Optimisation of sample preparation for DNA extraction from formalin fixed paraffin embedded tissues of unresolved sudden unexpected death cases

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In partial fulfilment of the requirements for the degree

MPhil (Biomedical Forensic Science)

Division of Forensic Medicine and Toxicology

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

Date of Submission: 10 December 2019

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Abstract

A retrospective case review revealed an increase in sudden unexpected death (SUD) admittance at Salt River Mortuary (SRM) between 2014 and 2018, and that 40 % of SUD occurred in young individuals between the ages of 1 and 40 years old (SUDY). Despite extensive investigations, the cause of death remained undetermined in 26 % of SUDY cases. These dormant cases may benefit from retrospective post-mortem molecular autopsies for investigation into genetic causes of death. Often, formalin fixed paraffin embedded tissues (FFPETs) are the only archival sources of DNA available for retrospective analyses. This study aimed to optimise DNA recovery from FFPETs for potential use in molecular autopsies of unresolved SUDY cases. To this end, DNA was extracted from FFPET sections using the QIAamp® DNA FFPE tissue kit; the thickness and number of sections were varied. DNA was assessed using spectrophotometry, real-time PCR and digital capillary electrophoresis. Results showed that finer sectioning (1-µm thick as compared to 3-µm and 5-µm thick), improved DNA concentrations, purities and DNA fragment lengths. Increasing the number of 1-µm thick sections from 30 to 100, significantly improved DNA yield. DNA was not significantly more degraded for FFPETs stored for up to three years, which holds promise in the effectiveness of the technique for aged samples. The DNA extraction method developed in this study yielded a median of 320 ng (287 ng - 698 ng) of DNA with 55 % of DNA fragments being at least 400 bp in size. These results are especially informative for downstream molecular analyses, indicating that genotyping or sequencing assays need to be designed to target amplicons less than 400 bp in size. The degraded nature of the FFPET samples also suggests that massively parallel sequencing might be suited for downstream molecular analysis for determining cause of death in unresolved SUDY cases.

Acknowledgements

"*You cannot connect the dots looking forward; you can only connect them looking backward*"
– Steve Jobs.

Looking backward on this year, I have come to appreciate the growth I've experienced and the strides I've taken, none of which would be possible without the invaluable wisdom and guidance of my fellows and peers.

I would like to thank my mentor and supervisor, Dr Laura Heathfield and co-supervisor Calvin Mole, for granting me the opportunity to make a contribution to the work done in the Molecular Forensics Research group. I must express my profound gratitude for the support, guidance and words of encouragement offered throughout the development of my skills and in writing this thesis.

A special thank you goes out to all those who collaborated and contributed to the success of this study. It could not have been completed without the patience of Michelle Perrins and Yolanda Davies in the tedious task of sample collection and the generosity of Helen Ilsley for access to her facilities.

Lastly, I would like to thank my colleagues who took this journey alongside me and provided the moral support that was well-needed.

I would like to acknowledge the University of Cape Town for the resources necessary to conduct my experiments and for the generosity endowed on me by the National Research Foundation in sponsoring my degree.

I dedicate this work to my family who supported me through every hurdle I faced and every setback I conquered. Your patience and commitment is exactly what I needed to achieve this feat.

Foreword

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA:	Analysis of variance
bp:	Base pairs
C_t:	Cycle threshold
CTAB:	Cetyl trimethylammonium bromide
DIN:	DNA integrity number DNA
DNA	Deoxyribonucleic acid
FFPET(s):	Formalin fixed paraffin embedded tissue(s)
H&E:	Haematoxylin and eosin
HIV:	Human immunodeficiency virus
HREC:	Human Research Ethics Committee
IPC:	Internal positive control
MW	Molecular weight
MPS	Massively parallel sequencing
NGS	Next generation sequencing
OAD:	Office Autopsy Database
PCI:	Phenol-chloroform-isoamyl alcohol
PCR:	Polymerase chain reaction
pH:	Potential hydrogen
Pro K:	Proteinase K
qPCR:	Quantitative polymerase chain reaction

SRM	Salt River mortuary
SUD:	Sudden and unexpected death
SUDY:	Sudden and unexpected death in the young
TMLL:	Tygerberg medico-legal laboratory
UCT:	University of Cape Town
USA:	United States of America
WES	Whole-exome sequencing
%:	Percentage
µl:	Microlitre
µm:	Micrimeter
℃:	Degree Celsius
n:	Sample number
ng:	Nanograms
p:	Probability
v:	Version
χ^2:	Chi-squared

Chapter 1: Literature review

1.1. INTRODUCTION

1.1.1. Post-mortem investigations

Sudden and unexpected death (SUD) is a devastating event and occurs when a seemingly healthy individual demises rapidly and without warning, and where cause of death is apparently unexplained. The unanticipated death occurs by non-violent means or is an instantaneous death within 24 hours of exhibiting any signs or symptoms of disease (World Health Organisation, 2016).

Previous studies have reported a temporal increase in SUD case admittance at two of the major mortuaries in South Africa, namely the Tygerberg medico-legal laboratory (TMLL) and Salt River mortuary (SRM) (Tiemensma, 2010; Mole, 2019). There was more than double the number of SUD cases admitted to TMLL in 2005 (7.5 %) than in 2001 (3.2 %) and approximately three times the number of SUD cases admitted to SRM in 2016 (19.2 %) than in 2007 (6.5 %) (Tiemensma, 2010; Mole, 2019). It can be deduced from this data that the burden of SUD in South Africa is becoming a more prominent issue with each passing year.

It is mandated in South Africa that SUD cases undergo medico-legal investigations to determine the cause of death (*Regulations regarding the rendering of forensic pathology service*, 2018). In the majority of these cases, the cause of death is ascertained through a combination of medico-legal autopsies, ancillary investigations and consideration of the case histories (Maluleke, 2018). It is at the discretion of the forensic pathologist to submit a specimen for ancillary testing, a choice which often depends on the case context (*Regulations regarding the rendering of forensic pathology service*, 2018). A study by de Jong (2017) found that approximately 63 % of cases admitted to SRM had requests for at least one ancillary test – of which, approximately 44 %, 16 % and 7% were blood alcohol-related, ballistic-related and histology, respectively (de Jong, 2017). Other ancillary tests, such as toxicology and pharmacology were performed at a far lesser rate (de Jong, 2017).

Previous studies have identified a number of causes and risks related to SUD of all ages, including HIV, hospital-acquired infections, such as pneumonia and tuberculosis as well as

heart disease or cardiac channelopathies (Tiemensma, 2010; de Luna & Elosua, 2012; Morris et al., 2016; Maluleke, 2018; Van Deventer, Rossouw & Du Toit-Prinsloo, 2016; Kruger et al., 2018; Heathfield, Martin & Ramesar, 2019). In certain cases, despite a complete autopsy and ancillary investigations, the cause of death might still be undetermined; these cases are referred to as 'negative autopsies'.

Cause of death determination is essential for bringing closure to the relatives of the deceased as well as for clinical developments in treatments for pathologies related to these deaths (Etheridge & Saarel, 2014). However, the proportion of negative autopsies in SUD cases were reportedly up to 14 % in South Africa and between 5 % and 30 % in other regions across the globe (Lim et al., 2010; Tiemensma, 2010; Morris et al., 2016; Oscar & Ramon, 2016). This highlights the importance of developing alternative ancillary investigations which could further aid in determining cause of SUD in instances where the current approaches are not sufficient.

1.1.2. Molecular autopsies

Genetic investigations have shown value as an investigative tool in autopsy-negative SUD cases, as they have aided in the identification of underlying genetic variants which could have caused or contributed to death (Madea et al., 2010; Campuzano et al., 2014; Hata, Kinoshita & Nishida, 2017; Heathfield, Martin & Ramesar, 2018). This technique of analysing the genetic material of an individual for purposes of cause of death determination has come to be known as the 'molecular autopsy' (Madea et al., 2010).

While molecular autopsies have not yet been introduced routinely in forensic investigations, research has demonstrated their success in aiding previously unresolved cases as well as in revealing medical information that could be used to screen at-risk relatives for the variants identified in SUD cases (Madea et al., 2010; Oscar & Ramon, 2016; Sanchez et al., 2016; Hata, Kinoshita & Nishida, 2017; Heathfield, Martin & Ramesar, 2018). Indeed, a systematic literature review showed that the cause of death was subsequently established in a median of 32 % of previously unresolved SUD in young individuals, between ages 1 – 35 years old, through the use of molecular autopsies (Heathfield, Martin & Ramesar, 2018).

It is largely hypothesised that concealed cardiac channelopathies contribute significantly to the high rate of autopsy-negative SUD, especially in young individuals (Campuzano et al., 2014). As a result, the majority of molecular autopsy research has been directed at identifying disease-causing genetic mutations within cardiac channels that do not present with morphological

abnormalities, most commonly Long QT Syndrome, Brugada syndrome and Catecholaminergic polymorphic ventricular tachycardia (Campuzano et al., 2014; Brion et al., 2015; Heathfield, Martin & Ramesar, 2018; Heathfield, Martin & Ramesar, 2019). Mutations in the voltage-gated ion channels encoded by the *KCNQ1, KCNH2* and *SCN5A* genes, which are responsible for encoding cardiac channels for potassium (K+) and Calcium (CA2+) ions, respectively, are among those implicated in sudden cardiac death (Tester et al., 2012; Hata, Kinoshita & Nishida, 2017).

Additional genetic variants, such as those related suicide as well as alcohol and drug metabolism have been identified as potential targets for molecular autopsy investigations (Cui et al. 2007; Heathfield et al, 2014). However, further research would be required to establish a panel of genes for these tests. In suicide cases, it may be of interest to investigate the expression of variant genes implicated in the increased susceptibility to suicidal behaviours (Cui et al. 2007). For instance, a mutation in the Regulator of G-protein Signalling 2 (*RGS2*) gene responsible for intracellular signalling has been shown to be associated with increased anxiety and aggressive behaviour, often leading to an increased risk of suicide (Cui et al. 2007).

Fresh samples, such as blood or fresh-frozen tissues collected as part of the prospective case investigation, are currently the gold standard for genetic testing on post-mortem samples (Middleton et al., 2013). However, a retrospective molecular autopsy may be beneficial in reducing the current burden of unresolved SUDY cases, where prospective sampling is no longer an option. In such instances, the most likely biological material available in the archival storage is in the form of formalin fixed paraffin embedded tissues (FFPETs), which would have been obtained for routine histopathology analyses.

FFPETs are preserved by treating tissue biopsies with formalin to fix the proteins and cellular structures within the tissues (Hewitt et al., 2008; Canene-Adams, 2013). Subsequently, the tissue is set in a block of immunohistochemistry-grade paraffin wax to facilitate the precise sectioning of the tissue (Canene-Adams, 2013). Formaldehyde, which is used in the fixative, forms chemical reactions with the DNA in the tissues resulting in denaturation or cleavage of the DNA strands (Srinivasan, Sedmak & Jewell, 2002). Furthermore, formaldehyde causes intra- and inter-molecular DNA and protein crosslinking (Srinivasan, Sedmak & Jewell, 2002; Do & Dobrovic, 2015; Donczo & Guttman, 2018). Thus, DNA yielded from FFPETs is typically of poor quality and highly degraded. The effect of formalin-fixation and paraffin-embedding on DNA in FFPETs has shown to hinder downstream molecular tests such as PCR

amplification, consequently reducing the success of identifying genetic variants for molecular autopsies (Srinivasan, Sedmak & Jewell, 2002; Huijsmans et al., 2010; Do & Dobrovic, 2015).

Despite the detrimental effects associated with FFPET regarding molecular analyses, this tissue still represents an extensive and largely untapped DNA repository (Kokkat et al., 2013). If the quality and quantity of DNA recovered from these tissues can be improved, through optimisation of the DNA extraction method, it would allow for deeper investigation into many cases which would otherwise remain dormant. As a result, retrospective molecular autopsies could be performed, which may ease the burden of unresolved SUDY cases at SRM.

1.2. RATIONALE AND AIM OF LITERATURE REVIEW

There are various potential approaches to optimising DNA recovery from different biological samples, including FFPETs. Previous studies have found that DNA quality and quantity recovered from FFPETs can be improved by adjusting variables such as the tissue type, chemical composition of the solvents and solutions and incubation temperatures for both commercially manufactured and conventional in-house methods (Huijsmans et al., 2010; Maraschin et al., 2017; Reid et al., 2017). The aim of this literature review was to identify variables which have been investigated in previous research and to summarise and critically assess the relevance of the effects of these variables on the quality and quantity of DNA recovered from FFPETs.

1.3. SEARCH STRATEGY

The articles for this review were retrieved by performing a detailed search of PubMed, Web of Science and Scopus databases using keywords as shown in Table 1.1. Following the initial search, PubMed, Web of Science and Scopus yielded 88, 176 and 310 articles, respectively. The title and abstract of each article were screened for relevance and were excluded if they pertained to non-human specimens, focused on RNA or protein recovery as opposed to DNA recovery, focused on the identification of the causative effects of genetic variants as opposed to DNA recovery or if they were not available in English (Table 1.2.). Peer-reviewed, original articles and case reports were included, whereas literature reviews were excluded to avoid over-representation of the data. Applying these criteria yielded 54 articles, 18 of which were replicated across the three databases. Hence, a total of 26 articles were included in this literature review.

Table 1.1. *Keywords used in the search of PubMed, Web of Science and Scopus databases for literature pertaining to the optimisation of DNA extraction from formalin fixed paraffin embedded tissues. The 'OR' command was used to run the alternatives; the 'AND' command was used to run the keywords.*

Keyword (AND)	***Alternatives (OR)***
Optimisation	Optimising; optimal; modification; develop
DNA extraction	DNA purification; DNA isolation
FFPET	Formalin fixed paraffin embedded tissue; FFPE

Table 1.2. *Filtering process for exclusion of journal articles produced from a literature search of PubMed, Web of Science and Scopus.*

Database	**Initial search**	**Exclusion criteria**					**After exclusion**
		Non-human	Non-DNA extraction optimisation	RNA/ protein	Genetic variants	Not accessible	
PubMed	88	24	32	6	15	0	11
Web of Science	176	11	91	15	42	2	15
Scopus	310	11	182	23	63	3	28

1.4. SEARCH RESULTS

The quantity and quality of DNA extracted from FFPETs appeared to be dependent on a number of variables which fell within the tissue preparation and DNA extraction workflow (Figure 1.1). These included pre-fixation (tissue type, age and degree of autolysis), fixation (type of fixative and duration of fixation), post-fixation (storage, tissue preparation and processing) and DNA extraction variables (Gilbert et al., 2007). Previous research seemed to focus on the tissue preparation and DNA extraction parameters.

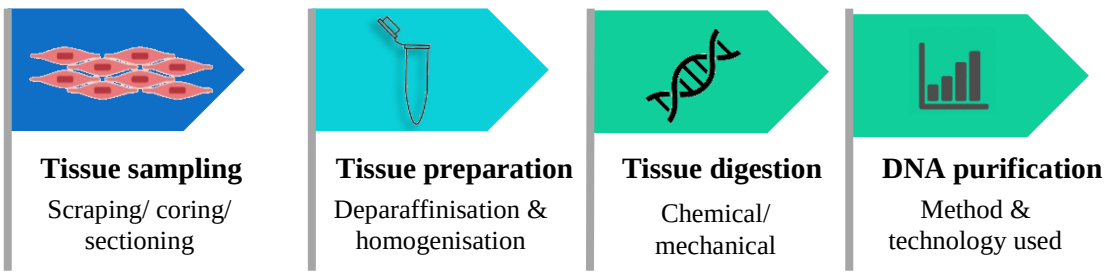

Figure 1.1. *Factors in the DNA extraction workflow which may affect the quality and quantity of DNA extracted from formalin fixed paraffin embedded tissues. Arrows represent the direction of the workflow.*

The articles reviewed in this study were critically analysed and information pertaining to the effect of a particular variable on DNA quantity and/or quality was highlighted. The table in Appendix A.1. summarises the results for the analysis of each of the 26 articles included in this review. The main findings have been synthesised in the text below.

1.4.1. Tissue sampling

The tissue processing methodology for FFPETs typically begins with the sampling of tissue blocks. Microtome sectioning is the most common sampling method for FFPETs and involves the removal of a thin layer from the tissue block using a steel blade. Alternatively, tissue can be removed from the block by needle biopsy (coring) or scraping with a sterile scalpel. Tissue coring only removes a small, often targeted portion of tissue, which would be advantageous for clinical diagnostic purposes as it allows for selection of specific portions of tissue that may be of interest (Patel et al., 2016). However, coring was not commonly used for forensic samples.

Overall, microtome sectioning was performed in 81 % of the studies in this review, whereas scraping and coring were only used in 23 % and 8 %, respectively (Appendix A.1.). Studies wherein microtome sectioning was used reported successful DNA recovery and PCR amplification of fragments ranging from 90 base pairs (bp) to over 600 bp in size. However, there was great variability in the tissue sectioning parameters, i.e. tissue thickness and number of sections. Thus, a direct comparison could not be made for these studies. Various methods called for a range of one to 15 sections of 3-µm to 50-µm in thickness. Further research is warranted in assessing the effect of variable tissue sectioning parameters on DNA recovery.

Two studies in this review directly compared the sectioning, coring and scraping methods with regards to the DNA yield and success of PCR amplification. One study reported that tissue cores yielded between 122 % and 132 % more DNA than tissue sections from clinical samples, which may be due to the coring process being more targeted than microtome sectioning (Bukhari et al., 2008; Chung et al., 2012). Regions on the tissue with the highest number of nuclei can be identified by cell staining and microscopy and selected for by coring (Patel et al., 2016). However, it was further reported that tissue cores collected from autopsy material had lower DNA yields than those collected from surgical biopsies (Patel et al., 2016). These findings reiterate that while coring may be an effective method for cell selection, it may be more suitable for clinical diagnostic studies than forensic investigations into cause of death determination.

Another study reported that while there was no difference in the DNA yield for equal amounts of tissues that had been sectioned or scraped, tissue sections had a slightly higher PCR success rate than scraped tissues (Bukhari et al., 2008). It appeared that microtome sectioning was considered a more suitable method than coring and scraping for DNA extraction from post-mortem FFPET samples for downstream molecular analyses. However, due to variability in the literature with regards to the tissue sectioning parameters, there is room for additional research into the effect of tissue thickness and number of tissue sections on DNA recovery and PCR success rate.

1.4.2. Tissue preparation

Once the FFPET blocks have been sampled either by sectioning, coring or scraping, the tissue is then processed to make it more amenable to DNA extraction and downstream molecular analyses. Tissue processing involves the removal of the paraffin wax, also termed 'deparaffinisation', followed by rehydration and homogenisation of the tissue. Deparaffinisation is arguably the most crucial step for successful DNA extraction as undissolved paraffin incorporated in the sample has been shown to inhibit PCR and cause inaccurate quantification results (Pikor et al., 2011).

FFPETs can be mechanically or chemically deparaffinised. The most common deparaffinisation method used in the studies included herein, was chemical solubilisation, where 50 % of studies made use of xylene and 35 % made use of organic solvents (Bonin & Stanta, 2013; Kocjan, Hošnjak & Poljak, 2015). Since xylene is highly toxic and hazardous, the use of organic alternatives, such as limonene, hexane, pentane or mineral oil, were increasingly more common in recent years (Gilbert et al., 2007; Lin et al., 2009; Bonin & Stanta, 2013).

Although a direct comparison cannot be made between studies which used xylene and organic solvent deparaffinisation methods, it was noted that DNA quantity, quality and PCR success rate was comparable for both methods. Furthermore, it was previously demonstrated that the organic solvent, pentane, produced comparable DNA yields with no difference in PCR success rate as compared to xylene (Gilbert et al., 2007).

Some DNA extraction methods omitted the deparaffinisation solution completely and instead opted for solubilising agents or mechanical deparaffinisation methods such as melting of the wax by applying heat or pressure to remove the paraffin directly into the lysis buffer (Coombs, Gough & Primrose, 1999; Chung et al., 2012; Bonin & Stanta, 2013; Zhong et al., 2013; Kocjan,

Hošnjak & Poljak, 2015). Heating and pressure cooking were used in 31 % and 8 % of the studies, respectively.

Pressure cooking was the most recent development (first used in 2012) and has only been used in two studies in this review (Chung et al., 2012; Zhong et al., 2013). These studies reported no significant difference between pressure cooking and xylene deparaffinisation with regards to the DNA yield and PCR success rate. However, there were a number of confounding factors which could have influenced this result (Gilbert et al., 2007; Zhong et al., 2013). For instance, the studies used a small sample size and did not control for tissue type, tissue sampling method, tissue processing and DNA purification methods. Additional research is therefore required to generate empirical data to assess whether pressure cooking is indeed a suitable alternative to xylene.

Four of the studies in this review (15 %) reported that heating methods yielded higher quantities of DNA with increased purity and up to 16 % increased PCR success rate as compared to xylene (Coombs, Gough & Primrose, 1999; Shi et al., 2002; Chung et al., 2012; Lagheden, Eklund, Kleppe, Unger, Dilner, et al., 2016). However, a limitation of their conclusions was that heating methods were often paired with other chemical (e.g. organic) solvents to facilitate the chemical reaction and further enhance the efficiency of deparaffinisation.

Manufacturers of certain commercial DNA extraction kits have largely shifted away from using xylene, and instead have developed organic deparaffinisation solutions paired with the use of heated incubation (Kocjan, Hošnjak & Poljak, 2015). Overall, this seemed to be the best approach to the deparaffinisation of FFPETs, due to its similar DNA recovery to xylene, but with the added advantage of carrying far fewer health and safety risks (Coombs, Gough & Primrose, 1999; Shi et al., 2002; Atanesyan et al., 2017).

1.4.3. Tissue digestion

Deparaffinisation is typically followed by proteolytic tissue digestion in attempts to overcome the DNA and protein cross-linking caused by formaldehyde. Digestion is most commonly achieved by incubation with proteinase K (pro K), a highly active broad-spectrum serine protease that cleaves both native and denatured proteins, releasing the DNA (Bonin & Stanta, 2013; Kocjan, Hošnjak & Poljak, 2015).

Many studies indicated that an extended incubation with pro K at an elevated temperature improved the efficiency of DNA extraction and PCR amplification of longer fragments (Shi et al., 2002, 2004; Dedhia et al., 2007; Gilbert et al., 2007; Huijsmans et al., 2010; Okello et al., 2010; Kocjan, Hošnjak & Poljak, 2015). The majority (71 %) of studies in this review recommended and made use of DNA extraction protocols with a short 15-minute incubation at 95 ℃ during the tissue lysis step, followed by overnight incubations at 55 – 65 ℃ (Huijsmans et al., 2010).

The short 15-minute pre-incubation at 98℃ has been shown to improve DNA yield and PCR efficiency, which is often recommended by manufacturers of commercial DNA extraction kits (Gilbert et al., 2007; Huijsmans et al., 2010). Studies have reported that higher incubation temperatures resulted in an increase in DNA concentration and purity. However, this result was dependent on additional factors such as the chemical composition and pH of the digestion buffer as well as the incubation time (Shi et al., 2002, 2004; Dedhia et al., 2007; Gilbert et al., 2007).

When a basic digestion buffer (pH 10 – 12) was used, incubation temperatures above 100 ℃ improved the DNA yield and PCR amplification of fragments up to 600 bp in length (Shi et al., 2002, 2004). However, when the pH was reduced below 9 with incubation temperatures at 55 ℃ – 65 ℃, DNA yield and fragment length were significantly reduced (Shi et al., 2002; Dedhia et al., 2007; Gilbert et al., 2007). This has been attributed to the fragmentation of DNA that occurs at a combination of low pH and elevated temperatures (Gilbert et al., 2007).

Similarly, the time of incubation was thought to be closely linked to the resultant DNA fragmentation associated with increased temperatures. Thus, a balance between time and temperature must be found to ensure minimal DNA degradation (Gilbert et al., 2007). Longer incubation times at lower temperatures (55 ℃ – 65 ℃) have been shown to improve DNA yield and successful amplification by quantitative polymerase chain reaction (qPCR) (Gilbert et al., 2007; Okello et al., 2010). While no significant difference in DNA yield was seen when tissues were incubated for 4 hours and 24 hours, some studies have shown that increasing the incubation time further to 48 hours significantly enhanced PCR success (Gilbert et al., 2007; Snow et al., 2014). This phenomenon is based on the principle that incubation time and temperature is directly related to the thermal energy input, which in turn causes partial reversal of the DNA-crosslinks (Gilbert et al., 2007; Bonin & Stanta, 2013). Hence, as the thermal energy input increases, DNA-crosslinking decreases thereby making it more amenable to PCR-amplification. When incubation time was increased above 48 hours, the increased PCR success rate became less significant and in some instances, was even be reduced (Gilbert et al., 2007).

This may be due to a plateau-effect in the ability to reduce DNA cross-linking, thereby limiting the success of PCR amplification (Gilbert et al., 2007).

The variable chemistries, lysis times and temperatures for the DNA extraction methods used in previous studies made it challenging to standardise the incubation parameters for an improved DNA extraction method. Typically, manufacturers optimise the commercial DNA extraction protocols to their specific chemistries. It may be useful to modify these protocols to suit the laboratory setting or sample conditions. When using a strong basic digestion buffer, it may be beneficial to use higher temperatures and longer incubation times for an increased DNA yield and PCR efficiency. When using digestion buffers with a lower pH, it is recommended to limit the time and temperature to a maximum of 48 hours at 55 – 65 ℃.

1.4.4. DNA purification

The approach to DNA extraction ranges from conventional heat-treatment and phenol-chloroform methods to commercially manufactured DNA extraction kits (Shi et al., 2004; Dedhia et al., 2007; Huijsmans et al., 2010; Wang et al., 2013). In 2015, there were 35 commercial DNA extraction kits specifically designed for FFPETs available on the market (Kocjan, Hošnjak & Poljak, 2015). Most commercial kits were based on a similar principle; 'silica or resin adsorption technology', which manipulates the binding affinities of silicates for DNA under various conditions of pH and salt concentration (Kocjan, Hošnjak & Poljak, 2015).

There are a number of advantages in using a commercial kit over a conventional in-house method. For instance, kits have reduced variability in their solutions, thereby increasing reproducibility. This might explain why commercial kits have been shown to improve DNA yield – as a result of a reduced observational error (Weirich et al., 1997; Bonin & Stanta, 2013). The main disadvantage of using commercial kits is that it is relatively costly. Furthermore, one study reported that despite the high cost of commercial DNA extraction kits, it did not yield sufficient DNA for PCR amplification (Dedhia et al., 2007). Overall, head-to-head comparisons between various laboratory-based and commercial kits produced variable results.

The variability in the literature centres around the effects of in-house and commercial methods on the DNA extraction efficiency. Thirteen of the studies in this review directly compared the in-house phenol-chloroform-isoamyl alcohol (PCI) and cetyl trimethylammonium bromide (CTAB) methods to various commercial extraction kits. There was a split conclusion, where five out of 11 of the studies reported that commercial kits had greater DNA yields than

conventional methods, while the remainder of the studies reported the opposite (Dedhia et al., 2007; Bonin et al., 2010; Huijsmans et al., 2010; Funabashi et al., 2012; Paireder et al., 2013; Wang et al., 2013; Oskina et al., 2017). This conflict of results may be attributed to the variability in the pre-analytical conditions, such as tissue processing methods and differences in the in-house protocols used in these eleven studies.

In addition to DNA yield, six studies reported that commercial kits yielded DNA with higher purity measurements and longer DNA fragments (Coombs, Gough & Primrose, 1999; Gilbert et al., 2007; Huijsmans et al., 2010; Chung et al., 2012; Paireder et al., 2013; Wang et al., 2013). PCI extraction had significantly larger variation in the reported DNA quantities and purities. Thus, despite variability in the reports on DNA concentration, the overall consensus appears to be that commercial DNA extraction kits produce more accurate and precise results and are therefore more reliable for downstream molecular analyses (Ludyga et al., 2012; Oskina et al., 2017).

In the majority of studies which reported an improved DNA yield and PCR efficiency, the QIAamp DNA mini/micro or QIAamp DNA FFPE tissue kits were used. These studies reported that the purity of the DNA and reproducibility of the results from in-house methods were poor as compared to the Qiagen kits (Ludyga et al., 2012; Oskina et al., 2017).

A comparison between the various commercial kits used across the studies in this review showed that the QIAamp DNA FFPE tissue kit, Wax-free DNA extraction kit, TrimGen's WXF, Ambion's RAD and Sigma's GEN were among those which appeared to produce relatively high DNA yields. Of these, the QIAamp DNA FFPE tissue kit has been reported to be most suitable for FFPET specific amplification and best for larger fragment amplicons. Other kits, such as the truXTRAC FFPE DNA extraction kit has shown to yield competitive amounts of DNA, but with larger variability depending on the sample type (Kresse et al., 2018).

The process of selecting a commercial DNA extraction kit is largely based on the aim and scope of the study and the laboratory requirements or restrictions. For instance, the tissue type, species, length of amplicon and potential downstream applications should be factored in when choosing between the DNA extraction technologies currently available. There are a number of commercial DNA extraction kits which have shown to yield DNA suitable for a multitude of downstream molecular processes (Kocjan, Hošnjak & Poljak, 2015; Atanesyan et al., 2017; Kresse et al., 2018). A molecular diagnostics focused laboratory might be highly motivated to reduce hands-on time and increase the speed and efficiency of DNA extraction. These

laboratories may be more interested in automatic DNA extractions than those with smaller-scale studies and non-clinical applications (Khokhar et al., 2012).

1.5. RATIONALE

There is great need for the development of an optimised DNA extraction protocol for use in a local context; specifically, for SRM. This would allow for further investigation, by means of molecular autopsy, into unresolved SUDY cases. In order to identify candidate cases for inclusion in this study and for molecular autopsies on SUDY cases at SRM, additional epidemiological and descriptive information is needed. This data, which is sorely lacking in the literature, can be obtained by retrospective analysis of SUDY cases at SRM.

Data obtained from the case review could prove essential for identifying pre-analytical variables that could affect DNA quality and quantity, such as tissue type, specimen storage time and circumstances surrounding death.

A previous unpublished study in the research group compared DNA recovery from FFPET blocks of various tissue types. The tissue type did not seem to affect DNA recovery, however, a trend was seen where FFPET sections with a greater cell count yielded higher DNA concentrations. Thus, when multiple tissue types are available for a single case, microscopic quantification of the cell content may aid in establishing the best FFPET block for DNA extraction.

In addition, the previous study showed that increasing the number of tissue sections of equal thickness was shown to improve DNA recovery when using three commercial DNA extraction kits, namely the NucleoSpin® DNA FFPE XS kit (Machery Nagel), ZR FFPE DNA MiniPrep™ kit (Zymo Research) and the QIAamp® DNA FFPE tissue kit (Qiagen). Results showed that the Qiagen kit yielded the best quality and quantity of DNA from FFPETs, followed by the Zymo Research kit.

For each of these kits, there was comparable DNA recovery when using mineral oil and the commercial deparaffinisation solution. Furthermore, xylene did not appear to improve DNA extraction from FFPETs.

Lastly, this previous study showed that neither DNA concentration nor DNA integrity was affected by storage time (up to two years). This research, together with the results from the

literature review provided insight into a number of variables that could affect retrospective DNA extraction from FFPETs.

Building on this research, further optimisation of DNA recovery from FFPETs would enable retrospective DNA analysis for investigations into genetic causes of death and potentially reduce the burden of unresolved SUDY cases at SRM. Due to a large backlog in forensic casework, it would be beneficial to further assess the applicability of using the optimised DNA extraction method for samples that have been stored over time. In this case, a longitudinal study would allow for comparison of the DNA quality and quantity for FFPETs that have been collected more recently and those stored for multiple years.

1.6. AIM AND OBJECTIVES

The aim of this study was to investigate the use of FFPETs as a source of DNA in SUDY cases for the intended future application of molecular autopsies.

The study objectives were to:

i. Retrospectively analyse the Office Autopsy Database to identify and briefly describe SUDY cases admitted to SRM.

ii. Optimise the DNA extraction procedure on FFPET samples from control SUDY cases admitted to SRM using the QIAamp® DNA FFPE tissue kit by adjusting the thickness and number of FFPET sections.

iii. Assess the effect of thickness and number of FFPET sections on DNA quantity, purity integrity and fragment size.

iv. Compare the concentration, purity, integrity and fragment size for DNA extracted from FFPETs that had been stored between one and three years.

Chapter 2: Methods

2.1. STUDY DESIGN

This study had a quantitative, cross-sectional design, with both retrospective and prospective components. First, forensic cases admitted to SRM between 1 January 2014 and 31 December 2018 were retrospectively reviewed. These data were primarily used as inclusion criteria to identify samples for the prospective, experimental phase of the study. Data from the retrospective study were further used to contextualise the outcomes of the study in terms of its pre-analytical factors.

The prospective phase of the study assessed the independent variables of FFPET section thickness, number of sections and storage time on the dependent variables of DNA concentration, purity, integrity and fragment size, within a population of SUDY cases from SRM.

This research was approved by the University of Cape Town (UCT), Faculty of Health Science Human Research Ethics Committee (HREC REF: 211/2019) (Appendix A.2.).

2.2. RETROSPECTIVE IDENTIFICATION OF SAMPLES

The Office Autopsy Database (OAD) (HREC REF: R036/2014) was used to identify cases and samples relevant to this study. Of the cases admitted to SRM for medico-legal investigation between 1 January 2014 and 31 December 2018 (n = 11 585 cases), 2940 cases (25 %) were admitted under the circumstances of SUD of all ages (Appendix A.3.). When assessed according to age, 1 168 cases were identified where the individual was between 1 and 40 years old at the time of death, herein to be referred to as SUDY.

The medico-legal case files of the SUDY cases were reviewed in greater detail. Variables pertaining to the age, sex, cause of death, date of death, death scene environment, date of autopsy and specimen collection for ancillary investigations were recorded. The demographic information and availability of FFPETs were used to identify ‘control’ samples for the optimisation of DNA extraction (n = 3) and ‘test’ samples ($10 \leq n \leq 25$) for assessing the applicability of the optimised method on more relevant samples; i.e. tissues that were stored over a number of years.

A case was considered for inclusion as a control sample if it was admitted to SRM between 1 January 2018 – 31 December 2018, the interval between death and autopsy was less than five days, cause of death was determined and histology specimens had been collected and no longer needed for the medico-legal investigation (Figure 2.1.). Three cases were identified and FFPET blocks from these cases were collected from the in the histopathology repository (HREC REF: R037/2014). The FFPET blocks of three different tissue types from each of these three cases were included for optimisation of DNA extraction. Hence, a total of nine FFPET samples were included in the control sample set.

A case was considered for inclusion as a test sample if it was admitted to SRM between 1 January 2014 – 31 December 2017, and cause of death was undetermined (Figure 2.1.). Due to the lack of availability of FFPET specimens for research purposes, only cases from 2016 were included in the test sample set. This comprised of a total of 32 tissue types from 12 cases.

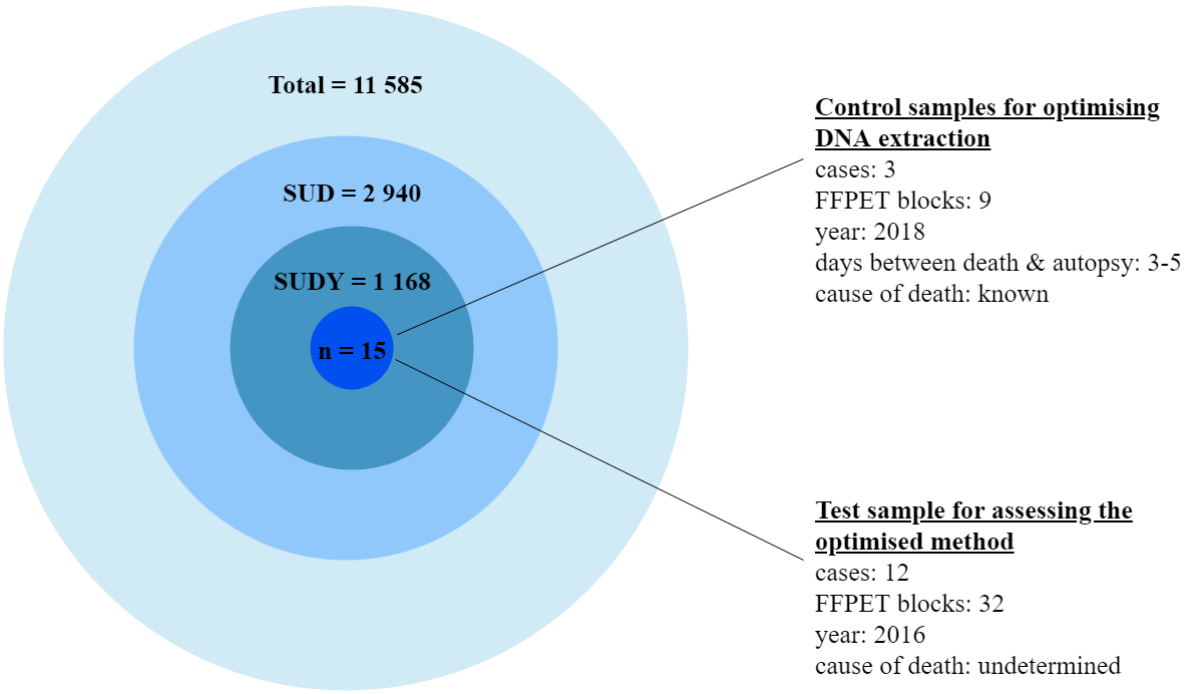

Figure 2.1. *Categorisation and sampling of forensic cases admitted to the Salt River mortuary between 1 January 2014 and 31 December 2018.*

2.3. TISSUE QUALITY ASSESSMENT

The FFPET blocks (representing the various tissue types available for each case) were given a quality score based on the presence of tears, total surface area and the total number of nuclei. To this end, a single 5-µm section was cut from each FFPET block using an Accu-Cut® 200 rotary microtome (Sakura Fintek, Amsterdam, UK) with disposable blades (Feather®, Osaka, Japan). To prevent cross-contamination, the first three sections of each block were discarded, the microtome was sterilised and the blade was changed between blocks.

Each tissue section was placed in a water bath set at 60 ℃ to facilitate its transfer to a Histobond glass slide (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), then dried overnight in an incubator set at 65 ℃. The following day, the tissue sections were stained with hematoxylin and eosin (H&E) (Appendix A.4.).

The samples were visualised using a Leica DM500 light microscope under 100 X magnification with LAZ EZ™ software (Leica, Heerbrugg, Germany). The total surface area and the total number of nuclei present in each slide were quantified on the microscopic images using ImageJ software v1.46r (National Institutes of Health, USA). The various tissue types for each case were ranked according to the number of tears and the number of nuclei per total surface area.

2.4. DNA EXTRACTION

Based on preliminary data, it was hypothesised that finer sections would yield greater DNA qualities and quantities and that increasing the number of sections of equal thickness would improve DNA recovery. To test this, the thickness and number of FFPET sections were varied in the control samples.

The FFPET blocks representing the tissue types with the highest quality score for the control samples, as quantified by microscopy, were sectioned to 5-µm, 3-µm and 1-µm thick (Figure 2.2.). The number of tissue sections was incrementally increased from 5 to 100 sections. This was done in a staggered manner to avoid depleting the tissues. The best combination of variables (thickness and number of sections) was established by quantification of DNA recovery for the three control cases, as described in section 2.5 below.

The DNA recovery for FFPETs that had been stored for a number of years was assessed. FFPET blocks representing the tissue type with the highest quality score for each of the test samples, as quantified by microscopy, were sectioned according to the optimised sectioning parameters.

FFPET sections were subjected to DNA extraction using the QIAamp® DNA FFPE tissue kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany) with the following modifications: 320 µl Deparaffinisation solution (Qiagen, Hilden, Germany) was used, tissue lysis was performed at 56 °C (900 rpm) for 20 hours on a Thermo-Mixer C (Eppendorf, Hamburg) and DNA was eluted into 50 µl ATE buffer.

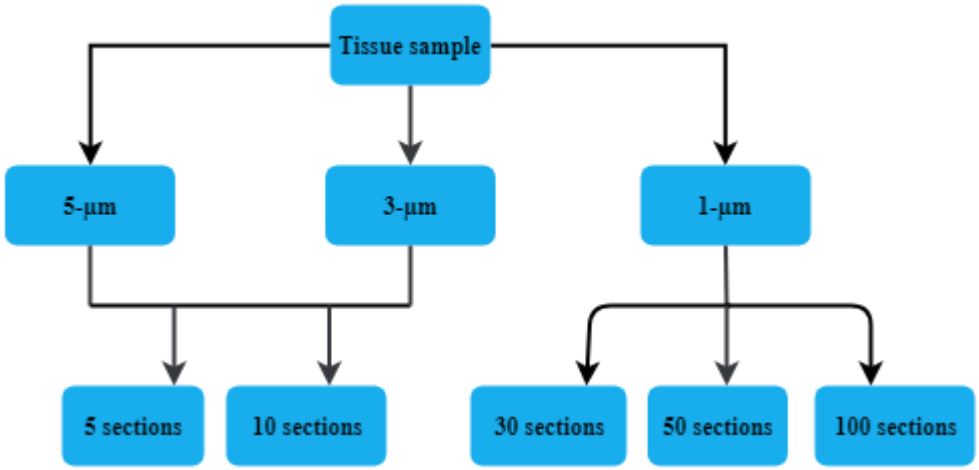

Figure 2.2. *Microtome sectioning parameters used for optimisation of DNA extraction from FFPETs.*

2.5. DNA QUANTIFICATION

2.5.1. NanoDrop™ Spectrophotometry

The purity of the samples was quantified using the NanoDrop™ 2000 spectrophotometer as per the manufacturer's instructions (Thermofisher Scientific, Wilmington, USA). Buffer ATL was used as a blank measurement and a 2-µl aliquot of each sample was used for quantification. The DNA purities were measured according to the A260/A280 and A260/A230 ratios. Samples were considered free of protein and organic compounds or chaotropic salt contaminants if the A260/A280 and A260/A230 ratios were above 1.8 and 2.0, respectively.

2.5.2. qPCR

The effective DNA concentration was quantified by qPCR using the Quantifiler™ Trio DNA quantification kit, according to the manufacturer's instructions (Thermo Fisher Scientific,

California, USA). Each sample was quantified alongside a five-step serial dilution of standard DNA (50 ng/μL to 0.005 ng/μL), processed on a 7500 Real-Time PCR instrument with the HID Real-Time PCR Analysis software v.1.2. (Applied Biosystems, California, USA).

This DNA quantification kit implements two human autosomal markers, one of 80 bp and another of 214 bp in size. The DNA concentration was automatically quantified using the C_t values for the small and large autosomal amplicons. An internal positive control (IPC) was present in each sample to indicate the proper functioning of the assay and the presence of PCR inhibitors. Samples with IPC C_t values greater than 31 were considered to have significant PCR inhibition (Brooks et al., n.d.; Applied Biosystems, 2017).

2.5.3. TapeStation

The integrity and fragment length of each sample was analysed by subjecting samples to digital capillary electrophoresis, using the 4200 TapeStation System with the Genomic DNA ScreenTape® assay, according to the manufacturer's instructions (Agilent Technologies, Waldbronn, Germany).

The DNA integrity number (DIN) was automatically generated for each sample by assessing the distribution of signal as compared to the lower DNA marker. DIN values range from 1 to 10; with 1 being highly degraded and 10 being completely intact DNA. Samples with DIN values below 3 were considered severely degraded (Petersen et al., 2016; Jung, Ji & Schmidt, 2017). The fragment sizes were automatically quantified against the Genomic DNA ladder and the DNA concentrations for the area under the peak of each sample was quantified against the known concentration of the lower marker.

2.6. Statistical analyses

Statistical analyses and visualisation of the data were performed using the Prism software v.8.3 (Graphpad, California, USA). Descriptive statistics were performed on all variables to determine the measures of central tendency and dispersion.

Categorical variables, including the number of cases, age, sex, environmental factors and days between death and autopsy were assessed using the χ^2 test. Each category included the total number of cases which were admitted in the 5-years assessed in the case review, and each of

the categories was analysed individually. P-values less than 0.01 were considered statistically significant.

Numerical data, including the DNA concentrations, purities and integrities were assessed using ANOVA. For comparisons between tissues of various thickness, the data were grouped into 5-µm, 3-µm and 1-µm sections. A one-way ANOVA with Tukey's multiple comparison's test was done on each of the groups. For assessing the effect of the number of sections on DNA recovery, a two-way ANOVA with Sidak's multiple comparison's test was done on the combined set of data. P-values less than 0.05 were considered statistically significant.

For assessing the effect of storage time on DNA concentration, purity and integrity, a Pearson's correlation was performed. R-values closer to 1 or -1 indicated a strong correlation, whereas values closer to 0 indicated a weak correlation.

Chapter 3: Results

3.1. CASE REVIEW

All data collected for the control and test samples were summarised in Appendix A.5. The main findings were synthesised in the text below and information for individual cases are available on request and upon approval from the Director of Forensic Pathology Services.

3.1.1. Outcomes of post-mortem investigations

A total of 1 168 SUDY cases were admitted to SRM over the 5-year study period. There was an increase in SUDY admittance from 137 cases in 2014 to 334 cases in 2017, with a slight decrease to 321 cases in 2018 (Appendix A.3.).

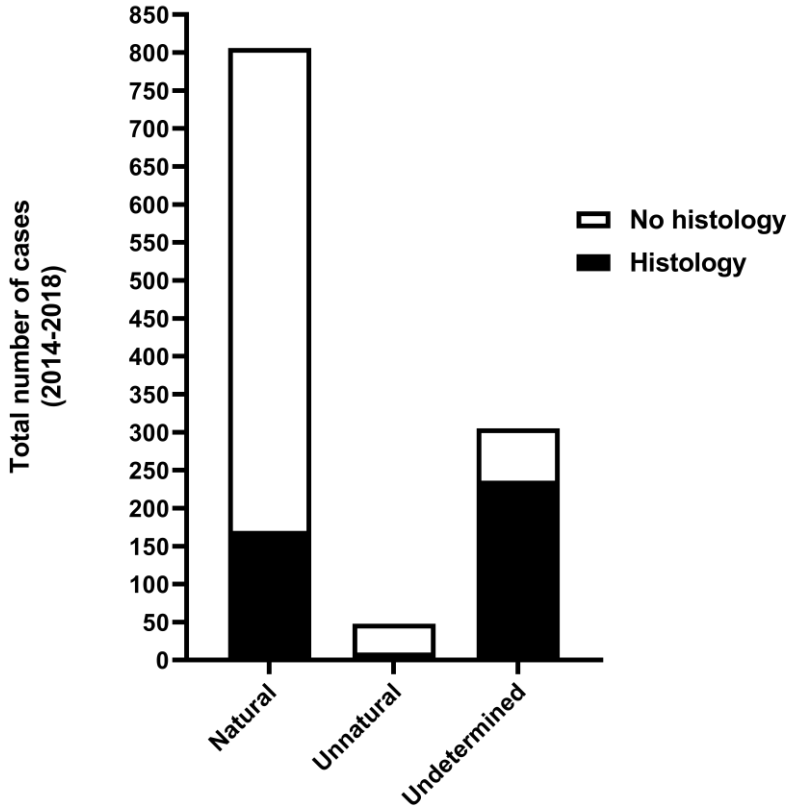

Figure 3. 1. *Outcomes of post-mortem investigations for the total number of SUDY cases admitted to Salt River mortuary between 2014 and 2018. (n=1168).*

The outcomes of the post-mortem investigations into these cases revealed natural and unnatural causes of death in 806 (69.01 %) and 48 (4.11 %) cases, respectively (Figure 3.1.). The cause of death in 305 (26.11 %) cases were undetermined at the time of the study.

Specimens for histopathology analysis were obtained at autopsy for 416 (35.62 %) SUDY cases over the 5-year period. A significantly larger proportion of undetermined SUDY cases (56.73 %) had histopathology specimens taken as compared to cases reported to have occurred from natural ($p < 0.0001$) and unnatural causes ($p < 0.0001$) (Figure 3.1.).

Histopathology specimens obtained at the time of autopsy are routinely fixed in formalin and embedded in paraffin for histological analysis. However, not all of these are available for research purposes. Natural causes of death were reported for two of the three control cases used for the optimisation of DNA extraction; the third being an unnatural death (Appendix A.5.).

3.1.2. Post-mortem environmental conditions

The death scene environment and time between death and autopsy were recorded for the total SUDY cases at SRM.

The majority of SUDY cases (69.52 %) at SRM were reported to have indoor death scene environments. A further 13.86 % and 16.61 % had outdoor and unspecified death scene environments, respectively.

The majority of cases (75 %) were autopsied within five days of death (mean = 4.08 days; median = 3 days). The largest interval between death and autopsy for SUDY cases at SRM was 97 days, however, this was identified as an outlier in the population.

3.1.3. Population demographics

There was a disproportionate number of cases across the age range between one and 40 years old. The majority of SUDY cases (75 %) at SRM were above the age of 25 years at death (Figure 3.2.). The age category of 39 – 40 years old had the highest number of deaths (n = 90) over the 5-year period. The three cases in the control sample included in this study were 23 years, 34 years and 39 years of age at death. The test sample was more diverse with regards to the age categories, ranging from 14 to 40 years (Appendix A.5.).

When comparing male to female subpopulations, a similar trend was seen as described above for the total number of cases. However, there were significantly more males than females overall (p = 0.0007). This trend was not seen for the control or test samples; as there were similar numbers of males and females included in this study (Appendix A.5.).

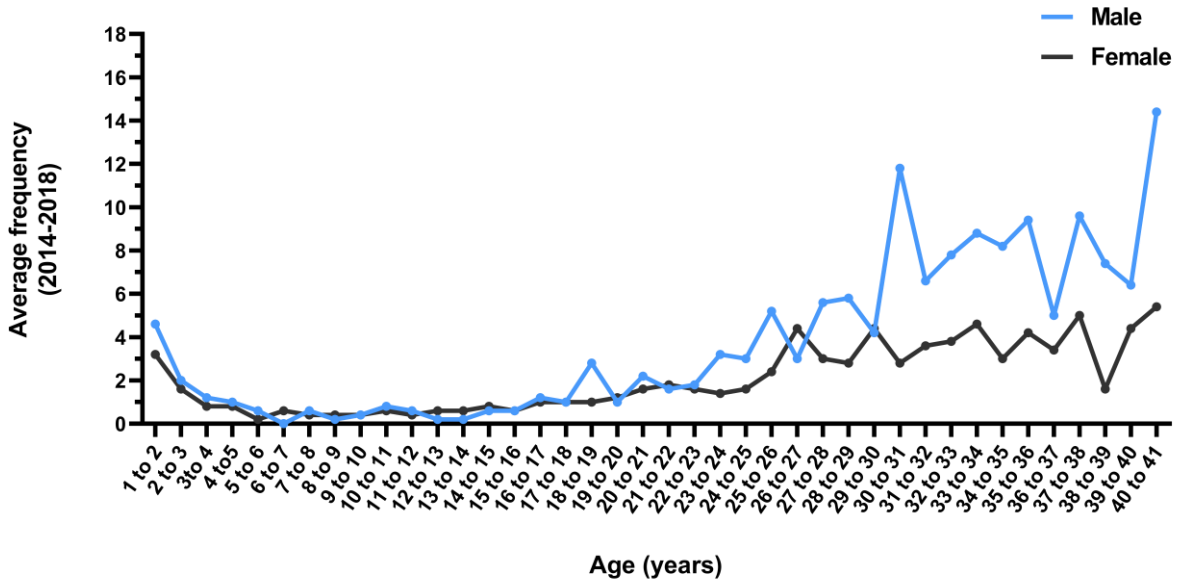

Figure 3. 2. *Age distribution according to sex for the total number of SUDY cases admitted to Salt River mortuary between 2014 and 2018. (n=1168).*

3.2. OPTIMISATION OF DNA EXTRACTION

3.2.1. Tissue thickness

Microscopic quantification of FFPET quality showed which tissue types had the greatest number of nuclei per total surface area. For the control sample set, brain and lung tissue were generally of better quality as compared to pancreas, kidney and heart tissues which were available (Appendix A.5.). The FFPET from the brain and lung from each of the control cases was then sectioned in varying thicknesses, and DNA was extracted to assess the relationship between tissue thickness and effect on DNA concentration, purity and integrity.

The median DNA concentrations (in a final volume of 50 µl) and total DNA yields were reported for each tissue thickness (Appendix A.6.). A comparison between DNA concentrations of 5-µm, 3-µm and 1-µm sections revealed that finer sections produced greater DNA yields (Figure 3.3.).

There was a noticeable, but not significant, difference in DNA concentrations of the small marker for 1-µm and 3-µm sections ($p = 0.08$). Moreover, there was a significant difference in DNA concentrations for 1-µm and 5-µm sections ($p = 0.0427$). For the large marker, the concentrations for the 1-µm and 5-µm sections were noticeably, but not significantly different ($p = 0.0617$), whereas DNA concentrations for the 1-µm and 3-µm sections were significantly different ($p = 0.0493$).

While there was ambiguity regarding whether the 3- and 5-µm thick sectioning significantly affected the DNA yield, it was clear that 1-µm sections produced superior yields to both.

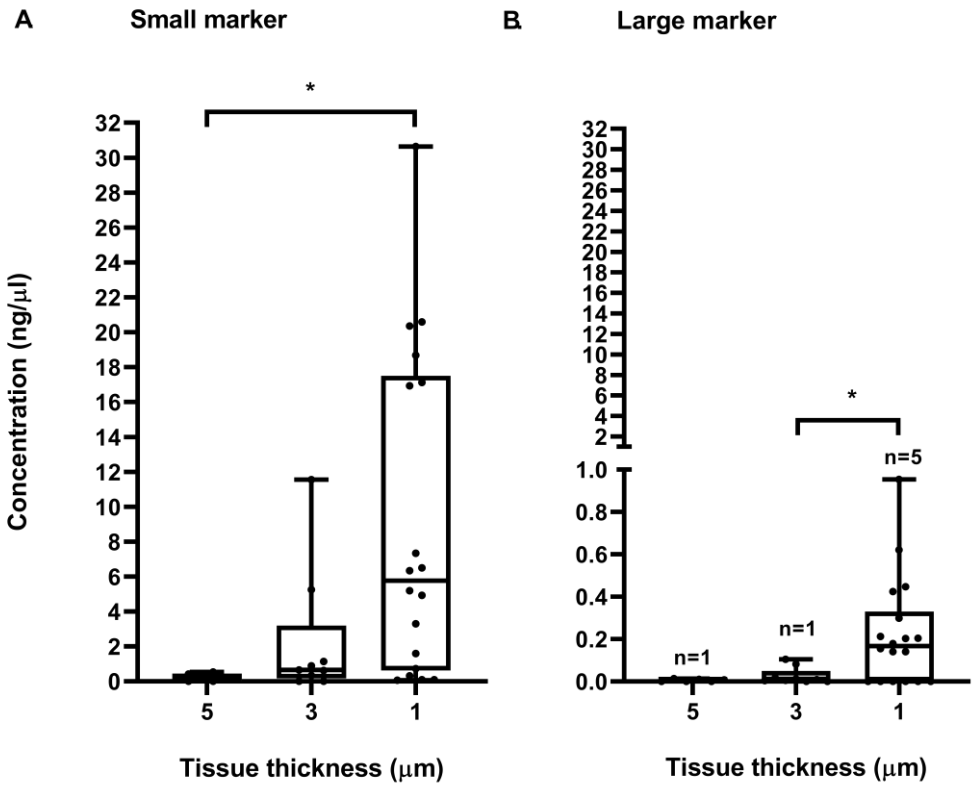

Figure 3. 3. *The effect of FFPET thickness on DNA concentration as quantified by qPCR of (A) 80 bp marker and (B) 214 bp marker. (n): number of samples which were too degraded to amplify. (*): $p < 0.05$.*

The DNA purities for FFPETs of varying thicknesses were also assessed. Results showed that there was a significant increase in both the A260/A230 ($p = 0.0093$) and A260/A280 (0.0005) purity ratios when the tissue thickness was reduced from 3-µm to 1-µm (Figure 3.4.). An even greater difference was seen when comparing the A260/A230 ($p = 0.0001$) and A260/A280 ($p < 0.0001$) ratios for tissues of 5-µm to 1-µm in thickness. Similar to the results for the DNA concentrations, there was no significant difference between the 3-µm and 5-µm sections for either purity ratio.

Overall, there was less variability in the A260/A280 ratios, with the majority of 1-µm sections being within the threshold for pure DNA. While there was more variability in the A260/A230 ratios, a large proportion of 1-µm sections were within or far above the threshold, suggesting that finer sections may have more efficiently reduced the levels of chaotropic salt contaminants.

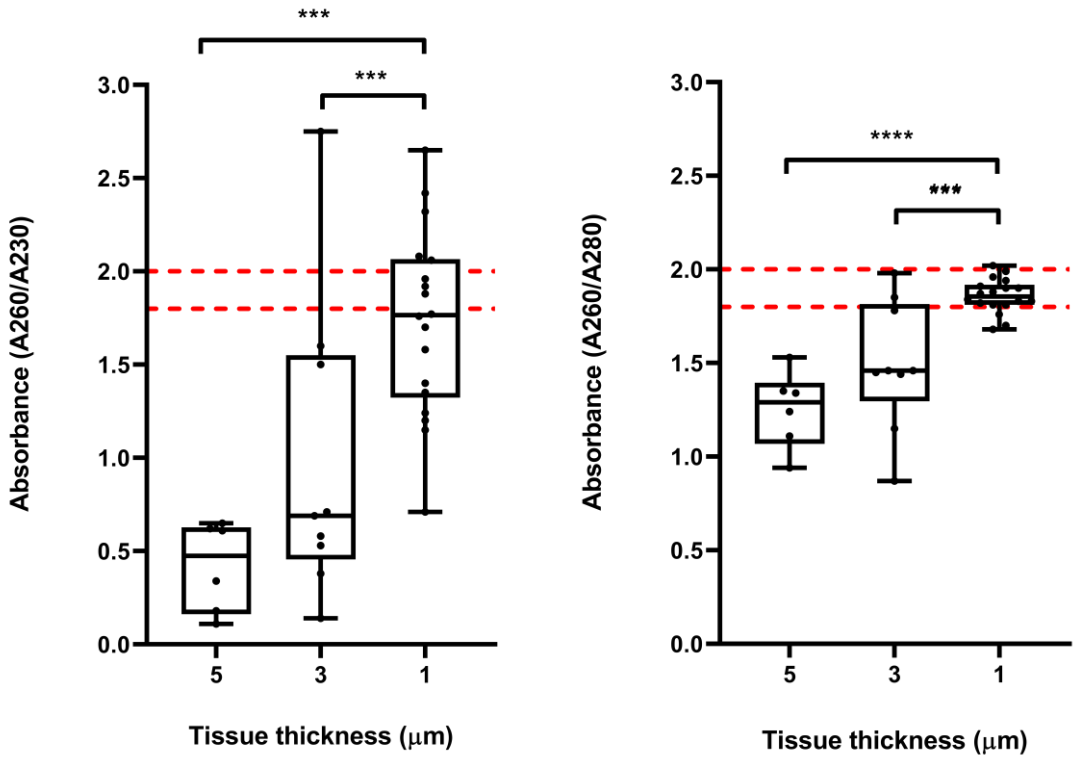

Figure 3. 4. *The effect of FFPET thickness on DNA purity as measured by NanoDrop™ spectrophotometry absorbance at A260/A230 and A260/A280. Red dotted lines indicate the threshold for pure DNA. (**): $p < 0.05$; (***): $p < 0.005$; (****): $p < 0.0005$.*

Results from the Genomic DNA ScreenTape® assay revealed that the tissue thickness significantly affected DNA integrity. The median DIN values were 4.1, 2.7, and 1.7 for 5-µm,

3-µm and 1-µm sections, respectively (Figure 3.5.). This indicates that while all samples were of poor integrity, there was significant difference in DIN when tissue thickness was reduced from 5-µm to 3-µm ($p = 0.0425$), from 3-µm to 1-µm ($p = 0.0265$) and from 5-µm to 1-µm ($p = 0.0001$). This is indicative of a higher level of DNA degradation for finer sections.

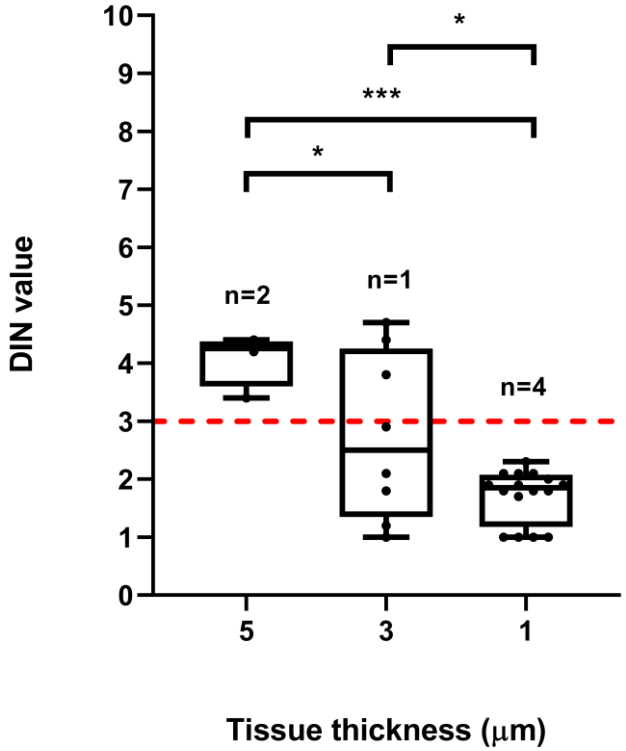

Figure 3. 5. *The effect of FFPET thickness on DNA integrity number (DIN) as quantified by the Genomic DNA ScreenTape® assay for highly degraded DNA. The red dotted line indicates the threshold for highly degraded DNA. (n): number of samples which fell outside of the range for DIN quantification. (*): $p < 0.05$; (***)*: $p<0.0005$.

There was a high degree of variability in the fragment lengths for all samples, despite the tissue thickness. Finer tissue sectioning improved the yield of larger DNA fragments. Specifically, 55 % and 22 % of 1-µm and 3-µm sections had fragments larger than 400 bp, respectively, whereas none of the 5-µm sections yielded fragments larger than 400 bp in size (Appendix A.7.).

Therefore, results from the assessment of tissue thickness revealed that thinner sectioning improved DNA concentration, purity and fragment length, but not DNA integrity. Using this information, 1-µm sections were deemed to be better overall and was thus used in subsequent optimisation steps and experiments.

3.2.2. Number of tissue sections

The effect of the number of tissue sections (30, 50 and 100 sections) on DNA recovery was reported, using 1-µm thick sections (Appendix A.6.).

While no difference in DNA concentration was observed for 30 and 50 sections, a further increase from 50 to 100 sections significantly improved the concentrations of the small marker ($p = 0.006$) (Figure 3.7.). Moreover, a significant difference was observed for 30 as compared to 100 sections for both the small ($p = 0.008$) and large ($p = 0.035$) DNA markers.

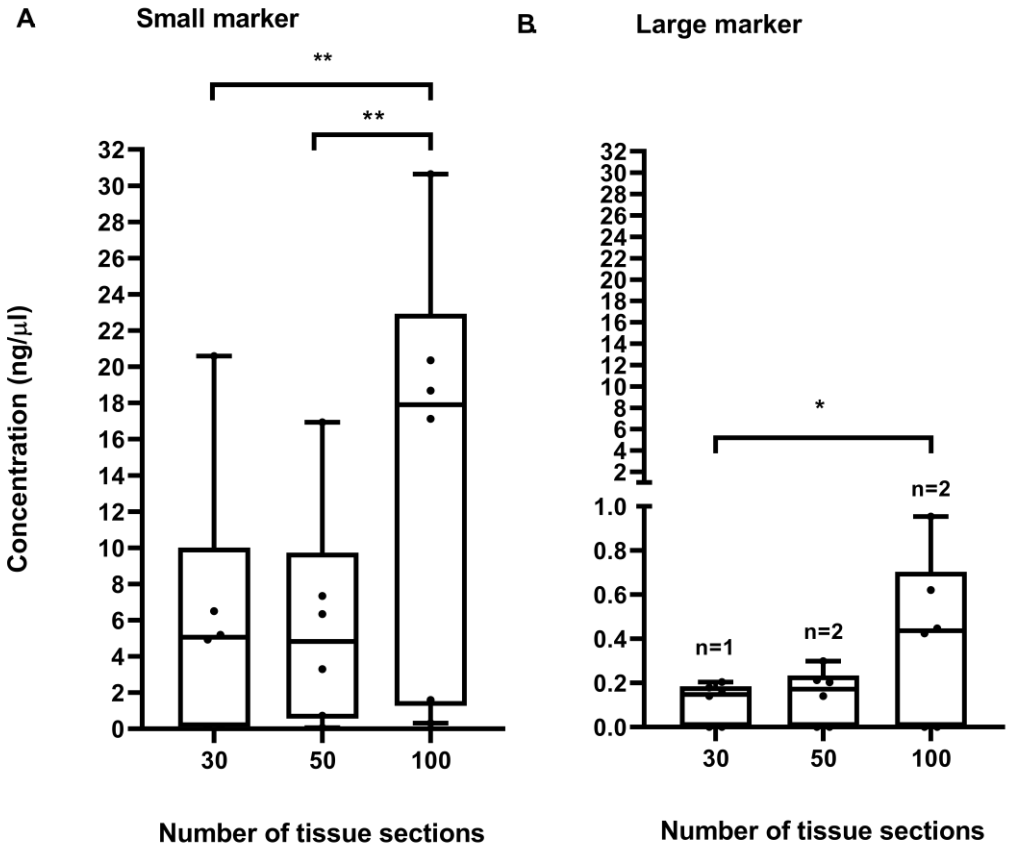

Figure 3. 6. *The effect of number of FFPET sections on DNA concentrations as quantified by qPCR of (A) 80 bp marker and (B) 214 bp marker. (n): number of samples out of six which were too degraded to amplify. (*): $p < 0.05$; (**): $p<0.005$*

An increase in the number of FFPET sections (1-µm thick) did not have any effect on the DNA purity (Figure 3.8). Nearly all A260/A230 ratios were within the threshold for highly pure

DNA. As was seen previously, there was far greater variability in the A260/A230 than the A260/A280 ratios, however, there was no significant difference in purity ratios for the various numbers of tissue sections.

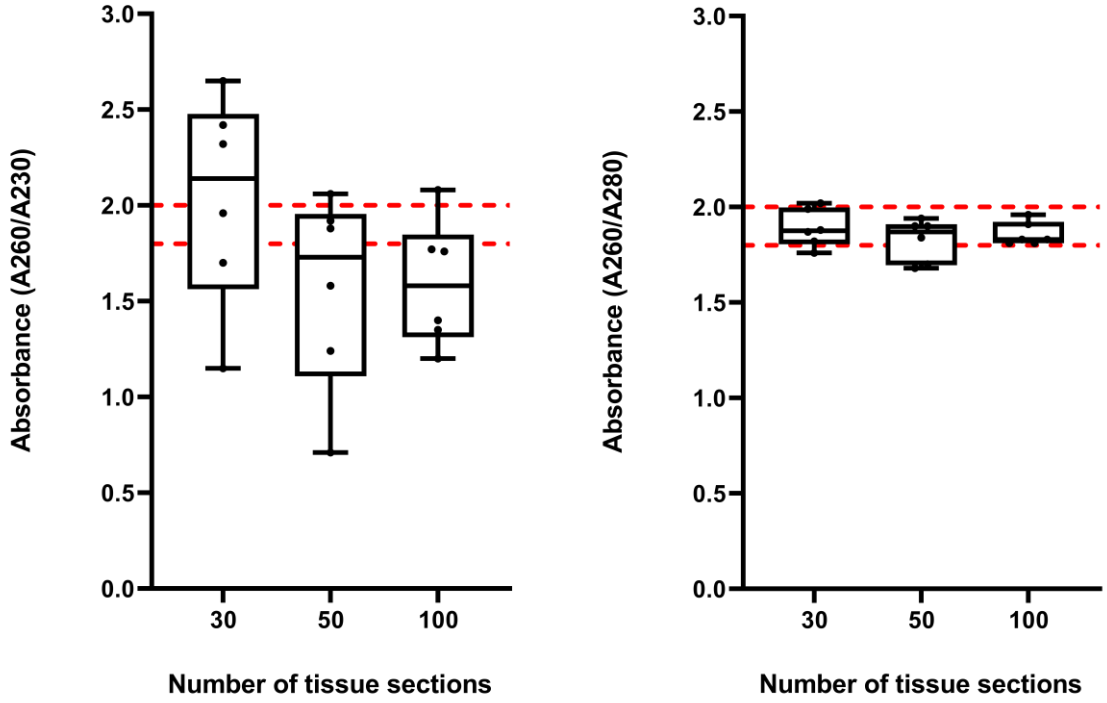

Figure 3. 7. *The effect of number of FFPET sections on DNA purity as assessed by NanoDrop™ spectrophotometry absorbance at A260/A230 and A260/A280. Red dotted lines indicate the threshold for pure DNA.*

All FFPET sections (1-µm thick) had DIN values below 3, indicating a high degree of fragmentation. There was no difference in the reported DIN values for 30, 50 and 100 sections (Figure 3.8.). Moreover, there were similar mean fragment lengths and proportions of fragments greater than 400 bp in size for the various numbers of tissue sections. Thus, increasing the number of tissue sections of equal thickness did not improve DNA fragment length.

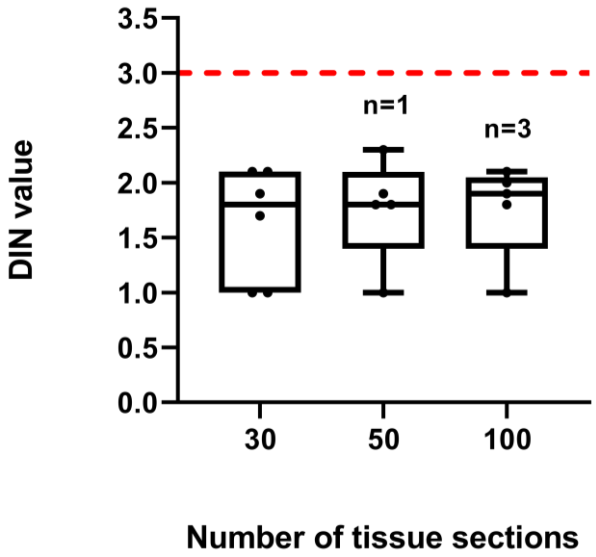

Figure 3. 8. *The effect of number of FFPET sections on DNA integrity number (DIN) as quantified by the Genomic DNA ScreenTape® assay for highly degraded DNA. The red dotted line indicates the threshold for highly degraded DNA. (n): number of samples out of six which fell outside of the range for DIN quantification.*

3.3. TESTING THE OPTIMISED DNA EXTRACTION METHOD

The best tissue thickness and number of tissue sections was established from assessment of the results for DNA concentration, purity, integrity and fragment length. It was established that 100 sections of 1-µm thick yielded the highest DNA concentrations, with the greatest purity ratios and a larger proportion of DNA fragments greater than 400 bp in size.

Using these variables, DNA was extracted from stored FFPET blocks representing the tissue with the best quality for each of the test samples (Appendix A.5.). The effect of storage time on DNA concentration, purity and integrity was reported.

Results showed that a median of 320 ng (287 ng – 698 ng) of DNA was recovered from FFPET samples that had been stored for up to three years (Appendix A.6.). The DNA concentrations were only slightly reduced for FFPETs stored for longer periods of time (Figure 3.8). However, the decrease over time was not statistically significant. Furthermore, when taking the confidence interval for the p-value into consideration, there was no strong evidence to indicate that the inverse correlation between time and DNA concentration was biologically relevant.

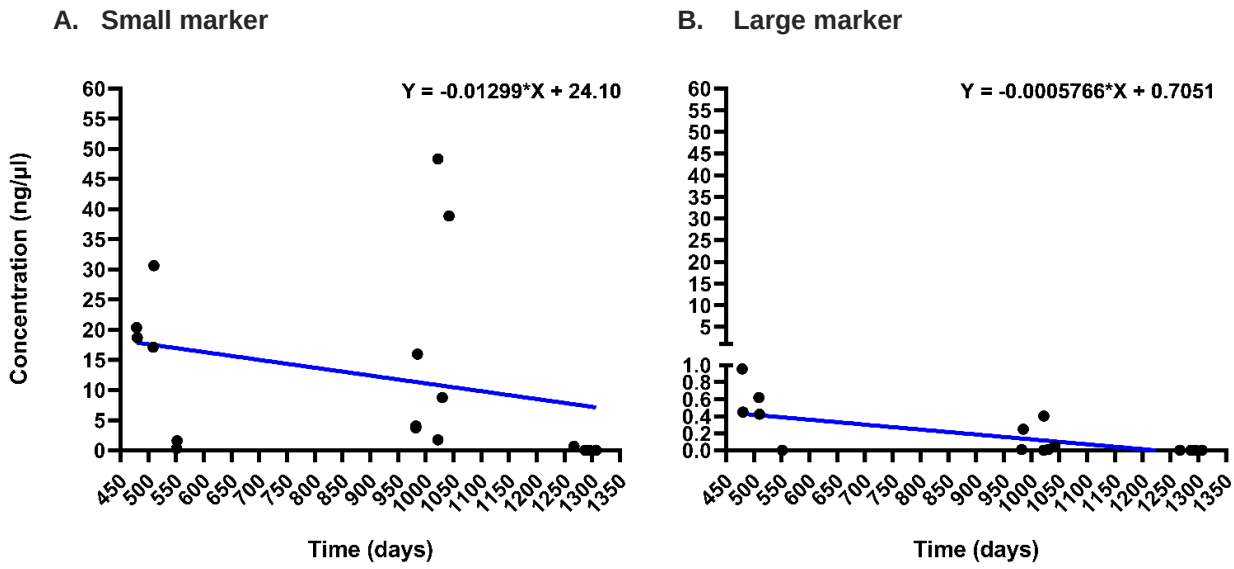

Figure 3. 9. *DNA concentrations for 100 FFPET sections of 1-µm thick stored over a period of 450 to 1 350 days (1-3 years), as quantified by qPCR for (A): 80 bp marker and (B) 214 bp marker. (n=16).*

There was a similar reduction in DNA purity with increased FFPET storage time (Figure 3.9). There was a weakly significant inverse correlation (p = 0.046) between time and A260/A230 measurements, again with a high degree of variability between samples.

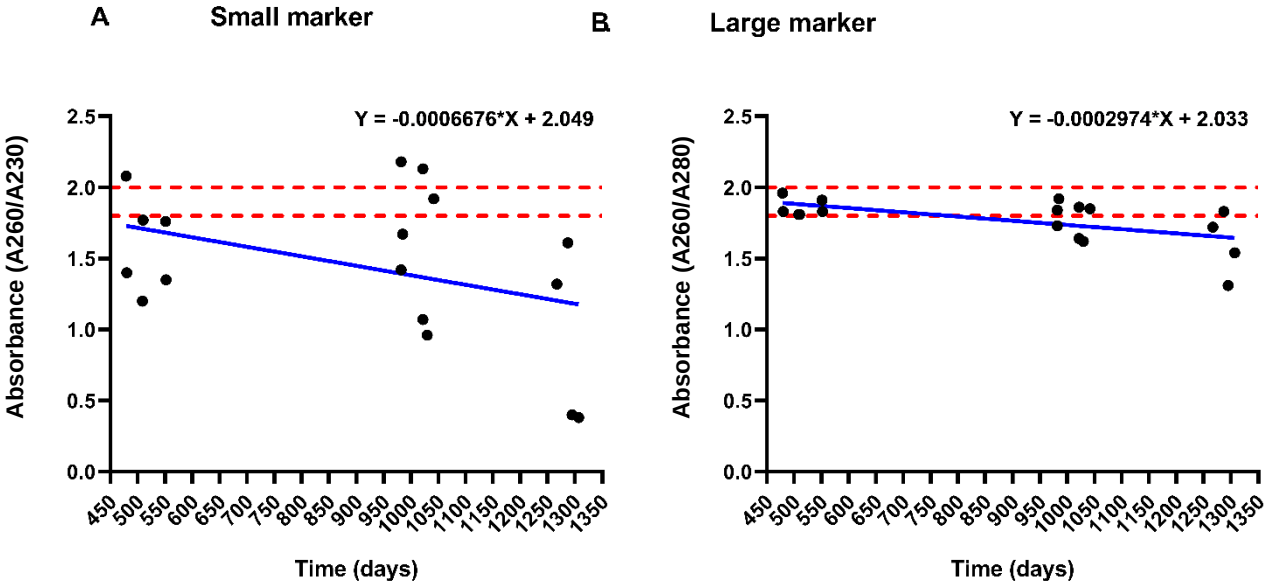

Figure 3. 10. *DNA purities for 100 FFPET sections of 1-µm thick stored for 450 to 1 350 days (1-3 years), as quantified by spectrophotometry absorbance at (A) A260/A230 and (B) A260/A280. (n=16)*

The integrity values for all FFPETs stored between one and three years were below DIN 3, indicating a high level of fragmentation. There was a slight decrease in DIN for samples stored over time (Figure 3.10.). The oldest samples had an approximate 50 % reduction in DIN than fresher samples overall. However, this slight decrease was not statistically significant.

In addition, 67 % and 45 % of cases from 2016 and 2018, respectively, had fragments greater than 400 bp in length (Appendix A.7.). However, the DNA degradation was not observed to be time-dependent.

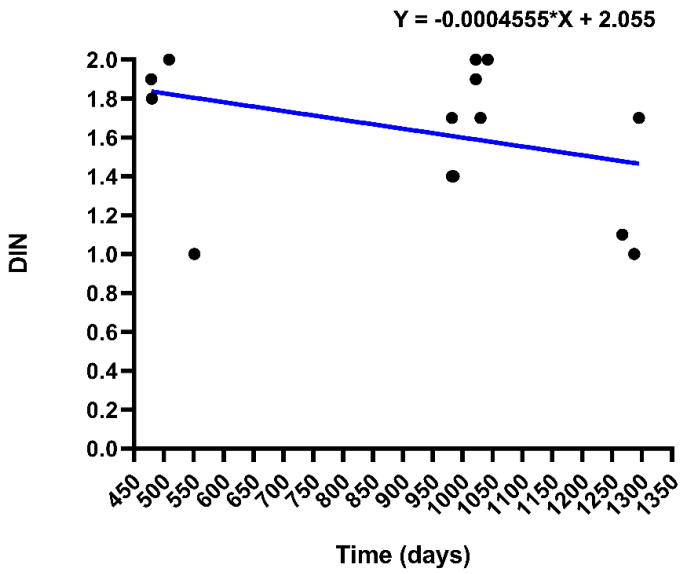

Figure 3. 11. *The effect of storage time (1-3 years) on DNA integrity number (DIN) as assessed by the Genomic DNA ScreenTape® assay for highly degraded DNA. (n=13).*

DNA recovered using the optimised sectioning parameters for FFPETs stored between one and three years did not appear to be affected by the pre-analytical conditions measured. There were no significant correlations between DNA recovery (in terms of concentrations, purities and integrities) and age or sex of the deceased. Furthermore, there was no correlation between DNA recovery the number of days between death and autopsy for specimen collection. However, due to lack of variability in the sample set and the small sample size, a comparison between death scene environmental conditions and DNA recovery could not be made.

Chapter 4: Discussion and conclusion

4.1. BURDEN OF UNRESOLVED SUD CASES

The SRM serves the West Metropole of the City of Cape Town in the Western Cape (South Africa). Upwards of 3 000 cases are received in this mortuary annually, which is relatively high as compared to most medico-legal centers in the Western Cape (de Jong, 2017).

This study showed that there was a large proportion of cases admitted to SRM under the circumstance of SUD, with approximately 40 % of these occurring in the young. The number of SUDY admissions at SRM continued to rise, as was demonstrated with the 2.3-fold increase in SUDY admittance over the 5-year study period. This is concerning, especially since investigations into SUDY cases admitted to SRM did not reveal cause of death in 26 % of cases. This may have been due, in part, to the proportional increase in the overall percentage of SUD cases admitted to SRM during the study period.

The number of unresolved SUDY cases at SRM was far greater than the range previously reported for negative autopsies in SUD of all ages nation-wide (Tiemensma, 2010; Morris et al., 2016). This might suggest the need for research into ancillary investigations that can be used to further investigate and resolve negative autopsies in SUDY at SRM, including post-mortem genetic testing for determination of cause of death (Du Toit-Prinsloo & Saayman, 2012; Etheridge & Saarel, 2014; Sanchez et al., 2016; de Jong, 2017).

However, it is of great concern that more than 50 % of all ancillary tests conducted during this study period had not been concluded and the results thereof not reported. Had this information been available, it could have been used to assist cause of death determination, ultimately reducing the number of unresolved SUDY cases. There are a number of reasons for incomplete ancillary testing, including backlogs in forensic case work and financial and staff constraints, which may extend or delay the time it takes for reports such as toxicological analyses to be returned by several months or even years (de Jong, 2017).

In South Africa, there is a shortage of genetic specialists that would be available for performing molecular autopsies on these unresolved SUD cases. This would place the additional pressure on forensic laboratories above and beyond the current burden on these institutions (du Toit-Prinsloo, 2012). Training of additional staff and equipping facilities for performing molecular autopsies would be subject to the current financial constraints and mandates of the government.

This poses a major logistical consideration that would need to be overcome before molecular autopsies can be made routine in post-mortem investigations of unresolved SUD cases.

The epidemiological information for the SUD population at SRM had not been evaluated in previous research. This study added some of the first quantitative data regarding the burden of unresolved SUD cases currently faced by SRM and highlights the need for additional research to investigate and identify risk factors in this population.

4.2. IMPROVED DNA RECOVERY

An improved method for DNA extraction from FFPETs of SUDY cases at SRM was developed. The combination of microscopic quantification of total nucleic acid content and adjusting the sectioning parameters, significantly improved DNA recovery in terms of the DNA concentrations, purities and amplification of larger fragment sizes.

The nucleic acid content per total surface area, as quantified by microscopy, was particularly useful in this study by eliminating FFPET blocks with little to no nucleic acid content, ultimately saving on time and resources. Once the appropriate FFPET block was selected for DNA extraction, optimisation of the tissue sectioning parameters, namely the thickness and number of FFPET sections, was shown to significantly improve DNA recovery.

4.2.1. Tissue thickness

The thickness of the FFPET sections used in DNA extraction was shown to have an effect on overall DNA recovery. Finer sectioning of FFPETs significantly improved DNA yield, with a median of 288 ng (36.5 ng – 856 ng) of DNA extracted from 1-µm thick sections, regardless of the number of sections used (Appendix A.6.). This represents an 8.8-fold and 26.8-fold increase from 3-µm and 5-µm sections, respectively.

The improved DNA yield with finer tissue sectioning is in accordance with the results of a previous study, which reported that sections thicker than 2-µm yielded significantly lower DNA quantities when the digestion time was less than 48 hours (Weiss et al., 2011). Authors purported that finer sectioning may improve tissue homogenisation and digestion, which in turn improves the DNA yield. Since additional factors, such as incubation time and temperature may play a role in the effect of tissue thickness on DNA yield (Weiss et al., 2011); these factors

were kept constant in this study (as informed from the literature review). It can therefore be deduced from the results of this study, that the improved DNA concentration was a direct result of finer FFPET sectioning.

In addition to the amount of DNA recovered, DNA purity was shown to improve with finer FFPET sectioning. All DNA samples extracted from 1-µm thick sections were above the threshold for pure DNA, with a median A260/A280 ratio of 1.8. This represents a 1.3-fold and 1.4-fold increase in A260/A280 ratios for 3-µm and 5-µm sections, respectively. The improvement in DNA purity with finer sectioning was more pronounced in the A260/A230 ratios, where 1-µm sections had a median ratio of 1.7 as compared to 0.7 and 0.5 for 3-µm and 5-µm sections, respectively.

Previous studies that used the QIAamp® DNA FFPE tissue kit also observed this trend with regards to DNA purity. Due to many confounding variables, it was not possible to draw direct comparisons between results from these studies, however, FFPET sections ≤ 8-µm, yielded DNA with A260/A280 ratios from 1.78 – 2.52, as compared to 10-µm thick FFPET sections which yielded DNA with A260/A280 ratios from 1.37 – 2.04 (Ludyga et al., 2012; Turashvili et al., 2012; Sengüven et al., 2014; Snow et al., 2014; Atanesyan et al., 2017; Watanabe et al., 2017; Carlsson et al., 2018). There was no indication in the literature of the effect of tissue thickness on A260/A230 ratios, as these were not commonly reported in the studies where the QIAamp® DNA FFPE tissue kit was used. Overall, these data indicated that finer tissue sectioning yielded DNA of greater purity, which is further supported by the results observed in this study.

Lastly, the thickness of FFPET sections used in DNA extraction was shown to significantly affect the DNA integrity. However, unlike DNA concentrations and purity, DIN was shown to decrease with finer sectioning. All samples used in this study had DNA integrity values below five (Figure 3.7). This result was expected since it has been established that the process of formalin fixation results in DNA fragmentation (Srinivasan, Sedmak & Jewell, 2002; Do & Dobrovic, 2015; Donczo & Guttman, 2018). When sectioning was reduced to 1-µm, the integrity values were further reduced below three. This suggested a higher degree of DNA fragmentation with finer sectioning.

However, when analysing the DIN values against the results from the digital capillary electrophoresis, it became evident that the decrease in DIN for 1-µm sections may be attributed to the larger proportion of fragments with a high molecular weight (MW). When the tissue thickness was reduced from 3-µm to 1-µm, there was a 2-fold increase in the proportion of

DNA fragments above 400 bp in size (Figure 3.10.). Since the DIN is a ratio of the concentration of fragments relative to the small fragment in the DNA ladder, the presence of large fragments actually reduces the DIN. Thus, the interpretation of DIN in the context of severely degraded tissue may need to be reconsidered.

The fragment sizes yielded from thinner sections in this study is in accordance with previous studies, which demonstrated successful amplification of fragments up to 400 bp in size (Wu et al., 2002; Lin et al., 2009; Hühns, Röpenack & Erbersdobler, 2015). While there was an apparently lower DIN (i.e. higher degree of fragmentation) in finer sections, there actually was a greater success for obtaining larger fragments than was seen for thicker FFPET sections.

Overall, by employing a DNA extraction method with tissue sectioning as thin as 1-µm, DNA recovery from FFPET was improved. As was indicated by the literature review, previous studies made use of FFPET sections between 3-µm and 50-µm. This study demonstrated that reducing the thickness of FFPET sections to 1-µm, significantly improved DNA concentrations, purities and fragment lengths.

4.2.2. Number of tissue sections

In considering the results of the research previously conducted by this research group, increasing the number of tissue sections of equal thickness was expected to improve DNA recovery from FFPETs. However, this strategy was not previously applied to FFPET sections as fine as 1-µm thick.

This study showed that there was significant variation in the DNA quantities for 1-µm thick sections, which appeared to be dependent on the number of sections used. The DNA concentrations were almost quadrupled when the number of tissue sections was just doubled (from 50 to 100 sections) (Figure 3.4.) This was expected, since increasing the number of tissue sections ultimately increases the input starting material for DNA extraction. This was supported by the comparison of two previous studies, one which yielded 1 280 ng of DNA from seven FFPET sections and another which yielded a median of 220 ng of DNA from two FFPET sections of equal thickness, using the QIAamp DNA FFPE tissue kit (Ludyga et al., 2012; Turashvili et al., 2012).

Interestingly, DNA concentrations were not improved with smaller increases in the number of FFPET sections; i.e. from 30 to 50 sections. This finding, which has not been reported in

previous literature, is an important factor to consider when assessing the eligibility of a FFPET block for molecular autopsy investigations. It was not clear from the data generated in this report, what the ideal number of tissue sections for optimal DNA recovery was. However, this data indicated that samples with extremely low DNA content would require large amounts of starting material to significantly improve DNA recovery from FFPETs. This could be a limiting factor in cases where only a small amount of FFPET is available. It may be beneficial to direct future research at further investigating the relationship between DNA yield and number of tissue sections on a more incremental level. This may be particularly useful in cases with limited FFPET, which are being considered for downstream molecular analyses.

Neither the purities nor the DNA integrities were affected by an increase in the number of tissue sections of equal thickness. Overall, there was more variability in the A260/A230 ratios, indicating less efficient removal of organic compounds or chaotropic salt contaminants. While the A260/A230 ratios were not commonly reported in studies where the QIAamp DNA FFPE tissue kit was used, two studies reported A260/A230 ratios between 1.6 and 2.58 (Ludyga et al., 2012; Turashvili et al., 2012). However, these studies did not control for tissue thickness nor any of the additional confounding factors reported, in the literature, to affect DNA purity.

While not much is known regarding the effect of FFPET sectioning on DNA integrity, it is largely reported in the literature that DNA integrity is affected by incubation times and temperatures, as it is related to DNA fragmentation (Shi et al., 2002, 2004; Dedhia et al., 2007; Gilbert et al., 2007; Huijsmans et al., 2010; Okello et al., 2010; Snow et al., 2014; Kocjan, Hošnjak & Poljak, 2015). The incubation temperatures in this study were kept constant. It would be beneficial to further optimise the method developed in this study to improve DNA integrity by adjusting the incubation times and temperatures, particularly when a large number of sections are used.

4.2.3. Storage time

The DNA concentration, purity and integrity were not significantly associated with storage time for the FFPET blocks, which had been stored for up to 3 years. This is in accordance with previous research, which reported minimal DNA degradation for samples stored for two to 47 years, including the pilot study previously conducted by this research group (Wu et al., 2002; Funabashi et al., 2012; Niland et al., 2012; Kokkat et al., 2013; Zhong et al., 2013; Nechifor-Boilă et al., 2015; Watanabe et al., 2017; Millán-Esteban et al., 2018).

The only statistically significant reduction over time was seen with the A260/A230 ratios. This would indicate less efficient removal of organic compounds or chaotropic salt contaminant in older samples. However, as was seen during the optimisation of DNA extraction, there was more variability in the A260/A230 than the A260/A280 ratios, which may have contributed to the relationship observed between purity and storage time. The sample size would need to be increased in order to make a stronger conclusion on the biological significance of this potential inverse relationship.

Overall, this study serves as a good indication on the effect of FFPET sampling on the DNA concentration, purity and integrity for samples stored up to three years for its potential use in retrospective molecular autopsies of SUDY cases at SRM. However, there is still room for improvement on the DNA recovery to meet the requirements of molecular autopsy investigations.

4.3. ELIGIBILITY FOR POSTMORTEM GENETIC TESTING

There are numerous sequencing technologies that can be employed to perform a molecular autopsy, each with associated advantages and limitations. A previous study suggested the use of a targeted approach to molecular autopsies. For instance, a four-gene panel has been identified for investigation into sudden cardiac death in the young (Semsarian, Ingles & Wilde, 2015). It is recommended to validate and confirm the presence of a genetic variant by Sanger sequencing and to use a multidisciplinary approach to interpreting the results in terms of causality to avoid false positives (Mcguire et al., 2014; Semsarian, Ingles & Wilde, 2015).

More recently, the development of massively parallel sequencing (MPS), commonly referred to as next-generation sequencing (NGS) technologies are becoming more popular for post-mortem genetic testing, as it is a high throughput system, making sequencing of genes more time- and cost-efficient (Brion et al., 2015). This technology allows for sequencing of more comprehensive gene panels and even whole-exome sequencing (WES) (Semsarian, Ingles & Wilde, 2015; Lahrouchi, Behr & Bezzina, 2016). In turn, screening for genetic variants are made easier and more robust in cases where no structural abnormalities were found at autopsy, thereby improving the likelihood of successful molecular autopsy. Hence, negative SUDY cases may benefit from NGS for cause of death determination above other strategies for molecular autopsies.

The quantity and quality of the DNA sample affects the ability to perform NGS (Brion et al., 2015; Arreaza et al., 2016). The minimum DNA quality and quantity requirements vary depending on the intended NGS application (Arreaza et al., 2016). Certain targeted DNA sequencing assays require as little as 1 – 10 ng DNA, while others require up to 200 ng DNA (Edinburgh Genomics, 2019). In this regard, by improving DNA recovery from FFPETs, the method developed in this study yielded sufficient DNA for an array of NGS applications. These results held true for samples that had been stored for up to three years (Figure 3.8.), which is a good indication that there is potential for using FFPETs in retrospective molecular autopsy investigations.

In addition to DNA quantity, certain quality criteria need to be met for successful NGS analysis. Optimally, DNA with a high molecular weight (MW) and low levels of fragmentation should be used (Arreaza et al., 2016). If the DNA does not meet these requirements, the library preparation and DNA sequencing results may be compromised. More specifically, insufficient DNA integrity can affect the read depth and cause allele dropout (Brion et al., 2015).

When using the DNA extraction method developed in this study, a greater proportion of high MW fragments were recovered. With this in mind, it appears that finer sectioning would expand the range of target genes that could be included in NGS analysis, consequently improving the success of molecular autopsy. In certain instances, the DNA size requirements can be adjusted if there is sufficient DNA and if the amplicon of interest is shorter than the smallest fragment in the sample (Corcoll et al., 2017; Edinburgh Genomics, 2019). This information can be used to assist in selecting appropriate markers and sequencing technologies for molecular autopsy. Taking the results from this study into consideration, it is recommended that if sequencing of fragments larger than 400 bp is required, it would be beneficial to reduce the thickness of FFPET sectioning as low as possible (in our laboratory, 1-µm), thereby improving the yield of larger DNA fragments.

It may be worthwhile to explore ways to further enhance the proportion of DNA with a high MW, as this would improve DIN values for DNA extracted from FFPETs. Previous studies have reported a correlation between the DIN and NGS quality criteria. The coverage and on-target sequencing for WES using the HiSeq 2500, 300 and 4000 systems were reportedly reduced for samples with DIN values below 3 (Petersen et al., 2016; Jung, Ji & Schmidt, 2017). The High Throughput Sequencing Unit at the German Cancer Research Center has established a threshold of DIN 7. If samples are below this threshold, there is no guarantee of successful library preparation when using the Agilent SureSelect^{XT} automated workflow (Jung, Ji &

Schmidt, 2017). However, another study, which reported a direct correlation between DIN and NGS library functionality showed that samples with a DIN value as low as 2.05 produced functional libraries (Millán-Esteban et al., 2018). When using the DNA extraction method developed in this study, all FFEPTs yielded DNA with DIN below 3. This highlights the need for further optimisation of DNA extraction to improve the DIN values for DNA extracted from FFPETs.

In addition to the variables tested in this study, there are a number of pre-analytical variables which could contribute to DNA quality. It has been hypothesised that age, sex, death scene environmental factors and time between death and tissue collection play a role in DNA degradation, consequently reducing DNA integrity (Niland et al., 2012; Bass et al., 2014; Reid, Martin & Heathfield, 2019). In this study, there were no significant differences between these demographic variables and the DNA metrics that were measured. Hence, no inferences could be made on the effect of these variables on DNA recovery from FFPETs, but this should be assessed in future with a larger sample size.

The time interval between death and autopsy for samples stored since 2016 and 2018 were 3 – 5 days and 1 – 9 days, respectively (Appendix A.5.). There was no clear indication that the variation in this time interval affected the DNA concentration or quality. Certain samples with 1- and 2- day intervals had the highest DIN values and DNA concentrations, while other samples with 2-day intervals had poor DIN readings and low DNA concentrations. The only sample with a 9-day interval had a relatively high DIN value and DNA concentration as compared to samples with a 1-day interval. Therefore, it is unlikely that the difference in time interval had much of an effect on the DNA quality or quantity reported in this study.

It was hypothesised by Reid et al. (2019) that the combination of a number of post-mortem variables, such as the time between death to specimen collection and death scene environmental factors, have a collaborative effect on DNA integrity (Reid, Martin & Heathfield, 2019). The majority of FFPETs used in this study were collected from cases where the deceased was exposed to an indoor environment at death. By virtue of being enclosed structures, indoor scenes typically offer more protection against these environmental factors (Reid, Martin & Heathfield, 2019). Due to the lack of variability in the samples in this study, there was insufficient data to identify the effect of environmental conditions on DNA integrity. A larger sample size and more diverse sample set would be required to investigate this further.

4.4. ETHICAL, LEGAL & LOGISTIC CONSIDERATIONS

Despite its success and range of applications in demonstrated research, molecular autopsies are not currently performed as part of forensic post-mortem investigations in South Africa. Several legal, ethical and logistical issues need to be considered before molecular autopsies can be performed or can become routine in a medico-legal setting in South Africa. The use of genetic material in itself poses many ethical concerns regarding consent, confidentiality and reporting of the results. These concerns are then further complicated when considering genetic testing on deceased individuals who by their nature are not able to provide consent. Thus, the onus may fall on the next-of-kin to make decisions on behalf of the deceased (McGuire et al., 2014; Moore et al, 2016,). However, it can be argued that in order to prevent the relatives of the deceased from obstructing the progress of a forensic investigation, that consent should not be required for post-mortem genetic testing (de Vaal, 2018). Ultimately, a balance needs to be found so as to not violate the family's expectation of control over the handling of the deceased while still allowing the forensic investigation to proceed without any hindrances (McGuire et al., 2014).

The concept of informed consent and confidentiality may further be complicated by the fact that DNA is inherently familial and results from the molecular autopsy may reveal hereditary diseases that could affect the family members of the deceased. This poses questions as to whether the results from the molecular autopsy should be revealed to those family members and whether they should receive genetic counselling regarding their susceptibility to the hereditary condition (McGuire et al., 2014; Moore et al, 2016). While there are currently no regulations which govern these decisions, careful consideration must be taken in order to perform these investigations in an ethical manner. On one hand, there is justification in the favour of public health to perform genetic testing on at-risk individuals, however, in-depth discussions need to be had surrounding the potentially negative emotional impact it may have on family members of the deceased (McGuire et al., 2014; Moore et al, 2016, Heathfield, et al., 2017).

Furthermore, there are concerns regarding the use of genetic testing in sensitive cases such as infant deaths or suicides (Heathfield, et al., 2017; Clarke, A. & Wallgren-Pettersson, C., 2018). Despite evidence that a genetic predisposition can be determined for these cases, there is concern in attributing a disease or genetic disposition when the penetrance is not well understood. The mere presence of gene or genetic mutation does not automatically indicate the expression of a dysfunctional trait or condition (Clarke, A. & Wallgren-Pettersson, C., 2018).

Hence, results from a molecular autopsy would merely indicate the probability of an individual exhibiting a particular trait or disease. In certain illnesses, the gene is always or very highly associated with the expression of a particular trait. However, in other instances, the gene is either lowly expressed or the expression probability is not known (Heathfield, et al., 2017; Clarke, A. & Wallgren-Pettersson, C., 2018). This again emphasises the need for specialist interpretation of results as misinterpretation can render the results would have little to no value.

4.5. LIMITATIONS

The strict selection criteria and the unavailability of FFPETs from certain ongoing forensic cases seemed to limit the sample size for this study. In future, it would be valuable to increase the sample size to reduce the possibility of type II error and strengthen the statistical significance of the results. A larger sample size would further allow for assessment of the biological relevance of results observed, such as the inverse correlation between time and A260/A230 ratios. This would provide a clearer understanding of the implications of various factors on DNA recovery.

There was little variability in the sample set with regards to the pre-analytical conditions such as age, sex and post-mortem time and environmental conditions. The absence of this information in the case files may have contributed to the lack of variability in the samples. The clinical history and family pedigrees, pathological data from the post-mortem and ancillary investigations and case context are all critical information for assessing the eligibility of a case for molecular autopsy (Semsarian, Ingles & Wilde, 2015; Cunningham, 2017). Therefore, the lack of information further complicates the ability to perform molecular autopsy investigations.

4.6. CONCLUSION

The combination of tissue screening by microscopy and the optimisation of DNA extraction by adjusting the sectioning parameters introduced here was a simple and effective method for improving DNA recovery.

Microscopic quantification of total nucleic acid content greatly improved the ability to identify FFPET blocks suitable for downstream DNA analyses. This was particularly useful for samples with little to no nucleic acid content. In these instances, microscopic quantification allowed for exclusion of samples for DNA extraction, thereby saving time and resources.

The sectioning parameters, namely, thickness and number of FFPET sections, were important factors in improving DNA recovery. Finer tissue sectioning enhanced the DNA concentration, purity and success of PCR-amplification of high MW fragments. Through the combination of finer tissue sectioning and an increased number of tissue sections, this study contributed to improving the DNA extraction method at for FFPET samples at SRM that had been stored for up to three years. This data provided insight into the quality and quantity of DNA we can gain from this method and insight into the eligibility for downstream molecular assays, such as NGS analysis. An improvement in the quality and quantity of DNA extracted from FFEPTs may have major implications in the investigation into unresolved SUDY cases by enhancing the ability to perform molecular autopsies for cause of death determination.

In addition to the quantitative criteria for performing molecular autopsies, thorough discussions into the ethical, legal and logistic aspects would be required before molecular autopsies can be introduced as a forensic investigative tool.

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Appendices

A.1. JOURNAL ARTICLES INCLUDED IN LITERATURE REVIEW

Table A.1.1. *Chronological literature review of studies aimed at optimising DNA extraction to improve DNA recovery from FFPET. The variables under investigation in each study are indicated with symbols, □: tissue sampling; ○: tissue preparation; ▲: tissue digestion and ■: purification method. The DNA extraction kit used was indicated as follows:*

1: QIAamp® DNA Minikit (Qiagen); 2: HighPure DNA Preparation kit (Roche Life Science); 3: Ultraclean® Bloodspin® kit (Mo Bio Laboratories); 4: QIAamp® DNA Micro kit (Qiagen); 5: DNeasy Tissue kit (Qiagen); 6: NucleoSpin® Tissue kit (Macherey Nagel); 7: EasyMAG nucliSens (bioMérieux); 8: Gentra column-kit (Qiagen); 9: GenElute® Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich); 10: TRI-Reagent® solution-DNA (Sigma-Aldrich); 11: RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific); 12: QIAamp DNA FFPE tissue kit (Qiagen); 13: AllPrep® DNA/RNA Mini Kit (Qiagen); 14: AllPrep® FFPE DNA/RNA Mini Kit (Qiagen); 15: Maxwell® 16 FFPE Tissue LEV DNA kit (Promega); 16: FFPE DNA extraction kit (Norgen Biotek); 17: Waxfree DNA kit (Trimgen); 18: RecoverAll DNA kit (Thermo Fisher Scientific); 19: Blood and Tissue kit (Qiagen); 20: Pinpoint Slide DNA Isolation System™ 20 (Zymo Research); 21: MasterPure™ DNA Purification Kit (Lucigen); 22: ZR FFPE DNA MiniPrep™ (Zymo Research); 23: GeneRead DNA FFPE Kit (Qiagen); 24: Agencourt FormaPure Kit (Beckman Coulter).

Year	Article	Tissue sampling (□)			Tissue preparation (○)				Tissue digestion (▲)			Purification method (■)		Effect on DNA quality and quantity
		Scrape	Core	Section	Xylene	Organic solvent	Heat	Pressure	<55°C	55-65°C	>65°C	In-house	Kit	
1999	(Coombs, Gough & Primrose, 1999)			✓	✓		✓			✓		✓	✓ 1	○ ■ PCR success • Chelex-100 was the most successful compared to simple boiling and PCI for heat-deparaffinisation. • Thermal cycling was most successful compared to microwaving and xylene. • The kit had similar success (60%) to the Chelex-100 + thermal cycling combination (61.3%).
2002	(Shi et al., 2002)			✓	✓		✓		✓	✓	✓	✓		○ ▲ DNA concentration • Higher temperatures improved yield (dependent on pH). Purity • Quality ratios were highest for 120°C-heated samples. PCR success • Greatest PCR success with 120°C-heated samples (pH 10-12).

Table A.1.1. *(continued).*

2002	(Wu et al., 2002)			✓			✓				✓		✓ 1; 2	○ ■ PCR success • PCR efficiency (90-386 bp) was 3-8X more for heated than unheated samples. • 60 % success for heated samples stored up to 8 yrs.
2004	(Shi et al., 2004)			✓			✓				✓	✓		▲ ○ DNA concentration • Higher temperatures had higher DNA yields. PCR success • Higher temperatures (100-120°C) improved PCR success (up to 600 bp).
2007	(Dedhia et al., 2007)			✓	✓					✓	✓	✓	✓ 1	▲ ■ DNA concentration • Heated samples had the highest DNA yield (160-211 µg) • The PCI method had the lowest DNA yield of 9 µg – 13 µg Purity • NanoDrop™ quality ratios for heated samples were sufficient for qPCR, but the modified QiaAmp method had the best quality ratios. PCR success • 100 % success for heat-treated samples (105 bp and 245 bp, but and 0% success for 653 bp).
2007	(Gilbert et al., 2007)			✓	✓	✓				✓	✓	✓	✓ 4	○ ▲ ■ DNA concentration • Deparaffinisation (xylene vs pentane) had no effect on DNA quantity • Increasing temperature of digestion incubation has no effect on DNA yield PCR success • Deparaffinisation had no effect on PCR success • 48 hour tissue digestion had an 8X increased PCR success than 24 hours • Increased temperatures (65°C vs 55°C) increase the PCR success, but not in all cases – highly variable • The kit had a higher success rate than organic extraction method

Table A.1.1. *(continued).*

2008	(Bukhari et al., 2008)	✓		✓	✓				✓			✓		□ PCR success • PCR success was greater for DNA from microtome sections (96%) vs scraping (93.75%) – result not statistically significant
2010	(Bonin et al., 2010)			✓	-	-	-	-	✓	✓		✓	✓ 4;5 ;6	■ DNA concentration • In-house methods had higher yields than kits. Purity • In-house methods had the higher levels of RNA and protein contamination than the kits. PCR success • Differences in quality measurements did not affect the PCR success.
2010	(Huijsmans et al., 2010)			✓		✓			✓			✓	✓ 1;7 ;8	▲ ■ DNA concentration • QIAamp kit had the highest yield PCR success • Inhibition in Gentra kit and in-house heat treatment • 100% success rate for all extraction methods except Gentra kit (400 bp) • 75% success rate for QIAamp and EasyMAG kits and 100% success rate for heat-treatment, but with low yields (600 bp)
2010	(Okello et al., 2010)	✓			✓	✓			✓	✓		✓	✓ 9;1 0;1 1	▲ ■ DNA concentration • Extremely low DNA yield (0.17 ng/µl) in TRD kit compared to GEN (2,42 ng/µl) and RAD (4,17 ng/µl) kits and in-house PCE (5,47 ng/µl). However, there was great variability in across the samples. • Longer incubation times led to an increase in DNA yield PCR success • Most PCR inhibition with WXF kit • Longer incubation times led to an increase in PCR inhibition

Table A.1.1. *(continued).*

2012	(Chung et al., 2012)		✓	✓		✓	✓	✓		✓		✓	✓ 1 2	□ ○ ■ DNA concentration • 181% increased yield for PCI compared to QIAamp kit – increase of 1.7X (cores) and 1.9X (sections). • Significantly higher DNA yield per tissue volume for cores compared to sections. Purity • Larger fragments extracted with QIAamp than PCI. • DNA purity was similar for pressure cooking and conventional PCI.
2012	(Funabashi et al., 2012)			✓	✓					✓		✓	✓ 1	■ DNA concentration • Significantly greater yield for PCE than for kit and salting out methods for up to 5 years (liver, spleen and brain) Purity • PCE had lower purity than the kit and salting out methods • Salting out contained the most impurities • Brain>liver>spleen PCR success • Recent samples showed higher intensities on an agarose gel than those stored for 1 and 5 years • Successful PCR of AMEL for kit only
2012	(Khokhar et al., 2012)			✓									✓ 13; 14; 15	■ DNA concentration • QIAamp FFPE kit yielded significantly more DNA than the other 2 kits Purity • All kits yielded pure DNA PCR success • 100% success rate for all kits for 268 bp fragment • Maxwell kit had the highest and most consistent success for 402 bp fragment • Poor success for all kits for 618 bp fragment - AllPrep FFPE DNA/RNA Mini Kit had the best performance (20%)

Table A.1.1. *(continued).*

2012	(Ludyga et al., 2012)			✓	✓					✓		✓	✓ 12; 16	■ DNA concentration • PCI had the highest DNA yield – but with high levels of variation between replicates DNA degradation • Archival tissue showed high levels of fragmentation, which was not seen in recently prepared FFPET Purity • Highest purity obtained with PCI and QIAamp kit PCR success • Highest PCR success with PCI, but with great variability
2012	(Turashvili et al., 2012)			✓	✓	✓			✓	✓			✓ 12; 17; 18	■ DNA concentration • Waxfree kit had the greatest yield followed by QIAamp, in-house PCI and RecoverAll kit DNA degradation Purity • Waxfree kit had the lowest purity PCR success • Waxfree had the highest success
2013	(Paireder et al., 2013)	✓								✓		✓	✓ 1;12	■ DNA concentration ✓ Greatest yields achieved with the QIAamp FFPE DNA kit compared to the mini kit and in-house CTAB methods DNA degradation ✓ DNA from all extractions were partly degraded
2013	(Wang et al., 2013)			✓		✓				✓		✓	✓ 4	■ DNA concentration • Genomic DNA extracted for ~67% cases using QIAamp – none for lab-based PCR success • 76% success rate for the kit compared to 33% for the in-house method (257 bp)

Table A.1.1. *(continued).*

2013	(Zhong et al., 2013)	✓			✓			✓					✓ 19	DNA concentration: • Linear increase in DNA concentration as the number of tissue sections increased when pressure cooked for between 5 and 30 mins • There was no significant difference in quantity when proK was added Purity • Better A260/A280 quality ratios for pressure cooking as compared to xylene deparaffinisation PCR success • 100% Successful amplification of 193 bp fragments for FFPET stored between 0.2 – 20 years • 21 % success for amplification of 546 bp for tissues stored up to 9 years
2014	(Snow et al., 2014)	✓	✓		✓					✓			✓ 12; 20	■ DNA concentration • No significant difference when using different sampling methods • QIAamp had a 4.6X increased yield compared to Zymo column extraction kit • Longer proK digestion had no effect on yield Purity • No difference between the two methods
2015	(Nechifor-Boilă et al., 2015)			✓	✓					✓			✓ 2 1	DNA concentration & Purity: • No difference for tissues stored 2 - 8 years

Table A.1.1. *(continued).*

2016	(Laghedeen, Eklund, Kleppe, Unger, Dillner, et al., 2016)			✓	✓		✓		✓	✓		✓ 19	○ PCR success • 16 % increased success with using heat as compared to xylene • Less PCR inhibition when using heat (2 %) as compared to xylene (18 %)
2017	(Atanesyan et al., 2017)			✓		✓	✓		✓	✓		✓	✓ 11; 12; 17; 22	■ DNA concentration • Highest yield with the in-house method followed by the QIAamp, Waxfree, Zymo research & RecoverAll Total Nucleic Acid kits Purity • All kits had pure DNA • Increased DNA purity with increased incubation temperature
2017	(Oskina et al., 2017)			✓		✓	✓			✓		✓	✓ 2; 12	■ DNA concentration • Higher yield with less variability in commercial kits compared to in-house methods DNA degradation • DNA from all kits were highly degraded Purity • Roche kit had the highest PCR inhibitors and the in-house method had the least PCR success • For 217 bp fragments, QIAgen had the lowest success rate and the in-house had the highest – not statistically significant

Table A.1.1. *(continued).*

2017	(Watanabe et al., 2017)			✓		✓				✓			✓ 12; 17	■ DNA concentration • Yield between 2.5 - 10X higher for Waxfree method as compared to QIAamp • QIAamp had a higher yield for fragments between 129 - 305 bp than the Waxfree • No difference for samples stored up to 12 years DNA degradation • Waxfree DNA was more fragmented than the QIAamp • Age-dependant DNA fragmentation - decrease between 0.5-3 years and 9-12 years Purity • Purity was higher for QIAamp than the Waxfree • No difference in samples stored up to 12 years
2018	(Carlsson et al., 2018)	✓											✓ 2; 12; 14;	■ DNA concentration • Roche and Allprep kits had similar yields to QIAamp Purity • QIAamp had higher purity than Roche and Allprep kits
2018	(Kresse et al., 2018)			✓									✓ 14; 23; 12; 24;	■ DNA concentration • truXTRAC kit had the highest yield, but the most variation • there was no difference in yield when comparing the three Qiagen kits DNA degradation • truXTRAC kit had the highest DIN values • There was no difference between truXTRAC kit and QIAamp DIN. • Lowest integrity was given for the GeneRead kit PCR success • truXTRAC kit had better amplificability
	Total	8	2	21	15	9	8	2	8	20	5	17	24	28

A.2. OFFICIAL ETHICS APPROVAL LETTER

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
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Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

11 June 2019

HREC REF: 211/2019

Ms L Heathfield
Division of Forensic Medicine & Toxicology
Reception, Entrance 3, level 1
Falmouth Building-FHS

Dear Ms Heathfield

PROJECT TITLE: OPTIMISATION OF DNA RECOVERY FROM FORMALIN FIXED PARAFFIN EMBEDDED TISSUE OF UNRESOLVED SUDDEN UNEXPECTED DEATH CASES. (MPHIL CANDIDATE: MS R VILJOEN)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 June 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Ms Rabia Viljoen will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

Signature Removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

A.3. SUD CASE ADMITTANCE

Table A. 3. 1. *Total SUD and SUDY cases admitted to Salt River mortuary between 2014 and 2018.*

Year	Number of cases	Number of SUD cases	Number of SUDY cases
2014	3461	590	138
2015	3692	627	172
2016	3657	727	203
2017	3885	1106	334
2018	4043	1107	321
Total	11585	2940	1168
Average	3747.6	831.4	233.6

Table A. 3. 2. *Categorisation of cause of death for SUDY cases as determined by post-mortem investigation and the number of cases where histology specimens were taken at Salt River mortuary between 2014 and 2018.*

Year	Natural		Unnatural		Undetermined	
	No histology	Histology	No histology	Histology	No histology	Histology
2018	215	54	14	6	92	79
2017	250	44	13	2	67	52
2016	124	26	8	1	70	58
2015	124	24	8	0	40	30
2014	93	22	5	1	36	17
Total	806	170	48	10	305	236
Average	57.04	12	3.12	1.6	18.4	18.96

A.4. H&E STAINING PROTOCOL

Following heat fixation of tissue sections to a glass slide, proceed to H&E staining as follows

Rehydration

1. Immerse the slide in xylene for 5 minutes
2. Dip slide in 100 % ethanol ten times
3. Slowly dip the slide in 96 % ethanol ten times
4. Immerse the slide in 70 % ethanol supplemented with 3 % ammonium for 30 minutes

Staining

5. Dip the slide in 1 % acetic acid 6 times
6. Wash the excess acetic acid off in a tap water bath for 5 minutes
7. Immerse in Haematoxylin for 10 minutes
8. Wash off the excess Haematoxylin in a tap water bath for 1 minute
9. Blue in Scott's water for 1 minute
10. Immerse in Eosin-phloxine for 5 minutes
11. Briefly rinse of excess Eosin in a tap water bath

Dehydration

12. Dip in 70 % ethanol 3 times
13. Dip in 90 % ethanol 3 times
14. Dip in 100 % ethanol 10 times (repeat this step twice more in fresh ethanol)
15. Clear in xylol by dipping 10 times (repeat this step twice more in fresh xylol)

Place a cover slip on the slide using mounting media.

A.5. CASE REVIEW DATA FOR SAMPLES

Table A. 5. 1. *Database of pre-analytical variables collected from the Office Autopsy Database at Salt River Mortuary for cases included in the control and test samples. Tissue types were selected based on a quality score obtained from microscopic quantification of the total nucleic acid content per FFPET section.*

Sample	Autopsy date	Death date	Days between death & autopsy	Sex	Age (years)	Ancestry	Tissue type	Death scene environment	Reported cause of death
Control 1	2018/04/30	2018/04/25	5	M	39	African	Lung/brain	Inside	Liver pathology
Control 2	2018/03/27	2018/03/24	3	F	34	African	Lung/brain	Inside	Hypertensive cerebral haemorrhage
Control 3	2018/02/12	2018/02/08	4	M	23	African	Lung/brain	Unspecified	Heat stroke
Test 1	2016/12/07	2016/12/03	4	F	16	African	Lung	Inside	Under investigation
Test 2	2016/12/06	2016/12/02	4	F	30	coloured	Kidney	Inside	Under investigation
Test 3	2016/12/01	2016/11/28	3	F	23	African	Brain	Inside	Under investigation
Test 4	2016/10/26	2016/10/21	5	M	16	African	Kidney	Inside	Under investigation
Test 5	2016/10/21	2016/10/20	1	F	14	African	Kidney	Inside	Under investigation
Test 6	2016/10/13	2016/10/11	2	M	37	African	Brain	Inside	Under investigation
Test 7	2016/10/07	2016/09/28	9	M	39	coloured	Kidney	Unspecified	Under investigation
Test 8	2016/03/08	2016/03/05	3	M	21	African	Kidney	Outside	Under investigation
Test 9	2016/02/16	2016/02/14	2	F	33	African	Brain	Inside	Under investigation
Test 10	2016/01/26	2016/01/24	2	F	40	coloured	Liver	Inside	Under investigation
Test 11	2016/01/19	2016/01/15	4	F	18	African	Brain	Inside	Under investigation
Test 12	2016/01/05	2016/01/02	3	M	32	African	Kidney	Inside	Under investigation

A.6. DNA RECOVERY

Table A. 6. 1. *The median DNA recovery for FFPET sections of varying thickness. 5-µm and 3-µm samples were paired (n=3) for optimisation. Yield was quantified by qPCR amplification of the (S) small and (L) markers, purity was assessed by spectrophotometry and DIN assessed by digital capillary electrophoresis.*

Tissue section			DNA yield				DNA purity		DNA integrity
Thick (µm)	**No.**	**Nuclei (per section)**	**S concentration (ng/µl)**	**L concentration (ng/µl)**	**S mass (ng)**	**L mass (ng)**	**A260/ A230**	**A260/ A280**	**DIN**
5	5	62300	0.1083	0.0010	5.42	0.05	0.34	1.35	4.3
	10	62300	0.32	0.01	16.08	0.5	0.61	1.24	3.9
	All	62300	0.2150	0.0042	10.75	0.21	0.48	1.29	4.2
3	5	62300	0.63	0.01	31.30	0.5	0.58	1.46	3.8
	10	62300	0.8978	0.0135	44.89	0.68	1.11	1.62	2.1
	All	62300	0.6580	0.0091	32.91	0.46	0.69	1.46	2.5
1	30	56700	5.06	0.1479	253	7.395	2.32	1.88	1.8
	50	56700	4.821	0.1717	241	8.585	1.73	1.87	1.8
	100	86067	17.90	0.4363	895	21.82	1.52	1.83	1.7
	All	71011	5.766	0.1627	288	8.36	1.67	1.84	1.8

A.7. DIGITAL CAPILLARY ELECTROPHORESIS

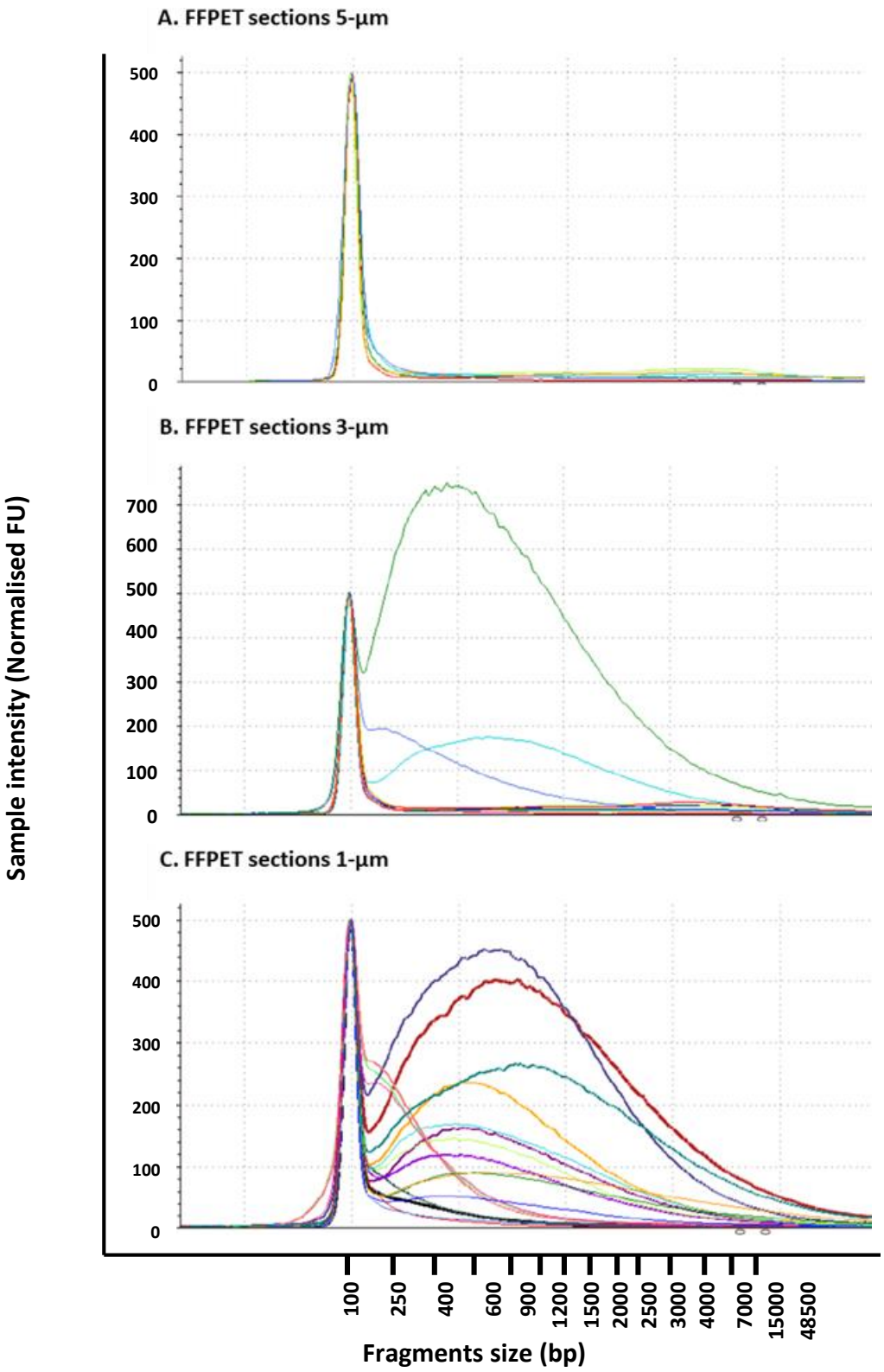

Figure A. 7. 1. *Distribution of fragment lengths for DNA extracted from FFPETs of varying thickness as quantified by the TapeStation System with the Genomic DNA ScreenTape® assay for highly degraded DNA. FFPET blocks were sectioned to (A): 5-µm (B): 3-µm and (C): 1-µm thick.*

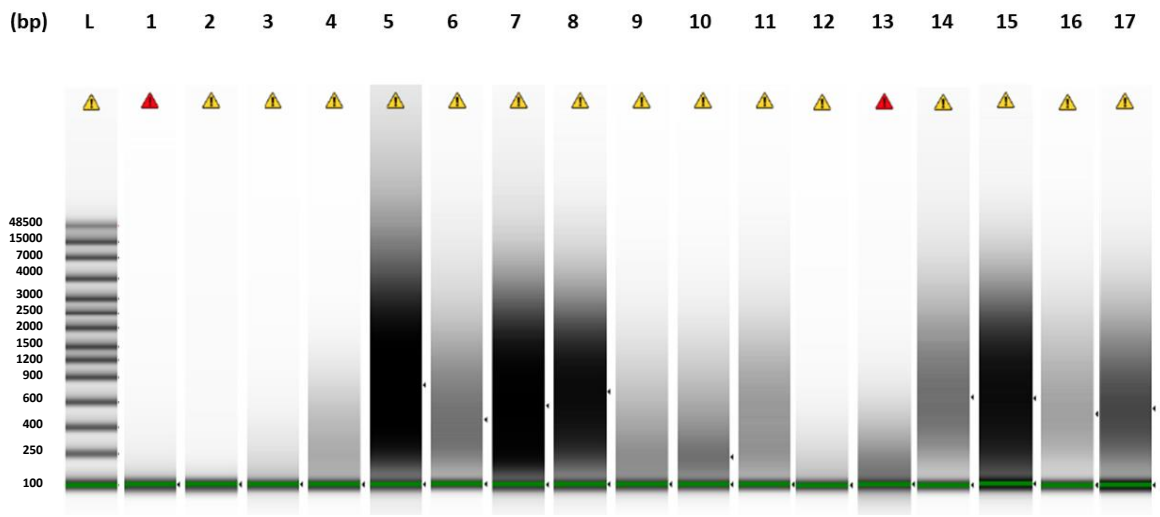

Figure A. 7. 2. *Digital capillary electrophoresis of DNA extracted from FFPET stored for 1-3 years using the 4200 TapeStation System with the Genomic DNA ScreenTape® assay. Samples are listed in chronological order as follows:*

Lane L: D1000 molecular weight marker (ladder).

Lanes 1-11: test samples stored for between 2 and 3 years.

Lanes 12-17: control samples stored for between 1 and 2 years.