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Roosa Ruotsalainen

Evaluation of Formalin-Fixed Paraffin-Embedded Tissue Samples for Germline DNA Analyses

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This study was conducted for Helsinki University, Department of Pathology in co-operation with Helsinki Biobank. This research was a part of a wider study about comparison of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples and white blood cells for genotyping. The purpose of this research was to compare the quality of DNA extracted from FFPE tissue specimens from different tissue types, preparation year of the FFPE sample, pathology laboratories and storage facilities, to determine if FFPE specimens were suitable for germline DNA analyses.

The study sample consisted of 113 FFPE tissue specimens from patients that have been diagnosed with cancer between years 1975 and 2019. Tumor sample slides were first examined with a microscope to confirm the cancer diagnosis, followed by a selection of suitable non-malignant tissue samples. The corresponding non-malignant FFPE tissue samples were punched to get material for DNA extraction. FFPE tissue punches were pretreated and the DNA was extracted with an automated QIAsymphony SP equipment. The quality and quantity of the extracted DNA was assessed at the Functional Genomics Unit (FuGU) in Helsinki.

Quality control parameters showed differences in DNA quality of the samples. The overall DNA quality was low for FFPE samples, but it was better for more recent specimens (2010 onwards) compared to older specimens. Some minor differences were seen between different tissue types, as well as different pathology laboratories, which might be due to differences in sample material from different hospitals. Sample storage facility had no effect on DNA quality.

It can be concluded that for germline DNA analyses, more recent samples should be used. FFPE samples containing lymphoid tissue were a good source of DNA. For future exploitation of the DNA samples of this study, the most representative samples with the best quality could be utilized in the genotyping project. For lower quality samples, a commercial FFPE DNA repair kit could be tested and applied before further applications.

Keywords	FFPE tissue, DNA, quality

Tekijä Otsikko Sivumäärä Aika	Roosa Ruotsalainen Parafiiniin valettujen kudosnäytteiden käytettävyyden määrittäminen ituradan DNA-analyyseihin 23 sivua + 3 liitettä 11.11.2019
Tutkinto	Laboratorioanalyytikko (AMK)
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Tämä opinnäytetyö tehtiin Helsingin yliopiston patologian osastolla yhteistyössä Helsingin Biopankin kanssa. Tutkimus oli osa laajempaa projektia, jossa tutkitaan parafiiniin valetuista kudosnäytteistä saatavan DNA:n sopivuutta genotyyppaukseen, vertaamalla sitä veren valkosoluista eristetyn DNA:n tuottamiin genotyyppeihin. Tämän opinnäytetyön tarkoituksena oli verrata parafiiniin valetuista kudosnäytteistä eristetyn DNA:n laatua eri kudoksista, eri vuosilta, eri patologian laboratorioista ja eri säilytyspaikoista kerättyjen näytteiden välillä, ja arvioida näytteiden käytettävyyttä ituradan DNA-analyyseihin.

Tutkimukseen käytetyt 113 näytettä olivat tervettä kudosta potilailta, joilla on syöpädiagnoosi vuosilta 1975–2019. Syöpäkudosnäytteen näytepreparaatit mikroskopoitiin syöpädiagnoosin varmistamiseksi, minkä jälkeen valittiin sopivat terveet kudosnäytteet. Parafiiniin valetuista kudosnäytteistä irrotettiin kudoslieriöitä DNA-eristystä varten. Kudoslieriöt esikäsiteltiin ja DNA eristettiin automatisoidulla QIAsymphony SP -laitteistolla. Eristetyn DNA:n laatu testattiin Functional Genomics Unitissa (FuGU), Helsingissä.

DNA:n laatuanalyysien tulokset vaihtelivat näytteiden välillä. Yleisesti parafiiniin valettujen kudosnäytteiden DNA:n laatu oli laatuarvoiltaan matalaa, mutta uudemmissa näytteissä (2010 eteenpäin) laatu oli parempaa kuin vanhemmissa. Pieniä eroja nähtiin eri kudosten välillä, kuten myös eri patologian laboratorioiden välillä. Erot todennäköisesti johtuvat eri näytemateriaaleista sairaaloiden välillä. Eri säilytyspaikat eivät vaikuttaneet näytteiden laatuun.

Johtopäätöksenä tuloksista voidaan sanoa, että genotyyppaus-projektiin käytettävien parafiiniin valettujen kudosnäytteiden tulisi olla mahdollisimman tuoreita parhaan mahdollisen DNA:n laadun varmistamiseksi. Parafiiniin valettu imukudos osoittautui hyväksi lähtömateriaaliksi DNA-eristykseen. Tutkimuksessa eristetyistä DNA-näytteistä voidaan valita edustavimmat näytteet genotyyppaus-projektin hyödynnettäväksi. Laadullisesti huonompia näytteitä voitaisiin yrittää parannella kaupallisilla DNA:n korjaus -kiteillä ennen jatkotutkimuskäyttöä.

Avainsanat	Kudosnäyte, parafiiniblokki, DNA, laatu

Contents

List of Abbreviations

FFPE	Formalin-Fixed Paraffin-Embedded
DNA	Deoxyribonucleic acid
GWAS	Genome-Wide Association Studies
SNP	Single nucleotide polymorphism
PRS	Polygenic risk score
H&E	Hematoxylin and eosin
DIN	DNA integrity number
dsDNA	Double-stranded DNA
ssDNA	Single-stranded DNA
SD	Standard deviation

1 Introduction

The aim of this research was to study the quality of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue specimens. This study was a part of a wider research concerning comparison of FFPE sample derived DNA to a white blood cell derived DNA for genotyping at the University of Helsinki, Department of Pathology. This research was conducted in co-operation with Helsinki Biobank.

The most common source of DNA in research is blood for its easy obtainment and high yield of DNA. Blood provides an excellent source for high-quality DNA and DNA can be extracted relatively effortlessly from whole blood or separated blood leukocytes. However, when a research requires DNA from a wide range of patients with different diseases, recruitment of participants gets tedious and expensive. In Finland, there are ten biobanks, out of which six are hospital biobanks that have at least a few million FFPE tissue samples in total in their possession which could be exploited in DNA analyses [1; 2]. Nevertheless, DNA extraction of FFPE tissue for genotyping has not been properly validated and has been used only in few reports.

The specific aims of the study were to evaluate the DNA quality of FFPE tissue samples in terms of comparing different tissue types, different preparation years, different pathology laboratories and different storage facilities. Further, the aim was to search for sample specific quality characteristics which could indicate better success in further DNA applications. Altogether 113 non-malignant tissue samples from cancer patients were used for germline DNA extraction.

2 DNA Applications in Modern Genetic Research

2.1 Personalized Medicine

Contemporary medical research is aiming increasingly into personalized medical care with constantly improving scientific and technological advances. The genetic data stored in individual's DNA combined with the person's health information can open a variety of new possibilities for improving health and preventing diseases. Genomic information could give a better understanding of causes, initiators and drivers of a disease besides the probable success or failure of medication. [3.]

For an individual personalized medical care means better chances to improve one's own health, more specific diagnoses for diseases and a possibility to choose the most effective treatment or drug. Personalized medicine could also help to prevent the most common chronic diseases and treat them individually. Screenings could be targeted more specifically to those who are most prone to the disease. [4.]

2.2 Genome-Wide Association Studies

A genome-wide association study (GWAS) is a method which involves rapid genome screening of a large number of people for genetic variants associated with a specific disease or trait. In a typical GWAS, around 500,000 variants are screened in a process called genome-wide genotyping. When new associations are detected, the information can be used to create better tools for detecting, treating and preventing the disease. These studies have been especially beneficial for discovering genetic variants that contribute to common diseases such as asthma, diabetes, cancer, mental health illnesses and heart diseases. These so called complex diseases are results of genetic and environmental factors. This type of research is offering a base for personalized medicine. GWAS gathers its data from biological samples, and also tissue samples such as FFPE specimens could be utilized. [5.]

A common type of genetic variation is single nucleotide polymorphism (SNP) which is a single base-pair change in the DNA sequence. In theory, GWAS is conducted by

comparing a DNA sequence of a large number of individuals with a specific disease (cases) to a DNA sequence of a large number of healthy individuals (controls) to identify differences in SNPs. If a certain SNP is more common in cases than controls, the SNP is said to be associated with the disease. In practice, diseases may have some considerably more complicated genetic and environmental determinants which need to be evaluated. [5; 6.]

GWASs have successfully identified SNPs associated with type 2 diabetes, heart diseases, obesity, Parkinson's disease and many other medical conditions. Also genetic variations that influence, for instance, response to anti-depressants have been identified. SNPs which are associated with a disease or trait are collected into a publicly available GWAS database (GWAS Catalog). By 24.9.2019, altogether 157 336 variant-trait associations have been reported in the GWAS Catalog. [5; 7.]

With GWAS statistics of a study population, a polygenic risk score (PRS) can be calculated for each individual that has genome-wide genotype data available. The PRS is calculated by counting the number of risk alleles (trait-associated alleles from a GWAS) weighted by their effect sizes (as reported in the GWAS). PRS can be used to predict a person's risk for a disease or trait. Finnish Institute of Health and Welfare is currently conducting studies to evaluate the functionality and introduction of PRS to Finnish health care. When properly studied, PRS could be used for example in health counselling in combination with behavioral and environmental risk estimation with the same costs as regular laboratory tests in public health care. [8; 9.]

2.2.1 FinnGen

FinnGen is an example of a large, nationwide GWAS project. It started in 2017 and it aims to analyze 500 000 DNA samples from Finns to increase and improve knowledge of disease causes, diagnostics, prevention and development of new drugs and treatments. The FinnGen-project is led by the University of Helsinki and it involves Finnish universities, hospital districts, biobanks, Finnish Institute for Health and Welfare as well as international pharmaceutical companies. [10.]

Due to the unique settlement and population history, the Finnish genome differs from all the other ethnicities in the world. Finland has been founded by a small founder population. The population lived in isolation with very little immigration for thousands of years, during which the population size dropped several times due to, for example, famine. It has been estimated that due to the unique structure of Finnish genomes, 20 000 Finns equal to hundreds of thousands, if not millions, of non-Finnish individuals in terms of statistical power to find trait associations in DNA. One of the advantages of the FinnGen study is the use of Finnish samples. [11; 12.]

3 Formalin-Fixed Paraffin-Embedded Tissue Samples

3.1 Preparation and Primary Use of FFPE Samples

Development of personalized medicine requires a considerable amount of research on the genetic determinants of diseases. Helsinki Biobank hosts a collection of FFPE tissue samples from approximately 700,000 patients of the HUS Helsinki University Hospital pathology archives [13]. This sample collection contains patients with a wide range of diseases, available for research. This existing, well annotated material could be used to study genotype-phenotype correlations, especially in diseases, where prospective blood DNA samples are not available.

The main sources for FFPE tissue are biopsies and samples taken from surgical resection specimens. A biopsy is usually a small sized tissue sample taken by a clinician from a specific lesion or a diseased organ for primary diagnosis, whereas a surgical resection specimen is obtained from the removal of an entire lesion or an organ which is subsequently sampled by the pathologist for either primary diagnosis, or more often diagnostic confirmation and staging. Tissue specimens can also be taken for therapeutic purposes.

Despite the manner of tissue sampling, all excised tissues need to be fixed immediately to pause the ongoing processes in the tissue to prevent post-mortem changes. Fixation is performed to make the tissue firmer and to preserve the vital structures and proteins within the tissue against their own enzymatic lysing process (autolysis). [14; 15.]

For FFPE specimens the fixative is 10% formalin (~4% formaldehyde). Samples are immersed in formalin in room temperature for 16–32 hours, depending on the size of the tissue. After fixing, the sample is dehydrated with ethanol to remove water derived from the fixative. Dehydration ends with xylene to clarify the sample. Last step is embedding the tissue in paraffin to harden it to make it adequate for cutting (figure 1). [16; 17.]

Figure 1. Prepared FFPE tissue specimens

FFPE samples are cut into thin, 2–5 μm, sections with a microtome to prepare slides for staining. Appropriate staining protocol depends on the expected diagnosis, considering that different stainings emphasize different components of the tissue. Hematoxylin and eosin staining (H&E) is the most common, as it stains cell nuclei in blue with hematin while eosin differentiates connective tissue fibers and matrices, and cell cytoplasm with different shades of pink and red. [18.]

After staining a pathologist can proceed with the interpretation of the slides under a microscope. After FFPE specimens' primary use in diagnostics, the samples are stored in the archives with negligible further exploitation.

3.2 DNA from FFPE Tissue and Its Challenges

With all the clinical information of the patient, FFPE tissue samples could be utilized to study the patient's genome for disease determinants. To study the genome, high-quality DNA is needed.

The protocol for preparing FFPE tissue samples affects the quality of DNA. Formalin fixation induces DNA crosslinking, where two nucleotides form a covalent bond between

each other instead of normal hydrogen bond [19]. Fixation also causes fragmented DNA and DNA modifications at a rate of one modification per 500 bases. Under-fixation can lead to degraded nucleic acids, while over-fixation can promote more extensive crosslinking and more severe fragmentation. [20; 21.]

Hence, the protocol for DNA isolation from FFPE tissue must be efficient in purifying highly fragmented DNA and reversing formalin fixation modifications. Many companies offer prefabricated kits and assays for DNA extraction from FFPE tissues which vary from column-based technologies to magnetic beads for binding DNA. In all protocols, the critical phase is the pretreatment of FFPE specimen. Before DNA can be extracted, the tissue needs to be completely devoid of paraffin for its inhibitory features leading to poor sample quality and unsuccessful amplification in downstream applications. [20; 22.]

It has been reported that FFPE samples give lower quality DNA compared to, for example, fresh frozen tissues or blood. Quality of DNA can be described with a DNA integrity number (DIN) which represents the integrity of genomic DNA, or in other words determines the sample's degradation level. DIN values range from one to ten, where one illustrates extremely fragmented DNA and ten indicates highly intact DNA. Fragment length denotes the most abundant number of intact base pairs in extracted DNA, which then correlates with DIN; the longer the fragments, the more intact the DNA and higher the DIN value. Although DNA extracted from FFPE tissues typically have lower quality, it has been successfully used in downstream applications [23.].

4 Materials and Methods

4.1 Selection and Preparation of the Specimens

The samples included in this study belong to patients that have been diagnosed with cancer between years 1975 and 2019. Specimens were collected from Meilahti, Jorvi, Hyvinkää, Kätilöopisto, Lohja, Maria's hospital and Women's clinic (Helsinki) pathology laboratory. Slides and FFPE samples were collected with Helsinki Biobank from either hospital archives or a temporary storage facility for older specimens in Lehtisaari, Helsinki.

Collected slides were first examined with a light microscope with the help of a pathologist to confirm the cancer diagnosis, as shown in figure 2.

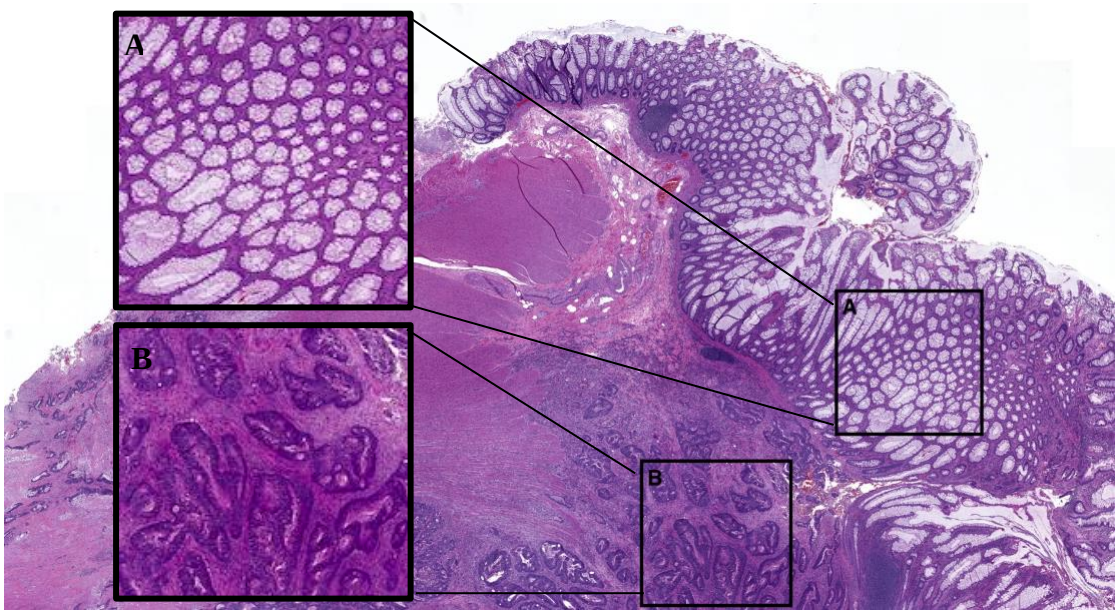

Figure 2. Colonic wall invaded by cancer. A: Normal mucosal glands. B: Cancerous area with tumoral glands. H&E staining, 20x magnification. Imaging of the slides was performed with Pannoramic 250 Flash III equipment (3D HISTECH Ltd.)

Once the diagnosis of cancer was confirmed, a specimen devoid of cancer from the same patient was selected for punching. Selected slides were annotated; the most cell abundant area was marked on the slide as a target for punches. Lymph nodes which contain a large number of cells (lymphocytes) with hardly any stroma have been

considered as the most suitable source for DNA (figure 3). Normal colonic and endometrial mucosa also represent a potentially good source of DNA due to their relatively high concentration of lymphocytes and thus were also selected for punching.

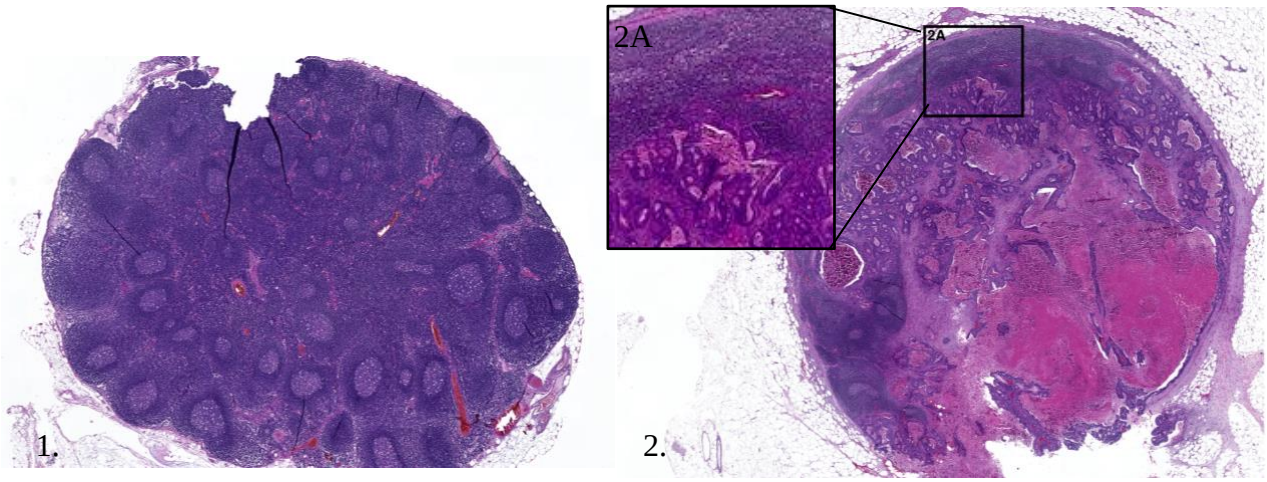

Figure 3. 1. Normal lymph node with no metastasis (H&E staining, 29x magnification), selected for punching. 2. Metastatic lymph node; 2A: The normal structure is replaced by tumoral glands with a remaining peripheral rim of normal lymph node tissue (H&E staining, 17x magnification), excluded from punching due to presence of cancer. Imaging of the slides was performed with Pannoramic 250 Flash III equipment (3D HISTECH Ltd.)

Corresponding FFPE specimens for selected slides were collected for punching. For most of the samples the storage facility is determined by their preparation year. Specimens prepared before year 2008 are stored in a temporary storage facility in Lehtisaari, Helsinki, with uncontrolled ambient temperature and humidity. Samples prepared from year 2008 and after are stored in hospital archives, with controlled temperature and humidity. As an exception, all specimens from Hyvinkää pathology laboratory are stored in a hospital archive, but in this study their results were reported separately for old (1975-2007) and recent (2008-2019) specimens, in order to be consistent with specimens from other pathology laboratories. Summary of study samples arranged by their location, tissue type and preparation year is presented in table 1.

Table 1. Distribution of samples by location, tissue type and preparation year

	1975-2007		2008-2019	
	Lymph Node	**Tissue**	**Lymph Node**	**Tissue**
Meilahti	10	4	21	13
Jorvi	10	4	11	11
Hyvinkää	3	3	11	4
Others	3	5	-	-

Punching of FFPE tissue samples was executed with Tissue-Tek Quick-Ray Tissue Microarray System (Sakura Finetek Japan Co., Ltd). Altogether 102 FFPE samples were punched four times with a 1 mm tip. In addition, total of 11 FFPE samples were punched twice with a 1.5 mm tip, in order to compare the DNA yield between two different punching strategies. Punches were targeted for the most cell abundant area of the tissue based on annotations made during specimen selection.

No exact quantification method was available for estimating the amount of input tissue and thus the height of the tissue in the punch was approximated by eye, to calculate the estimated tissue volume with a formula for volume of a right circular cylinder

$$V = \pi r^2 h, \tag{1}$$

where r is the radius of the punch and h is the height of the tissue in the punch. Excess paraffin was removed with a scalpel before placing the punches in sample tubes. Samples were anonymized for next phases.

4.2 DNA Extraction and Quality Analysis

For this study, the DNA extraction was performed with QIAsymphony SP automatic equipment (QIAGEN GmbH) with the QIAsymphony DSP DNA Mini Kit (QIAGEN GmbH). The extraction was based on magnetic particles, which purify and isolate the DNA. DNA binds to magnetic particles while unwanted components such as proteins are washed away. When washing is complete, magnetic field is released and DNA can be eluted. Pretreatment was carried out with QS GeneRead kit (QIAGEN GmbH), which

contained all needed phases including deparaffinization and lysing the tissue, as well as uracil-n-glycosylase treatment for repairing damages made by formalin fixation.

Pretreatment for FFPE tissue punches was performed by following the QS GeneRead DNA FFPE Treatment Kit Handbook's protocol [21] (QIAGEN GmbH). Protocol was modified by adding 320 µl deparaffinization solution (in step 4), and adding 40 µl of proteinase K instead of 20 µl (in step 6). Further, samples were incubated over night at 56 ºC instead of 1 h (in step 8).

DNA was extracted from pretreated samples with QIAsymphony SP equipment using QIAsymphony DSP DNA Mini Kit (QIAGEN GmbH). Applied equipment protocol was Tissue_LC_200_V7_DSP and the elution volume was 100 µl.

Quality analysis for the extracted DNA was performed at the Functional Genomics Unit (FuGU) in Biomedicum, Helsinki. The measured parameters were DNA concentration for quantity and DIN value and fragment length for quality. Directional concentration (double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) combined), DIN and fragment length were analysed with TapeStation 4200 System (Agilent Technologies Inc.) with Agilent Genomic DNA ScreenTape Assay (Agilent Technologies Inc). More accurate concentration for only dsDNA with eight-point standard curve was measured with Quant-It technology using Quant-It dsDNA Assay (Invitrogen).

For all quality control parameters, standard deviation (SD) and coefficient of determination (R_2), where applicable, were calculated.

5 Results

A table of DNA quality analysis results is presented in appendix 1 for 1 mm punches and in appendix 2 for 1.5 mm punches. In appendixes, the origin (in terms of pathology laboratory) of the sample is indicated in the sample ID:

- JK = Jorvi pathology laboratory
- YP = Meilahti pathology laboratory
- QK = Hyvinkää pathology laboratory
- MP = Maria's Hospital pathology laboratory
- NP = Women's clinic (Helsinki) pathology laboratory
- OK = Lohja pathology laboratory
- KH = Kätilöopisto pathology laboratory

Specific results concerning the effect of the tested parameters (different tissue types, different preparation years, different pathology laboratories and different storage facilities) on DNA quality are presented below.

5.1 Approximated Input Tissue Volumes

Calculated approximated input tissue volumes for 1 mm punches and 1.5 mm punches are presented in appendix 3 and correlation between the amount of input tissue (in mm^3) and DNA concentration is presented in figure 4.

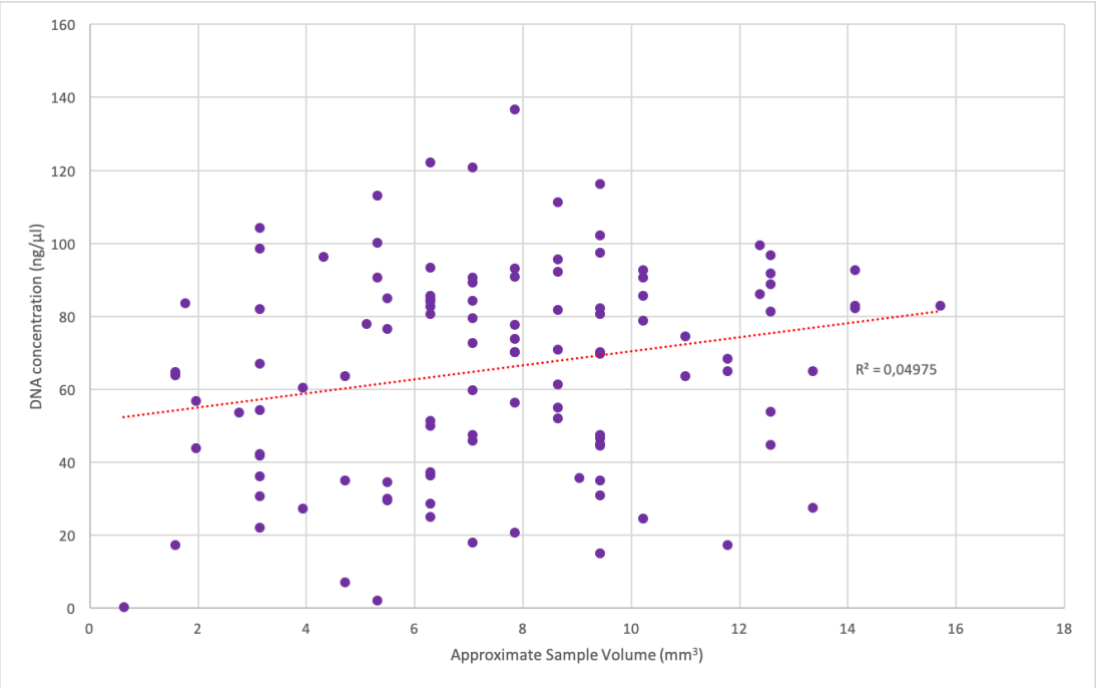

Figure 4. Correlation between tissue volume and DNA concentration

No correlation (R_2=0.05) was seen between the estimated amount of input tissue (in mm_3) and DNA concentration.

5.2 DNA Quantity and Quality in Different Tissue Types

DIN values were low as expected for FFPE specimens. Fragment length was also low as expected, and the average fragment length was quite similar for all different tissue types. Summarized results for different tissue types is presented in table 2 and in figure 5.

Table 2. Tissue specific DNA quality parameters

	Number of Samples	Average DNA conc. (ng/µl) (SD)	Average DIN value (SD)	Average fragment length (bp) (SD)
Appendix	5	72,6 (16,7)	2,72 (0,47)	1179 (434,7)
Colon	24	45,6 (21,0)	2,84 (0,81)	1255 (497,8)
Reproductive Tract	14*	62,7 (38,2)	2,77 (0,48)	1207 (321,6)
Lymphoid Tissue (1mm punch)	59**	71,1 (26,0)	2,65 (0,46)	1108 (330,6)
Lymphoid Tissue (1.5 mm punch)* **	11	81,9 (29,6)	2,39 (0,63)	922 (270,8)

*13 + 1 (endometrium + fallopian tube)

**58+1 (lymph node + tonsil)

***Samples chosen for 1.5 mm punches were 11 lymph nodes from Meilahti from years 2012–2018. Selected samples were another FFPE specimen from 11 patients that already had another specimen for 1 mm punching.

Surprisingly, 1.5 mm punches gave relatively lower DIN values and shorter fragment length compared to 1 mm punches. However, average concentration was higher in 1.5 mm punches compared to 1 mm punches, even though the approximate tissue volumes (in mm^3) were similar in 1 mm and 1.5 mm punches. In all further analyses, 1.5 mm punches were analyzed together with the 1 mm punches.

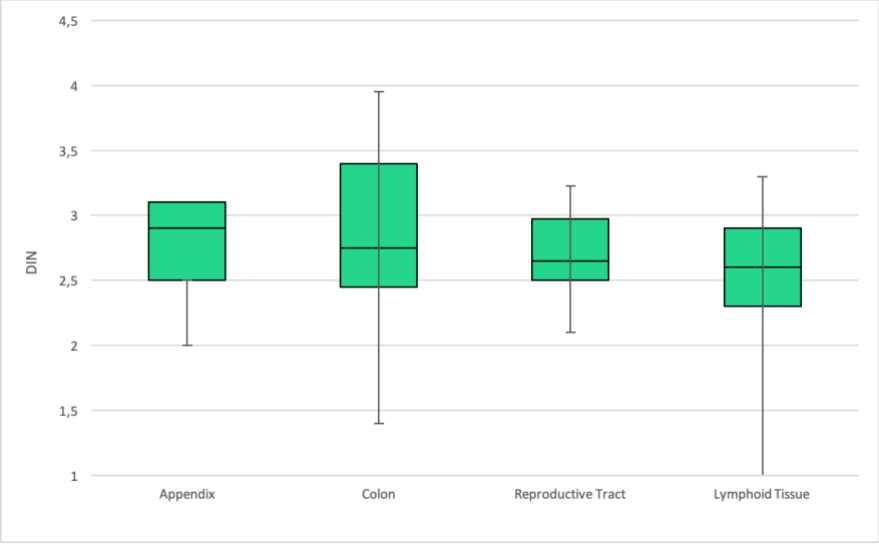

Figure 5. Range and median DIN values in different tissue types

The highest concentration of DNA was received from appendixes and the lowest from colonic mucosa. While giving the lowest concentrations, colon samples had also a wide variance and surprisingly gave the highest DIN values. The widest variance in DIN values was seen in lymphoid tissue. Endometrial tissue provided the most consistent DIN values, but the DIN values were relatively low.

5.3 Effect of Sample Preparation Year on DNA Quality

Sample preparation year showed no correlation (R_2=0.02) with DIN value (figure 6).

Figure 6. Correlation between sample preparation year and DNA quality

Among the 113 samples, most (68%) had DIN over 2.5. In specimens prepared before 2008, 13% had DIN over 3.0. Based on the correlation between sample preparation year and DNA quality (figure 6) samples prepared after 2010 seem to get higher DIN values, with 35% having DIN value over 3.0.

5.4 DNA Quality in Samples from Different Pathology Laboratories

DNA quality in samples from the three main locations (Meilahti, Jorvi and Hyvinkää) is presented in figure 7. Overall correlation between DIN values and preparation year was

modest (R_2=0.33 in samples from Hyvinkää) or negligible (R_2=0.00 in samples from Meilahti and R_2=0.07 in samples from Jorvi).

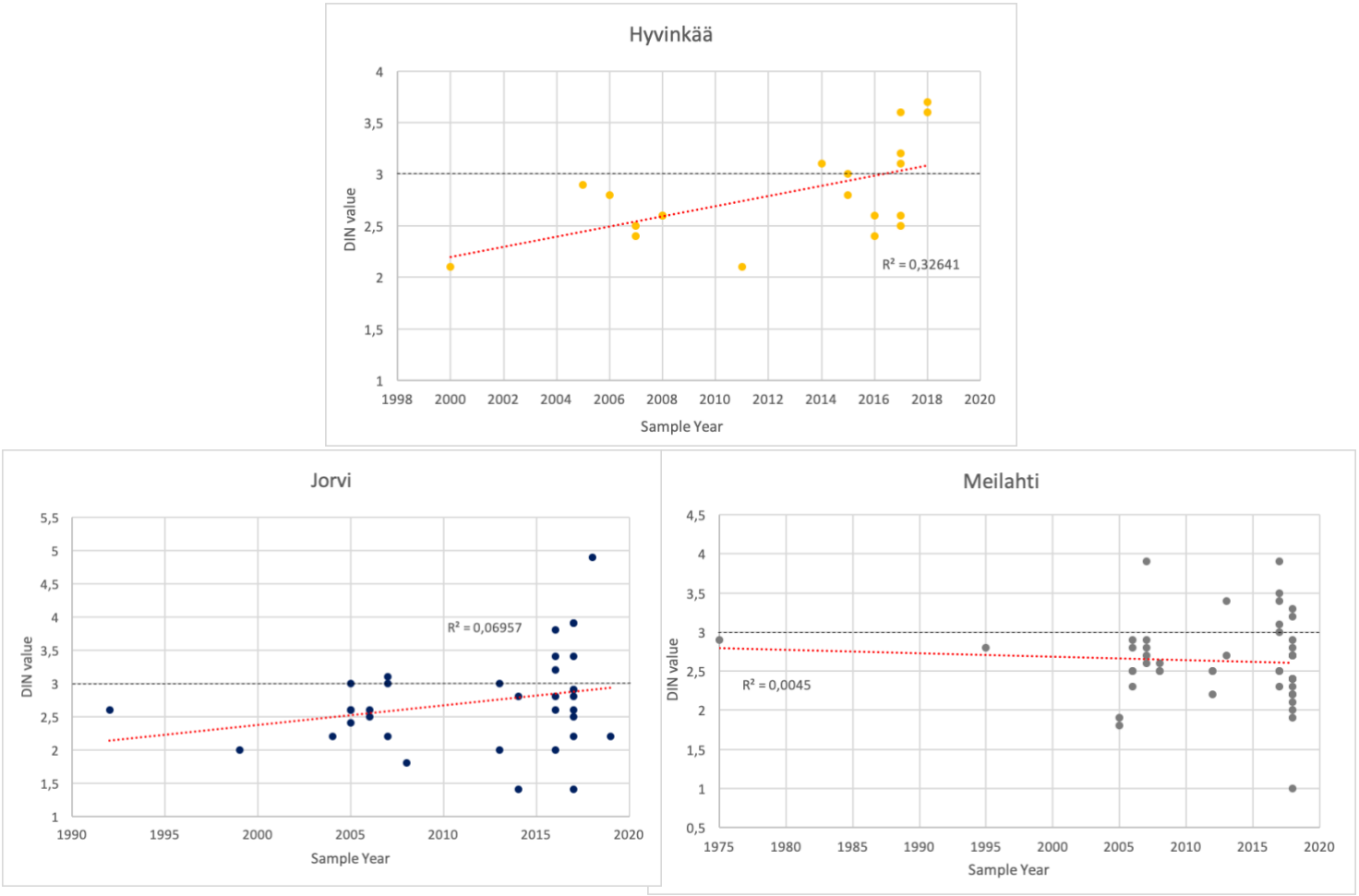

Figure 7. Correlation between sample preparation year and DNA quality in samples from Meilahti, Jorvi and Hyvinkää pathology laboratory

DIN values for most samples from Meilahti pathology laboratory varied between 2.0 and 4.0 (SD 0.53), while most samples from Jorvi had DIN value range of 2.0–4.5 (SD 0.72) and samples from Hyvinkää had a DIN value range of 2.0–4.0 (SD 0.47). DIN values begin to exceed 3.0 in samples prepared after 2010 and the highest DIN values for samples from all pathology laboratories were obtained for samples prepared in 2015 or later.

6 Discussion

The purpose of this study was to figure out whether tissue type, preparation year, preparation laboratory or storage facilty had an effect on the quality of DNA extracted from FFPE samples. The results show that more recently prepared samples seem to have overall better DNA quality than the older specimens.

It seems that the modifications for the GeneRead pretreatment protocol resulted in increased DNA yield. The original 160 µl volume of deparaffinization solution is designed for maximum two 10 µm FFPE tissue sections, which combined are tens of times thinner than one tissue punch. Doubling the amount of deparaffinization solution in this study resulted in specimens devoid of paraffin. Doubling of proteinase K from 20 µl to 40 µl and incubating the samples overnight resulted in completely lysed tissue specimens. DNA extraction was successful for all FFPE punches which clearly had tissue. Only 4% of the samples had too low DNA concentration for completing DNA quality analysis.

Tissue volumes were estimated by eye and recorded in an attempt to set a threshold for the minimum amount of input tissue in mm^3 for successful DNA extraction. Based on the results, setting a precise threshold was not possible, since the estimated amount of tissue (in mm^3) and measured DNA concentration had no correlation. This could be a result of difference in tissue types and other components, such as adipose tissue. One punch can consist of only adipose tissue with very few nuclei, while another punch can consist of purely lymphoid tissue with a high concentration of nuclei. Also, estimating the amount of input tissue sample by eye is not reliable.

The smallest sample, which was estimated to have less than 0.5 mm of tissue height in the punch and corresponding 0.7 mm^3 of tissue volume, gave no DNA. Based on this, one punch should have more than 0.5 mm for tissue height in the punch for successful DNA isolation. In general, FFPE tissue samples are much thicker than 0.5 mm and most patients have multiple blocks available, thus the most representative specimens could be selected for punching.

Overall low DIN values were expected for DNA extracted from FFPE specimens. As formalin fixation effects DNA negatively for example by causing fragmentation, it was

expected that fragment length and DIN value would be relatively low. Average DIN value for all tissues was below 3.0. Previously, Bonnet E, et al. [23] compared the quality of DNA extracted from fresh frozen tissue samples and FFPE tissue samples, receiving similar DIN values (range 2.0–4.0) and fragment lengths (1000–2000 bp) for FFPE samples. The results of this study for FFPE (DIN range 1.4–4.9; average fragment size 1138 bp) are in concordance with the results of Bonnet E, et al. [23]. An another thesis work [24] studying DNA extraction from malignant FFPE tissue had a DIN value range of 1.4–5.1, again in line with these results. In general, decent DNA quality was received from all studied tissue types, which indicates that lymphoid tissue including a high concentration of lymphocytes is a valuable source for DNA.

Sample preparation year showed some correlation with DNA quality. DIN values began to rise above 3.0 after 2010 in all locations. It is evident that the storage facility has no effect on DNA quality. Considering year 2010 as the cut-off, storage facilities have no effect on the samples, since the storing is divided from year 2007. Fixing should preserve FFPE samples for decades, so it is unclear why more recent samples had overall better quality. There might have been a change in pathology laboratories' fixing protocols somewhere around that time, or simply fixation does not stop all the changes in the tissue but just decelerate them.

When looking at the effect of sample age on DNA quality by location, what is stated above can be seen individually. The highest detected DIN values (DIN 3.5–5) in this study were for samples from Jorvi prepared after 2015, while most samples had DIN values below 3.5 in all locations. Higher quality of Jorvi samples might be a result of colonic tissue samples, which were mostly collected from Jorvi and gave the highest DIN values. This endorses the finding that for better quality sampling, FFPE specimens from year 2010 and forward should be utilized.

Bonnet E, et al. [23] also compared three different DNA extraction methods, one of which was GeneRead, the same method used in this research. Bonnet E, et al. [23] reported higher overall DNA quality (higher DIN values and fragment size) and better success rate in downstream applications with QIAmp FFPE DNA Tissue Kit (QIAGEN GmbH). QIAmp FFPE DNA Tissue Kit (QIAGEN GmbH) could be a considerable option for pretreatment

and DNA isolation; unfortunately, QIAmp FFPE DNA Tissue Kit (QIAGEN GmbH) is not applicable for automated QIAsymphony SP equipment at the moment.

When the University of Helsinki genotyping project proceeds, DNA samples extracted in this study could be utilized. The preliminary results of the genotyping project show that the higher the DIN value of the extracted DNA, the better the call rate and the higher the concordance with white blood cell derived DNA genotypes. If DNA samples extracted in this study are used for the genotyping project, the most representative samples should be chosen, for example samples with DIN value over 3.0 and with a preparation date from 2010 and forward. For poorer quality samples there are commercial FFPE DNA repair kits available, which could be studied and applied if the specimen is needed for further analyses.

7 Conclusion

In general, low quality of DNA extracted from FFPE tissue was expected. Damage for DNA caused by formalin fixation of the tissue cannot be repaired entirely, but there are methods for making DNA more intact and reducing the effect of fixation on DNA quality. For further analyses, it can be concluded that samples prepared on and after 2010 have better overall quality than older specimens. It can also be concluded that tissue containing lymphocytes is an advantageous source for DNA.

Overall, DNA quality results for FFPE samples were in line with previous publications and showed some differences according to preparartion year of the sample and origin (pathology department). For all specimens DIN value ranged between 1.4 and 4.9, which has been reported to be adequate for downstream analyses, such as next-generation sequencing. [23; 25.]

If older than 2010 specimens are needed, a commercial FFPE DNA repair kit could be applied for better results. For more precise results, more research should be done for older samples containing different tissue types. Also, it could be useful to figure out whether modifications to pathology laboratories' fixing protocols have been made in around year 2010.

For the genotyping project FFPE samples of lymphotyces containing tissue from year 2010 until this year 2019 should be prioritized, to make sure the best possible quality for DNA. In the future, FFPE specimens can be an important and abundant source for DNA for genome research with a magnitude of application possibilities.

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DNA Quality Analysis Results (1 mm Tissue Punches)

				TapeStation				Qubit
Sample ID	Sample Year	Sample Origin	Approx. Sample Vol. (mm^3)	DIN	Conc. (ng/µl)	Fragment length (bp)	Range (bp)	Conc. (ng/µl)
JK1	2013	colon	6,2832	2	51,6	668	651-786	28,6
JK10	2016	colon	9,4248	3,4	91	1633	1252-1704	45,1
JK11	2017	LN	5,4978	2,6	142	996	901-1081	76,7
JK12	2017	LN	9,4248	2,2	148	818	790-917	80,6
JK13	2017	colon	8,6394	3,4	181	1698	1369-1757	81,8
JK14	2017	colon	7,8540	3,9	153	1839	1376-1905	73,9
JK15	2017	LN	3,1416	3,9	83,2	2044	1674-2388	54,2
JK16	2017	LN	8,6394	2,5	199	995	959-1212	92,1
JK17	2017	appendix	10,2102	2,9	162	1280	1280-1332	90,7
JK18	2017	LN	9,4248	2,8	246	1206	999-1267	97,5
JK19	2017	colon	8,6394	2,9	69,8	1380	1002-1577	52,0
JK2	2013	LN	6,2832	3	131	1497	1262-1566	80,5
JK20	2017	colon	9,4248	1,4	52,4	383	373-419	30,9
JK21	2018	colon	3,1416	4,9	87,6	2333	2258-2333	41,8
JK22	2019	colon	8,6394	2,2	85,6	936	894-971	61,2
JK3	2014	colon	9,4248	1,4	20	390	376-427	14,9
JK4	2014	LN	3,9270	2,8	100	1303	948-1460	60,4
JK5	2016	LN	6,2832	3,2	129	1510	573-1537	84,0
JK6	2016	LN	7,8540	2,8	136	1206	449-1242	90,8
JK7	2016	colon	4,7124	3,8	74,4	1730	1655-1839	35,1
JK8	2016	LN	12,5664	2	145	679	551-775	81,3
JK9	2016	LN	10,2102	2,6	158	1063	998-1197	85,7
JKA	1992	endometrium	5,4978	2,6	52,9			29,9
JKB	1999	appendix	7,0686	2	79,5	622	596-650	47,5
JKC	2004	LN	6,2832	2,2	166	717	686-749	85,6
JKD	2005	LN	7,8540	2,6	162	1111	1062-1162	77,6
JKE	2005	LN	7,8540	2,4	133	944	834-1052	70,1
JKF	2005	endometrium	9,4248	3	79,5	1400	1350-1452	46,5

JKG	2005	endometrium	7,0686	2,6	101	1099	1070-1371	72,7
JKH	2006	LN	3,9270	2,6	62,2	1071	1061-1306	27,3
JKI	2006	LN	6,2832	2,5	97,2	1046	999-1086	51,3
JKJ	2007	LN	3,1416	3	112	1372	1322-1437	66,9
JKK	2007	LN	10,2102	2,2	120	853	815-918	92,6
JKL	2007	LN	1,5708	3,1	96,3	1382	1319-1448	63,9
JKM	2007	LN	0,6283		1,83			0,3
JKN	2008	LN	11,7810	1,8	94,3	553	531-587	64,9
KH1	2000	endometrium	12,5664	2,1	142	793	710-894	96,7
MP1	2003	LN	4,7124	2,6	102	1099	1059-1162	63,7
MP2	2004	LN	10,2102	2,6	157	1115	1015-1330	78,8
MP3	2004	LN	3,1416	2	39,9	572	478-615	22,1
NP1	1997	endometrium	1,5708	3,3	26,8	1616	1548-1659	17,4
NP2	1998	fallopian tube	11,7810	2,7	30,5	1202	1148-1261	17,2
NP3	1999	endometrium	13,3518	2,3	102	844	808-882	64,9
NP4	1999	endometrium	6,2832	2,3	50,9	931	890-976	25,1
OK1	2001	appendix	6,2832	3,1	142	1775	1652-1924	84,6
QK1	2008	LN	6,2832	2,6	194	1196	1154-1230	93,3
QK10	2017	LN	4,3197	3,6	173	1757	1557-1818	96,4
QK11	2017	LN	3,1416	3,1	168	1418	1365-1472	98,6
QK12	2017	LN	8,6394	2,6	203	1105	872-2646	95,6
QK13	2017	LN	8,6394	2,5	234	962	770-2527	111,2
QK14	2017	colon	6,2832	3,2	58,6	1647	1575-1692	37,4
QK15	2018	endometrium	7,8540	3,7	373	1725	617-1820	136,8
QK16	2018	endometrium	6,2832	3,6	317	1633	557-1784	122,1
QK2	2008	colon	7,0686	2,6	42,1			18,1
QK3	2011	LN	3,1416	2,1	44,8	761	755-880	30,6
QK5	2014	LN	3,1416	3,1	144	1447	1381-1514	104,2
QK6	2015	LN	1,9635	3	57,5	1354	1293-1419	43,8
QK7	2015	LN	9,4248	2,8	174	1188	921-1357	116,3
QK8	2016	LN	7,0686	2,6	256	1123	872-1175	120,7
QK9	2016	LN	9,4248	2,4	201	902	871-1007	102,2

QKA	2000	LN	10,9956	2,1	140	780	773-817	63,7
QKB	2005	endometrium	7,0686	2,9	122	1454	1426-1454	45,9
QKC	2006	LN	5,1051	2,8	182	1318	952-1590	77,9
QKD	2007	colon	12,5664	2,5	144	1001	963-1197	88,9
QKE	2007	LN	12,5664	2,4	190	874	748-915	91,7
YP1	2008	LN	2,7489	2,5	90,8	986	940-1035	53,7
YP10	2017	LN	5,4978	3	195	1224	1017-1311	84,9
YP11	2017	colon	6,2832	3,9	109	1791	1775-1872	50,0
YP12	2018	LN	6,2832	3,3	275	1492	1245-1643	82,6
YP13	2018	colon	3,1416	2,8	50	1249	1189-1299	36,1
YP14	2018	LN	7,8540	2,9	165	1195	956-1426	70,3
YP15	2018	colon	9,0321	2,7	58,5	1207	1163-1316	35,7
YP16	2018	endometrium	15,7080	2,7	225	1044	869-1140	82,8
YP17	2018	LN	7,8540	2,4	232	880	839-987	93,2
YP18	2018	colon	7,8540	2,3	140	903	836-1014	56,2
YP19	2018	LN	4,7124		1,43			7,0
YP2	2008	colon	7,8540	2,6	40,5			20,8
YP20	2018	colon	8,6394	2,7	92,5	1230	971-1540	55,0
YP21	2018	LN	3,1416	2	188	608	169-845	82,1
YP22	2018	colon	5,4978	1,9	77,4	637	551-709	34,5
YP23	2018	LN	9,4248	2,2	167	757	686-843	82,3
YP3	2012	appendix	9,4248	2,5	151	922	852-1027	69,8
YP4	2012	LN	12,5664	2,5	157	969	695-1037	53,8
YP5	2013	colon	13,3518	2,7	48,5	1077	1026-1119	27,6
YP6	2013	LN	8,6394	3,4	173	1609	1349-1686	70,9
YP7	2017	appendix	9,4248	3,1	156	1298	1259-1496	70,2
YP8	2017	colon	7,0686	3,4	290	1567	1318-1639	89,2
YP9	2017	endometrium	14,1372	2,5	211	949	846-1117	82,9

YPA	1975	tonsil	9,4248	2,9	161	1176	1056-1223	47,5
YPB	1995	LN	7,0686	2,8	216	1258	1061-1336	84,4
YPC	2005	LN	10,2102	1,8	50,9	518	479-555	24,6
YPD	2005	LN	5,4978	1,9	51,8	516	498-540	29,6
YPE	2006	LN	11,7810	2,5	130	1011	837-1171	68,3
YPF	2006	LN	3,1416	2,3	71,5	872	841-912	42,3
YPG	2006	colon	9,4248	2,8	56,3	1137	1087-1180	35,0
YPH	2006	LN	9,4248	2,9	74	1287	1229-1348	44,6
YPI	2006	endometrium	6,2832	2,5	65,7	1003	957-1042	36,5
YPJ	2007	LN	1,5708	3,9	111	1969	1882-2051	64,7
YPK	2007	colon	12,5664	2,8	97,6	1164	1120-1221	44,7
YPL	2007	LN	10,9956	2,6	155	1123	1081-1354	74,6
YPM	2007	LN	1,9635	2,7	72,6	1060	1030-1113	56,8
YPN	2007	LN	7,0686	2,9	130	1317	948-1732	79,5

DNA Quality Analysis Results (1.5 mm Tissue Punches)

				TapeStation				Qubit
Sample ID	Sample Year	Sample Origin	Approx. Sample Vol. (mm^3)	DIN	Conc.(ng/µl)	Fragment length (bp)	Range (bp)	Conc. (ng/µl)
YP1.2	2008	LN	7,0686	2,5	108	958	915-1003	59,7
YP4.2	2012	LN	14,1372	2,2	196	738	261-1753	82,2
YP7.2	2017	LN	7,0686	2,5	158	977	825-1024	90,6
YP8.2	2017	LN	12,3700	2,3	215	813	295-867	99,4
YP10.2	2017	LN	5,3014	3,5	276	1566	1470-1636	113,1
YP12.2	2018	LN	1,7671	3,2	183			83,7
YP14.2	2018	LN	14,1372	2,4	141	936	301-1061	92,5
YP17.2	2018	LN	12,3700	2,1	160	687	262-727	86,1
YP19.2	2018	LN	5,3014	1	5,3			2,1
YP21.2	2018	LN	5,3014	2,4	223	953	876-1151	90,6
YP23.2	2018	LN	5,3014	2,2	178	666	604-706	100,2

Approximate Sample Volumes

Project ID	Block Type	Punches	Observations	Approx. Height of the Tissue (mm)	Approx. Sample Vol. (mm^3)	Project ID2	Block Type2	Punches2	Observations2	Approx. Height of the Tissue (mm)2	Approx. Sample Vol. (mm^3)2
JKA	endometrium	4 x 1 mm		7	5,49778714	QK9	LN	4 x 1 mm		12	5,49778714
JKB	appendix	4 x 1 mm		9	7,06858347	QK10	LN	4 x 1 mm		5,5	7,06858347
JKC	LN	4 x 1 mm		8	6,28318531	QK11	LN	4 x 1 mm	thin	4	6,28318531
JKD	LN	4 x 1 mm		10	7,85398163	QK12	LN	4 x 1 mm		11	7,85398163
JKE	LN	4 x 1 mm		10	7,85398163	QK13	LN	4 x 1 mm		11	7,85398163
JKF	endometrium	4 x 1 mm		12	9,42477796	QK14	colon	4 x 1 mm		8	9,42477796
JKG	endometrium	4 x 1 mm		9	7,06858347	QK15	endometrium	4 x 1 mm		10	7,06858347
JKH	LN	4 x 1 mm		5	3,92699082	QK16	endometrium	4 x 1 mm		8	3,92699082
JKI	LN	4 x 1 mm		8	6,28318531	YPA	tonsil	4 x 1 mm		12	6,28318531
JKJ	LN	4 x 1 mm	thin	4	3,14159265	YPB	LN	4 x 1 mm		9	3,14159265
JKK	LN	4 x 1 mm		13	10,2101761	YPC	LN	4 x 1 mm		13	10,2101761
JKL	LN	4 x 1 mm	very thin!	2	1,57079633	YPD	LN	4 x 1 mm		7	1,57079633
JKM	LN	4 x 1 mm	almost no tissue!	0,8	0,62831853	YPE	LN	4 x 1 mm		15	0,62831853
JKN	LN	4 x 1 mm		15	11,7809725	YPF	LN	4 x 1 mm	thin	4	11,7809725
JK1	colon	4 x 1 mm		8	6,28318531	YPG	colon	4 x 1 mm		12	6,28318531
JK2	LN	4 x 1 mm		8	6,28318531	YPH	LN	4 x 1 mm		12	6,28318531
JK3	colon	4 x 1 mm		12	9,42477796	YPI	endometrium	4 x 1 mm		8	9,42477796
JK4	LN	4 x 1 mm	thin	5	3,92699082	YPJ	LN	4 x 1 mm	very thin!	2	3,92699082
JK5	LN	4 x 1 mm		8	6,28318531	YPK	colon	4 x 1 mm		16	6,28318531
JK6	LN	4 x 1 mm		10	7,85398163	YPL	LN	4 x 1 mm	two blocks one tube	14	7,85398163
JK7	colon	4 x 1 mm		6	4,71238898	YPM	LN	4 x 1 mm	very thin!	2,5	4,71238898
JK8	LN	4 x 1 mm		16	12,5663706	YPN	LN	4 x 1 mm		9	12,5663706
JK9	LN	4 x 1 mm		13	10,2101761	YP1	LN	4 x 1 mm	thin	3,5	10,2101761
JK10	colon	4 x 1 mm		12	9,42477796	YP2	colon	4 x 1 mm		10	9,42477796
JK11	LN	4 x 1 mm		7	5,49778714	YP3	appendix	4 x 1 mm		12	5,49778714
JK12	LN	4 x 1 mm		12	9,42477796	YP4	LN	4 x 1 mm		16	9,42477796
JK13	colon	4 x 1 mm		11	8,6393798	YP5	colon	4 x 1 mm		17	8,6393798
JK14	colon	4 x 1 mm		10	7,85398163	YP6	LN	4 x 1 mm		11	7,85398163
JK15	LN	4 x 1 mm	thin	4	3,14159265	YP7	appendix	4 x 1 mm		12	3,14159265
JK16	LN	4 x 1 mm		11	8,6393798	YP8	colon	4 x 1 mm		9	8,6393798
JK17	appendix	4 x 1 mm		13	10,2101761	YP9	endometrium	4 x 1 mm		18	10,2101761
JK18	LN	4 x 1 mm		12	9,42477796	YP10	LN	4 x 1 mm		7	9,42477796
JK19	colon	4 x 1 mm		11	8,6393798	YP11	colon	4 x 1 mm		8	8,6393798
JK20	colon	4 x 1 mm		12	9,42477796	YP12	LN	4 x 1 mm		8	9,42477796
JK21	colon	4 x 1 mm	thin	4	3,14159265	YP13	colon	4 x 1 mm	thin	4	3,14159265
JK22	colon	4 x 1 mm		11	8,6393798	YP14	LN	4 x 1 mm		10	8,6393798
KH1	endometrium	4 x 1 mm		16	12,5663706	YP15	colon	4 x 1 mm		11,5	12,5663706
MP1	LN	4 x 1 mm		6	4,71238898	YP16	endometrium	4 x 1 mm		20	4,71238898
MP2	LN	4 x 1 mm		13	10,2101761	YP17	LN	4 x 1 mm		10	10,2101761
MP3	LN	4 x 1 mm		4	3,14159265	YP18	colon	4 x 1 mm		10	3,14159265
NP1	endometrium	4 x 1 mm	biopsy, small sample, very thin!	2	1,57079633	YP19	LN	4 x 1 mm		6	1,57079633
NP2	fallopian tube	4 x 1 mm		15	11,7809725	YP20	colon	4 x 1 mm		11	11,7809725
NP3	endometrium	4 x 1 mm		17	13,3517688	YP21	LN	4 x 1 mm	thin	4	13,3517688
NP4	endometrium	4 x 1 mm		8	6,28318531	YP22	colon	4 x 1 mm		7	6,28318531
OK1	appendix	4 x 1 mm		8	6,28318531	YP23	LN	4 x 1 mm		12	6,28318531
QKA	LN	4 x 1 mm		14	10,9955743	YP1.2	LN	2 x 1,5 mm		4	7,06858347
QKB	endometrium	4 x 1 mm		9	7,06858347	YP4.2	LN	2 x 1,5 mm		8	14,1371669
QKC	LN	4 x 1 mm		6,5	5,10508806	YP7.2	LN	2 x 1,5 mm		4	7,06858347
QKD	colon	4 x 1 mm		16	12,5663706	YP8.2	LN	2 x 1,5 mm		7	12,3700211
QKE	LN	4 x 1 mm		16	12,5663706	YP10.2	LN	2 x 1,5 mm		3	5,3014376
QK1	LN	4 x 1 mm		8	6,28318531	YP12.2	LN	2 x 1,5 mm	thin	1	1,76714587
QK2	colon	4 x 1 mm		9	7,06858347	YP14.2	LN	2 x 1,5 mm		8	14,1371669
QK3	LN	4 x 1 mm	thin	4	3,14159265	YP17.2	LN	2 x 1,5 mm		7	12,3700211
QK5	LN	4 x 1 mm	thin	4	3,14159265	YP19.2	LN	2 x 1,5 mm		3	5,3014376
QK6	LN	4 x 1 mm	thin!	2,5	1,96349541	YP21.2	LN	2 x 1,5 mm		3	5,3014376
QK7	LN	4 x 1 mm		12	9,42477796	YP23.2	LN	2 x 1,5 mm		3	5,3014376
QK8	LN	4 x 1 mm		9	7,06858347						