Indiana Medical History Museum Cases	1911-1942	Unbuffered formalin fixative of varying concentrations	Medical History Museum formerly Central State Hospital for the Insane

ies [4]. The quality of DNA preservation or even nucleic acid degradation is expected in tissues stored in formalin at room temperature for months or years. This is due to the acidification of formalin into formic acid over long periods of time. Use of 10% neutral buffered formalin stabilizes the tissue for a longer time period than non-buffered formalin. Preservation of tissue in paraffin blocks is variable and is dependent on the type of formalin fixative solution used, the time of tissue storage in the fixative, and the various grades of alcohols, or ethanol, before paraffin-embedding [4, 6-9]. Other fixatives that have been commonly used were non-buffered formalin in the early 1900's that was then blocked into celloidin [10, 11].

In this research study, approximately 180 samples of tissues from kidney, liver, heart, lung, spleen, and brain genomic quality were analyzed in tissues from post-mortem patients and surgical cancer cases. DNA extraction was performed on the following tissues to determine DNA quality and integrity: 1) Tissues donated by the Indiana Medical History Museum (IMHM), formerly the Indiana Central State Hospital for the Insane, that were probably fixed in unbuffered formalin of various concentrations and embedded in celloidin (FFCE) (70+ years); 2) Formalin-fixed paraffin-embedded (FFPE) post-mortem autopsy and surgical cases from the 1970s, 1980s, 1990s, and 2000s (10 to 40 years) from IU Pathology files; 3) Tissues that were fixed in formalin and stored in ethanol (FF/ethanol stored) from 1990s organ donors from National Disease Research Interchange (NDRI); 4) Tissues donated by the IMHM that were probably fixed and kept in unbuffered formalin of various concentrations (70+ years).

Material and methods

Tissue procurement

All tissue specimens were retrieved from various sources. See **Table 1**. These cases were collected with a current institutional review board-approved protocol. The surgical cancer cases were collected via an informed consent and an approved IRB protocol. The post mortem cases were collected using an approved consent for autopsy and IRB approved protocol. The Organ Donor tissues that were unsuitable for transplant were donated from the family using anatomical gift consent. The tissues fixed in unbuffered formalin of various concentrations, including those that were also embedded in celloidin, were donated by the Indiana Medical History Museum and the tissue and both the tissues and records were obtained using an

approved IRB protocol.

Tissue preparation

Tissues from the IU Department of Pathology Archives and Methodist Hospital were collected and processed according to pathology standard operating procedures. Samples had tissue placed in 10% neutral buffered formalin, fixed overnight, and transferred to 70% ethanol prior to processing to a paraffin block. The blocks were microtomed and stained with H&E. The organ donor cases were similar but perfused with UW solution before surgical harvest and then organ/tissue fixation in 10% neutral buffered formalin and stored in 70% ethanol. Celloidin-embedded samples from the Indiana Medical History Museum from the early 1900's were placed in unbuffered formaldehyde for an undisclosed amount of time and embedded in celloidin through an unknown process. The concentration of formaldehyde from that time period was unknown from sample to sample and probably varied from year to year (**Figure 1**).

Figure 1. Gross photograph of the museum jar containing both celloidin embedded tissue and small pieces of tissues in unbuffered formalin. These jars were sealed and not opened since placing the tissue specimens in them (approximately 80+ years).

Histology review

H&E slides for all FFPE tissues and cases were reviewed. Cases that had severe congestion, hemorrhage, and necrosis were eliminated from DNA analysis. In addition the gastrointestinal tract including pancreas were excluded from the study due to post mortem autolysis. The old celloidin-embedded cases from the Indiana Medical History Museum did not have H&E slides available for review.

DNA extraction on FFPE tissues (10 to 40 year old blocks)

The block was microtomed to produce 10-40 mg of tissue (about 15 sheets of 10 micron thick sections). The tissue was placed in 2 ml cryovials and rinsed with xylene. The vials were vortexed for 10 seconds and centrifuged at 13 rpm. The supernatant was removed, and ethanol rinse was repeated. The cryovials were left uncovered for 15 minutes before 1 ml of isopropanol was added. The vials were soaked for 14 hours. The samples were centrifuged and the supernatant was decanted. The tissue was homogenized for 30 seconds. 240 µL of M1 reagent and 250 µL of M2 solution from the Autogen kit AFKT-FXTD were added to all cryovials and PK solution was made with a concentration of M1 solution to 50 mg of PK. Ten µL of the PK solution was added to all tissue samples, and the vials were left to rotate in an incubator overnight at 55 C. 0.5 ml was removed from all samples and added to the Autogen Flexstar machine for DNA extraction. The procedure was completed according to the Autogen manufacturer's recommendations.

DNA integrity and yield were assessed by determining sample absorbance at 260 and 280nm on the Nanodrop 1000.

DNA extraction on celloidin-embedded tissue, tissue fixed in unbuffered formalin, and tissue storage in ethanol (70+ year old museum specimens)

Numerous attempts to extract DNA from these samples failed during the summer of 2010. No bands were obtained on the gels the first summer. The samples were then stored for 9 months in a -80 degree freezer. In the summer of 2011, the protocol was altered and we tried three pilot studies with about 10 to 12 samples

each and all three were successful. First the celloidin samples were cut into very thin 10-40 mg sections of tissue. These sections were placed into 2ml cryovials along with 1ml of isopropanol. The tissues were homogenized for a few minutes until the tissue was liquefied. Most cases we saw visually very small white round spheres which floated. These were pieces of celloidin. The homogenized samples were then soaked for 24 hours. Top remove all this matter. The samples were centrifuged at 12 rpm for 3 minutes. The supernatant was decanted and the digestion process began. 240 µL of M1 reagent and 250 µL of M2 solution from the Autogen kit AFKT-FXTD were added to all cryovials. The PK solution was made with a concentration of 1 ml M1 solution to 50 mg of PK. Ten µL of the PK solution was added to all tissue samples, and the vials were left to rotate in an incubator overnight at 55 C. If samples were not fully digested, they were placed in new fresh Proteinase K solution and, again placed in the incubator to rotate until properly digested. The samples were weighed and 0.5 ml were removed from all samples and added to the Autogen Flexstar machine for DNA extraction. The procedure was completed according to the Autogen manufacturer's recommendations.

Controls

Fresh post mortem tissues with a very short post mortem interval period were used as the control for this experiment. The tissue had never been fixed or frozen before extraction. The digestion process for these tissues was as follows: 240 µL of M1 reagent and 250 µL of M2 solution from the Autogen kit AFKT-FXTD were added to all cryovials. The PK solution was made with a concentration of 1 ml M1 solution to 50 mg of PK. Ten µL of the PK solution was added to all tissue samples, and the vials were left to rotate in an incubator overnight at 55 C. If samples were not fully digested, they were left in the incubator until properly digested. 0.5 ml were removed from all samples and added to the Autogen Flexstar machine for DNA extraction. The procedure was completed according to the Autogen manufacturer's recommendations.

DNA integrity

DNA integrity and yield were assessed by determining sample absorbance at 260nm and 280nm on the Nanodrop 1000. The absorbance readings were put into a ratio (260nm/280nm) that was deemed the optical density (OD) reading. If this ratio was above 1.6 the data was observed to be of good quality. The yield was assessed by micrograms per milliliter. If the yield was above 100 µg/ml the DNA was observable.

Agarose gel

A 1.2% agarose gel was made. 10% of the samples were run through electrophoresis for 3 hours. The gel was saturated with ethidium bromide and a fluorescent picture was taken to capture the image.

Results

Table 2 illustrates the summarized data of results of the extraction of the tissues from the various fixatives. Approximately 180 samples were evaluated in this study. The average DNA yield for the various fixatives was: 367.77 µg/mL FFCE, 590.7 µg/mL FFPE, 53.74 µg/mL formalin-fixed/70% ethanol-stored, 33.2 µg/mL unbuffered formalin-fixed tissues, and 2616.24 µg/mL fresh tissue. The average OD readings for FFCE, FFPE, formalin-fixed/70% ethanol-stored tissues, tissues fixed in unbuffered formalin and fresh tissue were 1.86, 1.87, 1.43, 1.48, and 1.82 respectively. High quality DNA has an OD reading between 1.60 and 2.1 on average. In our results, (**Figure 2**), tissue that was fixed in formalin and then embedded in paraffin or celloidin produced on average higher OD numbers than the other tissues tested, indicating that DNA extracted from FFPE or formalin -fixed celloidin-embedded tissue has on average good quality. Tissue extracted from FFPE yielded on average the highest concentration of DNA of all tissues tested, while unbuffered formalin-fixed, celloidin-embedded tissue had a slightly lower DNA concentration yield (**Figure 3**). Tissues fixed solely in formalin and stored in 70% ethanol, and tissue fixed and kept in unbuffered formalin of various concentrations for 70+ years produced very low OD reading and yield on average of the tissues tested. Tissues from the heart, lung, and kidneys had higher OD values and DNA concentrations compared to the organs outside of the thoracic cavity, which included the brain, liver, and spleen (**Figure 4**).

Discussion

Our results demonstrated that formalin fixed celloidin or paraffin-embedded tissues yielded

Table 2. A composite table of a few samples from the various groups of different types of fixation and embedding with the raw OD values and DNA concentration yields. Legend is: FFPE= formalin fixed paraffin embedded, Ethanol= fixed for approximately 24 hours, then stored long term in 70% ethanol, celloidin= tissue fixed in unbuffered formalin, embedded in celloidin and stored in what was probably unbuffered formalin, unbuffered formalin= small pieces of tissues stored in unbuffered formalin and not embedded in celloidin.

Fixative	Tissue	Yield	OD (260/280)
FFPE	Brain	170.84	1.66
FFPE	Liver	1463.55	1.91
FFPE	Spleen	747.89	1.86
FFPE	Heart	233.93	1.99
FFPE	Lung	376.74	1.93
FFPE	Kidney	667.44	1.85
Ethanol	Heart	4.15	1.2
Ethanol	Liver	34.54	1.28
Ethanol	Kidney	53.4	1.66
Ethanol	Lung	129.82	1.5
Ethanol	Spleen	82.86	1.45
Ethanol	Brain	11.69	1.49
Celloidin	Heart	154.56	1.92
Celloidin	Brain	175.24	1.9
Celloidin	Brain	150.87	1.96
Celloidin	Liver	258.41	1.88
Celloidin	Spleen	307.01	1.73
Celloidin	Spleen	272.76	1.86
Unbuffered Formalin	Tissue Unknown	10.9	1.39
Unbuffered Formalin	Tissue Unknown	18.6	1.57
Unbuffered Formalin	Tissue Unknown	45.72	1.74
Unbuffered Formalin	Tissue Unknown	39.04	1.45
Unbuffered Formalin	Tissue Unknown	6.07	1.47
Unbuffered Formalin	Tissue Unknown	47.26	1.22
N/A (fresh tissue)	Liver	4946.55	1.61
N/A (fresh tissue)	Kidney	3083.19	1.87
N/A (fresh tissue)	Lung	1707.44	1.96
N/A (fresh tissue)	Brain	3072.61	1.89
N/A (fresh tissue)	Heart	277.41	1.98

higher quality and concentration of DNA than tissues that were not processed and embedded after liquid fixation. DNA stored in an aqueous or liquid media for prolonged periods (approximately 15 and greater than 70 years) of time did not yield usable DNA. Many factors can influence the results of obtaining high quality DNA from fixed and paraffin embedded tissue. These are chemical composition of the fixative, duration of the fixative, impact of tissue hypoxia and autolysis, size of the tissue specimen and the length of storage time [12].

Although the FFPE tissue yielded DNA with higher OD numbers than the celloidin-embedded tissue, the difference in the OD readings of the two embedding procedures was slight. The cause of slightly lower OD readings from the celloidin-embedded tissue could be due to the fact that the samples taken from the Indiana Medical History Museum were on average 50 years older than the FFPE samples we tested. Also, there is a possibility that not all the celloidin was removed from the tissues which physically obstructed cell lysis and DNA release from the nucleus whereas the paraffin was washed from the FFPE tissue making cell lysis easier. A previous study did not remove paraffin

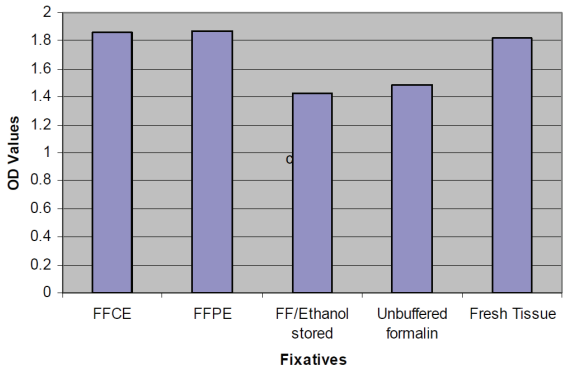

Figure 2. Optical Density values of the various tissues that were fixed, embedded, and stored under different methods for many years. The shortest time frame was 15 years for liquid storage, 70+ years for the unbuffered formalin storage, 15 to 40 years for the paraffin embedded tissue, and greater than 70+ years for the celloidin embedded tissue. Legend for the figure: FFCE=formalin fixed celloidin embedded, FFPE= formalin fixed paraffin embedded, FF/Ethanol stored= formalin fixed, unbuffered formalin (unknown concentration and buffering) and fresh tissue (roughly 14 hours post mortem and refrigerated 30 minutes after death).

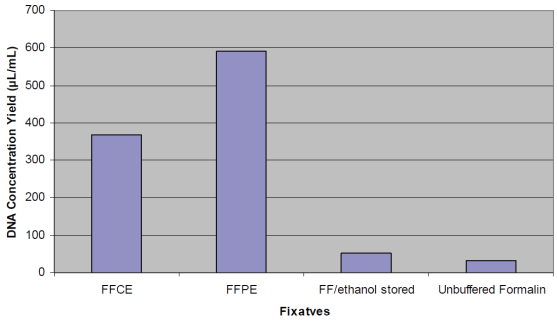

Figure 3. DNA concentration yield of the various tissues that were fixed, embedded, and stored under different methods. Legend for the figure: FFCE= formalin fixed celloidin embedded, FFPE= formalin fixed paraffin embedded, FF/Ethanol stored= formalin fixed, unbuffered formalin (unknown concentration and buffering) and fresh tissue (roughly 14 hours post mortem and refrigerated 30 minutes after death). The data for fresh tissue was excluded from the figure because the yield was so high that the raw data would not fit in the graph (> 2,500 ng/ml).

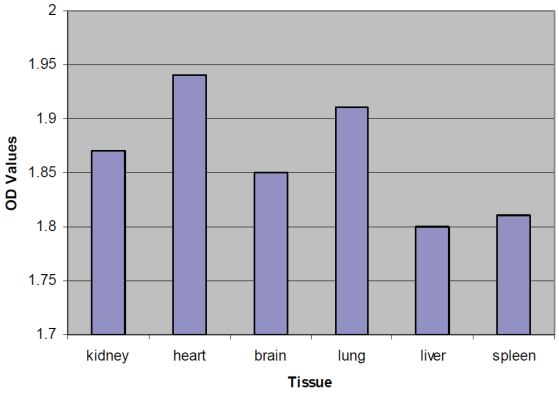

Figure 4. OD values of various tissues from different organs in the body. Some are from the abdominal cavity, the thoracic cavity and cranial cavity. Tissues from the abdominal cavity located around the pancreas had lower OD values due to pancreatic digestive enzymes leaking out during the time period from death to tissue removal at autopsy

but used a different extraction machine [13]. As with all archival tissues, we do not know if the tissue had been fixed for certain in formalin and the details of the fixation are not known. The formalin probably was not buffered as it is today, the formalin concentration was unknown, and the fixation time was highly variable. Also, the fixation temperature in this study was not known and the storage conditions were uncertain.

In addition, tissues from organs in the thoracic cavity, the heart and lungs, had higher quality (OD readings) DNA than those from the abdominal cavity, which were the liver, kidney, and spleen. This difference in post-mortem DNA quality could be due to the cellular post-mortem degradation of the liver, kidney, and spleen caused by the release of pancreatic enzymes and bacterial overgrowth that occurs quickly in the abdominal cavity compared to the thoracic cavity and the cranial vault. A previous study done on the extraction of RNA from 80 different tissues from four cases had similar findings in tissues from and around the gastrointestinal tract [9].

In a previous study involving DNA extraction from tissue stored for 80-plus years in liquid media, the results were similar to this study [14]. The quality and yield of usable DNA in the previous study was very low compared to the other methods of tissue processing and long-term storage. Formalin as a liquid medium breaks down into formic acid and degrades the DNA significantly more rapidly than it does tissue that is embedded in alternative media [4, 7, 8, 14-16]. This can occur in a few weeks. Both

formalin and 70% ethanol long-term storage (years) are not the appropriate media for obtaining usable DNA for genomic projects. The tissue embedding process for both celloidin and paraffin was more ideal for DNA preservation than long-term liquid storage, which is used in many museum specimens [12, 16].

In the FFPE evaluation of paraffin blocks in this study, post-mortem autopsy tissues from 4 different ten-year time intervals were evaluated to determine if there were any differences in the quality of DNA extracted from similar paraffin-embedded tissues, but from different decades. Even though the amount of time in tissue processing through various alcohols into xylene were different and there were different forms of paraffin used, there were no major variations in the quality and yield of DNA between the groups. Another study found similar results [17]. In addition, FFPE surgical cases were evaluated to analyze and compare the quality of DNA extracted from pre-mortem surgical cases with a time frame of tissue obtained from surgery into fixation of about 30 minutes, and post-mortem tissue samples with an average post mortem interval of about 14 to 16 hours. Again, no outstanding variations were seen in the DNA quality and yield between post-mortem and surgical tissue cases. In comparison to previous studies, no problems were encountered with the Autogen Flexstar DNA extraction method and DNA of a usable quality was obtained from the formalin-fixed paraffin-embedded blocks [6-8]. Previous studies often use proteinase K in DNA extractions. Several studies have shown that prolonged digestion with proteinase K gave higher yields of DNA than other methods [12, 16]. We strongly feel a long proteinase K digestion improved our DNA quality and yield with these samples on this study. A recent study had some difficulty extracting high yield DNA using six to seven new commercial kits from FFPE samples with a long time proteinase K digestion at 56 degrees [18].

Previous studies on DNA extraction from inner ear tissue embedded in celloidin revealed similar results to the results in this study. However, most of the other studies had very few cases and were mainly tissues from the temporal bone and inner ear [19, 20]. These studies also used tissues that were recently fixed and celloidin embedded, in contrast to this study that used tissue that had been stored for a long period of time(>70+ years). Most DNA extraction procedures in these studies used organic solvents to break down the celloidin compared to this study that did not use organic solvents [19, 20]. The methods used in this study had a 9 month storage period in a -80 degree freezer, thorough tissue homogenization, a long term PK digestion in a rotating incubator over 24 plus hours, and the Autogen Flexstar to obtain good DNA of a usable quality.

In conclusion, good usable DNA was obtained from celloidin and FFPE embedded tissues compared to fixation in liquid formalin and/or long term storage in 70% ethanol (>70 years). The long term storage in paraffin (10 to 40 years) did not seem to affect the DNA results. Celloidin -blocked tissues samples (>70 years) were extracted using non-organic solvent methods and yielded usable DNA of comparable quality to FFPE tissues.

Address correspondence to: Dr. Sandusky GE, IU School of Medicine, Dept. of Pathology, Medical Science Rm. 128, 635 Barnhill Dr., Indianapolis, IN 46202, USA Tel: 317-278-2304; Fax: 317-278-2018; E-mail: gsandusk@iupui.edu

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