Towards molecular autopsies: Internal validation of the Qubit™ 1X dsDNA HS Assay Kit

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Abstract

Molecular autopsies are post-mortem genetic analyses that can be used to aid in cause of death determination, especially in sudden unexpected death cases. Molecular autopsies involve analysing the decedent's DNA to identify potential pathogenic genetic variants. Accurate quantification of the DNA and associated sequencing libraries is essential, however, no validation studies have been published on the recommended fluorometric quantification methods. The aim of this study was to internally validate the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 Fluorometer as part of a molecular autopsy workflow for forensic applications. The Qubit™ 1X dsDNA HS Assay workflow was optimised and then used to assess the DNA concentration of control Lambda DNA, extracted DNA from forensic samples and DNA sequencing libraries. The accuracy, precision, dynamic range, and sensitivity were established in accordance with ISO 17025 standards. All parameters met the manufacturer's criteria of acceptance except for the precision of measurements for samples with DNA concentrations ≥ 0.5 ng/µl which were expected to be < 1% CV. The precision of the measurements (1.54% CV – 2.47 % CV), however, was deemed acceptable for our laboratory, as downstream DNA sequencing results surpassed quality thresholds. Additionally, DNA concentration measurements obtained from this DNA quantification workflow were similar to those obtained from other methods that have previously been validated in our laboratory. Overall, the Qubit™ 1X dsDNA HS Assay Kit was considered internally validated for DNA quantification in our laboratory and deemed fit for purpose. This study has enabled the use of this assay in the forensic setting for the first time and has advanced our progression towards implementing a molecular autopsy sequencing workflow in South Africa.

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Abbreviations

% CV	percentage coefficient of variation
COD	cause of death
CYP3A4	cytochrome P450 3A4
CYP3A5	cytochrome P450 3A5
ddNTP	dideoxyribonucleotide triphosphates
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
FFPE	formalin fixed paraffin embedded
HREC	Human Research Ethics Committee
HS	high sensitivity
ICC	intra-class correlation coefficient
ISO	International Organisation for Standardisation
KVLQT1	gene encoding a potassium channel protein
LOA	limit of agreement

LQTS	long-QT syndrome
M1 – M4	measurement 1 to measurement 4
M2 – M4	measurement 2 to measurement 4
M1 – M3	measurement 1 to measurement 3
MFRG	Molecular Forensic Research Group
MPS	massively parallel sequencing
NGS	next generation sequencing
OFPI	Observatory Forensic Pathology Institute
QMS	quality management system
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RYR2	gene encoding the ryanodine receptor 2 protein
SCN5A	gene encoding sodium channel proteins
SNV	single nucleotide variant
SOP	standard operating procedure
SUD	sudden unexpected death
SUDI	sudden unexpected death of infant

TE	Tris-EDTA
UCT	University of Cape Town
WES	whole exome sequencing
WGS	whole genome sequencing
WHO	World Health Organization

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Chapter 1: Introduction

1.1 Background

The medico-legal investigation into determining the cause of unnatural deaths is a requirement in most countries and in South Africa is mandated by the Inquests Act 58 of 1959 and the National Health Act 61 of 2003 (South Africa, 1959; South Africa, 2003). During a post-mortem investigation, samples may be obtained for ancillary analyses to provide insight into the cause of death diagnosis. The results from these analyses may form evidence and be subjected to scrutiny in a court of law. To ensure admissibility of such evidence in court and reliability of results, it is imperative that scientific analyses used in the forensic context are commensurate with international benchmarks for science and quality (*Frye v. United States*, 1923; *Daubert v. Merrell Dow Pharmaceuticals, Inc*, 1993; International Organization for Standardization, 2017). Method validation is therefore an important part of implementing workflows in a forensic setting.

The Observatory Forensic Pathology Institute (OPFI) is a state-of-the-art mortuary facility in Cape Town, which aims to holistically address medico-legal death investigation using an inter-disciplinary approach. A new ancillary investigation that will be offered in the OFPI is a molecular autopsy, to investigate genetic variants that may contribute to cause of death (COD) diagnosis. In preparation for this, the workflow needs to be researched, developed, optimised and validated.

This study focuses on the internal validation of one component of the larger molecular autopsy workflow; that is the use of the Qubit™ 1X double-stranded DNA (dsDNA) High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, Waltham, USA, Catalogue no. Q33230) (hereon referred to as Qubit™ Assay) to quantify DNA (and its derivatives) on the Qubit™ 4

Fluorometer (Thermo Fisher Scientific, USA, Catalogue no. Q33238) (hereon referred to as Fluorometer). The Qubit™ Assay fits into the larger molecular autopsy workflow which requires a fluorometric method to quantify 'libraries', which are collections of DNA fragments, for downstream DNA sequencing. This chapter will provide a theoretical background of molecular autopsies, sequencing techniques and DNA quantification methods, with a focus on the Qubit™ Assay. This will be followed by a motivation for the internal validation of the Qubit™ Assay and finally, the aim and objectives will be presented.

1.2 Molecular autopsies

Molecular autopsies are post-mortem genetic analyses that have been carried out since the late 1990s and are used to aid in COD determination, especially in cases where a full medico-legal autopsy was negative, and a COD was undetermined (Ackerman, Tester & Driscoll, 2001; Ackerman *et al.*, 1999; Martínez-Barrios *et al.*, 2023). The World Health Organisation (WHO) defines COD as the disease or injury that results in death. In South Africa, medico-legal autopsies are carried out by forensic pathologists for all deaths that are believed to be unnatural, and the findings thereof play an important role in determining the sequence of events that may have caused or contributed to an individual's death (Eriksson, 2016).

Molecular autopsies are conducted using forensic samples that have been collected during the decedent's autopsy. Blood, buccal swabs, bone, teeth, nail, and formalin fixed paraffin embedded (FFPE) tissue are all forensic samples that have been used as sources of DNA for molecular autopsies (Grassi *et al.*, 2023; Martínez-Barrios *et al.*, 2023). A review conducted by Heathfield, Martin and Ramesar (2019) found that the sample types used for molecular autopsies in sudden unexpected death of infant (SUDI) cases were blood or dried blood spots

(62.96% of cases), tissue (25.93% of cases), frozen tissue (11.11% of cases) or FFPE tissue (11.11% of cases). Blood is considered the gold standard sample type for DNA analyses (Middleton *et al.*, 2013); however, sometimes alternative samples are used as molecular autopsies may only be conducted years after the forensic autopsy due to laboratory backlogs or the molecular autopsy only being conducted retrospectively (Reid *et al.*, 2017; Viljoen *et al.*, 2022).

Additionally, molecular autopsies are not routinely performed in South Africa as the workflow is still being developed for standard practices. Furthermore, there is a lack of resources for molecular autopsies to be conducted routinely so they are performed when the need arises. In such cases FFPE tissue is often the only stored sample type available (Castiglione *et al.*, 2021; Grassi *et al.*, 2023). Numerous studies have successfully carried out molecular autopsies using archived tissue years after they were collected at autopsy (Ackerman, Tester & Driscoll, 2011; Dewar *et al*., 2017; Farrugia *et al*., 2015; Heathfield, Martin & Ramesar, 2018; Neubauer *et al*., 2017).

Molecular autopsies entail extracting DNA from collected forensic samples and analysing the deceased individual's DNA to identify any genetic variants that may be present (Figure 1.1) (Castiglione *et al.*, 2021). The variants are assessed to determine if they could have caused or contributed to the pathology which resulted in death (Martínez-Barrios *et al.*, 2023). Variants can broadly be defined according to their size as well as how they were formed (Nesta, Tafur & Beck, 2021). Variants include single nucleotide variants (SNVs), insertions, deletions, duplications, inversions and translocations. The variants can occur at a single base pair (e.g. SNVs) or they can include many base pairs (e.g. translocation) (Nesta, Tafur & Beck, 2021). Variants can also be categorised according to their pathogenicity which refers to how likely a variant is to cause a disease (Biesecker, Nussbaum & Rehm, 2018).

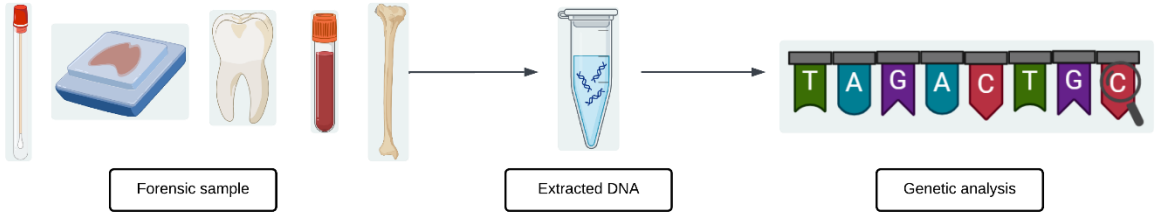

Figure 1.1. Molecular autopsy workflow.

The molecular autopsy workflow involves extracting DNA from forensic samples that were collected at autopsy. The extracted DNA is then sequenced and analysed to identify potential pathogenic variants.

1.2.1 Sudden unexpected death

One category of unnatural deaths that are subjected to a medico-legal autopsy is sudden unexpected death (SUD). A death is classified as a SUD when a seemingly healthy individual dies within 24 hours of the onset of symptoms (Campuzano & Sarquella-Brugada, 2023). Drory *et al.* (1991) conducted a study and identified 198 SUD cases at the Institute of Forensic Medicine in Tel Aviv (Israel) between 1976 and 1985, of which 162 underwent full medico-legal autopsies. A COD remained undetermined in 12% of the cases. Puranik *et al.* (2005) found that 4.3% of SUDs (427 cases) that had occurred between 1995 and 2004 remained undetermined after full autopsies were conducted at the Department of Forensic Medicine, Central Sydney Laboratory Service. A study investigating mortuary admissions at the Tygerberg Forensic Pathology Services (Western Cape, South Africa) between 2001 and 2005 found that 826 cases were classified as SUDs (Tiemensma & Burger, 2012). Of those SUD cases, 8.8% also remained undetermined after a full medico-legal autopsy (Tiemensma & Burger, 2012). It is evident that the number of SUD cases has rapidly increased through the years with the number of unresolved cases remaining an issue.

Molecular autopsies have reportedly resolved up to 35% - 44% of SUD cases which had negative medico-legal autopsy results (Heathfield, Martin & Ramesar, 2019; Tester & Ackerman, 2006). Post-mortem DNA testing has therefore provided a new avenue to investigate COD in cases originally deemed unsolvable. Ackerman's research group in the USA reported some of the first molecular autopsies and they were able to identify novel variants in the *KVLQT1* gene that caused long-QT syndrome (LQTS). This discovery led to a COD being determined after an initial negative medico-legal autopsy (Ackerman, Tester & Driscoll, 2001; Ackerman *et al.*, 1999). Similarly, Jin *et al.* (2005) determined the COD for 25 individuals as fentanyl toxicity due to affected drug metabolism which arose from variant alleles of the *CYP3A4* and *CYP3A5* genes. There are now countless studies and case reports of molecular autopsies being used to assist with COD determination in SUD cases, with most research to date being carried out in the global North (Buscemi *et al.*, 2011; Unuma *et al.*, 2023).

A central theme in the majority of these studies, is that the DNA findings from the molecular autopsies were used as the basis to screen the deceased's family members to identify any individuals who may also possess the pathogenic variants and therefore, be at risk (Ackerman, tester & Driscoll, 2001; Ackerman *et al*., 1999; Buscemi *et al*., 2011; Heathfield *et al*., 2020; Jin *et al*., 2005) . The value of molecular autopsies to surviving relatives was demonstrated by Ackerman *et al.* (1999) where the decedent's sister was identified as possessing the pathogenic variant that led to her brother's death but was deemed healthy due to normal electrocardiogram results. The findings from the molecular autopsy and her genetic screening led to her being put on beta-blocker therapy as a clinical intervention.

Molecular autopsies highlight how seemingly healthy individuals may unknowingly possess a pathogenic variant that can cause them to die "suddenly". They are therefore predisposed to fatal or near-fatal outcomes when a normally non-fatal injury occurs. Determining the genetic

contribution to an individual's death sheds light on how they died but also assists with identifying genetic risks for other family members who may also possess the same variant. Most research on molecular autopsies have focused on sudden cardiac death, especially channelopathies (Heathfield, Martin & Ramesar, 2019). However, molecular autopsies have been used to identify pathogenic variants that are involved in many different mechanisms of death. These discoveries highlight pathogenic variants that cause rare diseases which could be hereditary.

1.3 Sanger sequencing

DNA sequencing for molecular autopsies was originally carried out using a technique known as Sanger sequencing (Ackerman, Tester & Driscoll, 2001; Ackerman *et al.*, 1999). Sanger sequencing, or chain termination sequencing, was first described in 1977 when the authors sequenced the DNA of bacteriophage ΦX174 (Sanger, Nicklen & Coulson, 1977). The authors described a method whereby deoxynucleotide triphosphates (dNTPs) and fluorescently labelled dideoxyribonucleotide triphosphates (ddNTPs) were used to synthesise oligonucleotide chains that were complimentary to a target DNA region (Sanger, Nicklen & Coulson, 1977). The four different ddNTPs were fluorescently labelled with different colours and also did not contain the hydroxyl group on the 3' carbon, preventing extension of the oligonucleotide chain (Sanger, Nicklen & Coulson, 1977). ddNTPs were added to the reaction mixture at a higher frequency than the dNTPs to ensure that termination of the growing chain occurred at random (Sanger, Nicklen & Coulson, 1977).

Once copying of the target region through oligonucleotide chain synthesis was completed, the fragments of DNA were separated according to size using gel electrophoresis (Sanger, Nicklen

& Coulson, 1977). A fluorescent laser was used to excite the fluorophore in the fluorescently labelled ddNTP, starting with the smallest fragments, and the excited fluorophore released a specific colour signal according to the ddNTP it was (Butler, 2011; Clark, Pazdernik & McGehee, 2019). This process was repeated for all fragments from smallest to largest so that by the end the entire target region of the DNA had been sequenced.

Sanger sequencing advanced molecular genetics due to the technique's high fidelity and simple workflow. However, the technique does come with limitations. Sanger sequencing has a low throughput as only a single region of interest can be targeted at a time as demonstrated by Ackerman *et al.* (1999). In studies where Sanger sequencing has been used, it was observed that only a few genes, ranging from one to six genes, were targeted in molecular autopsies (Cann *et al.*, 2017; Evans *et al.*, 2013; Glengarry *et al.*, 2014; Kiehne & Kauferstein, 2007; Stattin *et al.*, 2016; Yamamoto *et al.*, 2011). Sequencing multiple genes using Sanger sequencing is costly and time consuming and is therefore not efficient enough or suitable for large-scale genetic screening.

1.4 Next generation sequencing (NGS)

Due to the limitations experienced with Sanger sequencing, molecular autopsies have shifted to using a more advanced sequencing technique known as NGS or massively parallel sequencing (MPS). The first NGS technology was launched in 2000 (Barba, Czosnek & Hadidi, 2014). NGS is able to sequence genes in parallel, allowing for more gene targets to be considered during molecular autopsies (Castiglione *et al.*, 2021; Neubauer *et al.*, 2017).

The increasing popularity of molecular autopsies has led to an approach that can screen for a large number of variants in a single reaction. Fortunately, NGS technology affords researchers and scientists the opportunity to sequence whole exomes (WES), whole genomes (WGS) or customised gene panels that comprise a collection of genes relating to a particular phenotype or pathway (Lahrouchi, Behr & Bezzina, 2016; Martínez-Barrios *et al.*, 2023). While whole genomes or exomes may be sequenced, virtual panels may be applied in the analysis phase of the workflow to facilitate interpretation of the relevant genes. For example, Neubauer *et al.* (2017) filtered 192 genes after carrying out WES and identified potentially causative variants in two of the genes (*SCN5A* and *RYR2*). Once a pathogenic variant has been found in the proband, targeted screening can be applied in living relatives which is considerably cheaper than NGS, provided one knows what the variant of clinical interest is.

There are numerous commercially available NGS platforms which each follow a specific protocol, however, all platforms follow the same general workflow for molecular autopsies (Figure 1.2) (Castiglione *et al.*, 2021). Li *et al.* (2015) demonstrated this workflow whereby DNA from a forensic sample was extracted, purified and **quantified**. Thereafter, library preparation was carried out where the DNA was fragmented, and adapters were ligated to the ends of the fragments. Finally, the library (collection of DNA fragments with ligated adapters) was amplified, and the enriched library was then **quantified** prior to sequencing.

The sequencing step differs according to the NGS platform used. The Ion Torrent platform conducts the DNA sequencing process by monitoring the release of hydrogen ions and subsequent pH changes while the Illumina sequencing approach makes use of fluorescently labelled dNTPs (Li *et al.*, 2015; Novroski, 2023; Zhou & Li, 2021). Once sequencing has been completed, the generated sequences are aligned to a reference genome to identify any variants present in the genome (Beedanagari & John, 2014). *In silico* tools are then used to assess the

pathogenicity of the variants (Lahrouchi, Behr & Bezzina, 2016). Sanger sequencing is still considered the gold standard due to its accuracy and reliability, therefore, NGS results are often validated using Sanger sequencing (Martínez-Barrios *et al.*, 2023; Sanchez *et al.*, 2016).

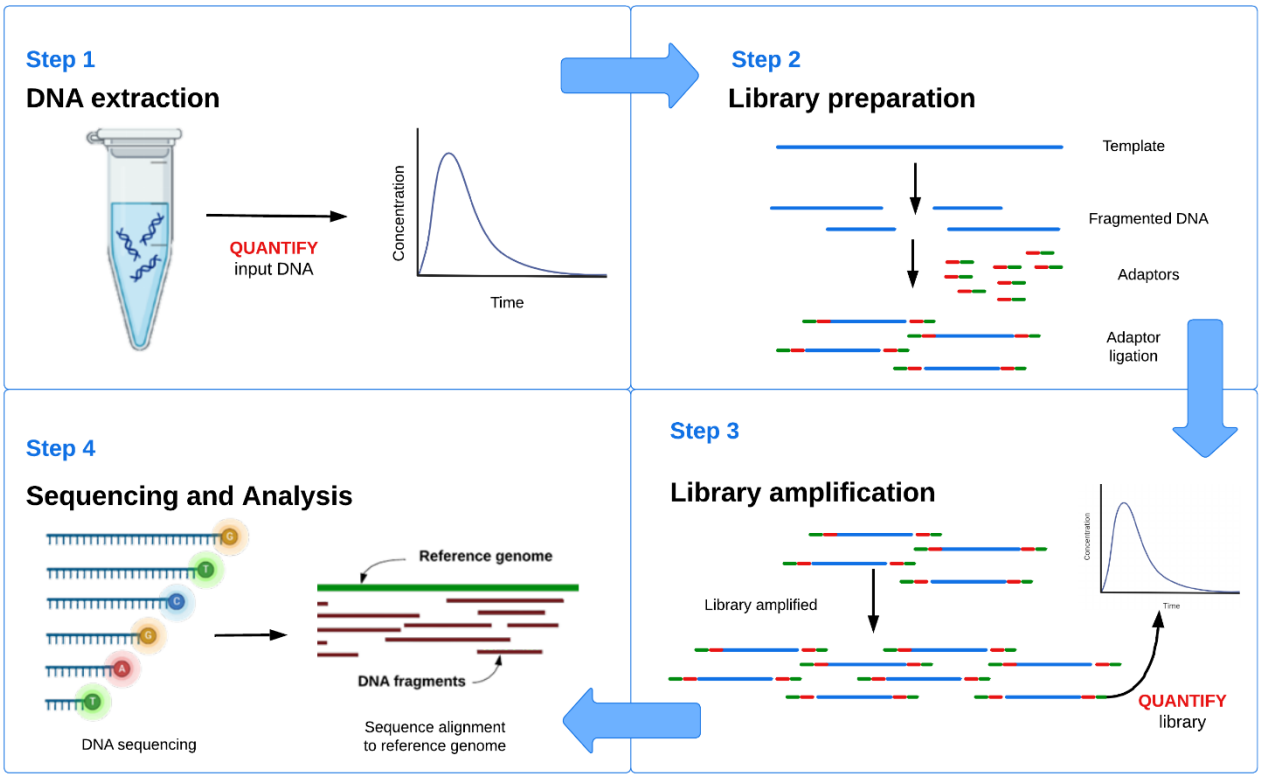

Figure 1.2 NGS workflow.

During the NGS workflow, DNA is extracted from forensic samples which undergo library preparation where the DNA is fragmented, and adaptors are ligated. The libraries are then amplified and quantified prior to sequencing. Sequenced libraries are aligned to a reference genome to identify potential pathogenic variants.

1.5. Quantification methods

The NGS workflow has two quantification steps, each serving different purposes. The first quantification step occurs after DNA extraction and is necessary as it ensures the correct amount of DNA will be used for optimal fragmentation and reproducible results (Simbolo *et al.*, 2013). The second quantification step is carried out after library preparation and prior to

sequencing and is necessary to ensure the optimum concentration of the prepared NGS library is introduced to the flow cell for sequencing (Young, Stewart & Dimalanta, 2017). Both these quantification steps play important roles in the success of the NGS workflow and choosing a suitable quantification method for each step can assist with achieving this success.

1.5.1 Quantitative PCR (qPCR)

qPCR is a fluorometric quantification method that has been validated for forensic use (Conte *et al.*, 2019; Swango *et al.*, 2007; Timken *et al.*, 2005). PCR was first described by Saiki *et al.* (1985) where the authors amplified the β-globin gene segment. The authors followed a workflow that involved binding of P-labelled probes to the target sequence, allowing for the DNA to be quantified by detection of a fluorescent signal (Saiki *et al.*, 1985). The PCR method was later adapted by Higuchi *et al.* (1992) to enable continuous quantification of PCR products as amplification took place (qPCR). qPCR has reportedly made use of intercalating fluorescent dyes or TaqMan probes that were added to the reaction mix (Adams, 2020; Dymond, 2013). The amount of fluorescence produced is proportional to the amount of amplified DNA product present (Adams, 2020; Tang, 2023).

qPCR is a suitable quantification method for the first quantification step of the NGS workflow, however, it is not routinely used during the second quantification step as a generic qPCR method makes use of primers that target a multi-copy locus target in the human genome which is present in extracted DNA but may not necessarily be present in the NGS library (Arya *et al.*, 2005). Additionally, the quantification of the generic target would not correspond to the total concentrations of the NGS library present as the library would have already undergone enrichment. This means that there would be multiple copies of each fragment in the library.

There are some customized qPCR kits available that contain primers that bind to the adapter sequence; however, this limits the NGS chemistries that can be used for molecular autopsies.

1.5.2 Fragment analysis

Fragment analysis is carried out alongside the quantification steps as part of quality assessment, using instruments such as TapeStation and Bioanalyzer (capillary-electrophoresis system) (Daviso, Simon & Pal, 2019). Fragment analysis is used to assess the size distribution of the NGS library fragments (Agilent Technologies, 2020). Panaro *et al.* (2000) described the method used for DNA fragment sizing and quantification using the Agilent 2100 Bioanalyzer which is comparable to the TapeStation system. Quantification was achieved by the separation of fluorescently labelled DNA fragments as they passed through a capillary (Agilent Technologies, 2020; Panaro *et al.*, 2000). The fragments gave off fluorescent signals as they were separated and were used to generate an electropherogram which displayed peaks which depicted the concentration of the DNA fragments as the area under the peaks.

Hussing *et al.* (2018) comparatively quantified NGS libraries using different quantification methods including Bioanalyzer and TapeStation. It was observed that quantification of NGS libraries' concentrations using fragment analysis methods, such as TapeStation, was not reliable and is thus not recommended (Hussing *et al.*, 2015; Panaro *et al.*, 2000). Dynamic range limitations of the assay hinder accurate quantification of DNA concentrations (Hussing *et al.*, 2015). Additionally, it has been observed that fragment analysis techniques report lower concentrations than other quantification methods (Hussing *et al.*, 2015; Panaro *et al.*, 2000). Overall, fragment analyses are useful for determining the size of NGS library fragments but are not considered accurate with measuring concentrations.

1.5.3 Qubit™ fluorometry

A fluorometric dsDNA assay can be used to overcome the limitations of quantifying DNA that are presented by qPCR and fragment analysis methods. Fluorometric dsDNA assays were developed to be able to measure a fluorescent signal emitted by an intercalating dye bound to the target dsDNA (Thermo Fisher Scientific, 2019). A fluorometer is used to detect the signal which is converted to a DNA concentration. Fluorometric assays have been recommended for NGS library quantification as the intercalating dye that is used is specific to dsDNA, making the method less susceptible to quantifying contaminants such as RNA or proteins (Illumina, 2018).

A fluorometric dsDNA assay that is able to quantify NGS libraries of dsDNA is the Qubit™ Assay (Thermo Fisher Scientific, 2023). Due to the principle of Qubit™ quantification, the assay is not specific to a quantification kit or library as is with qPCR and its primers (Thermo Fisher Scientific, 2019). Therefore, Qubit™ fluorometry is ideal for universal NGS library quantification.

Qubit™ fluorometry has been reported to be accurate, has a simple workflow and carries a low cost (Hussing *et al.*, 2015; Simbolo *et al.*, 2013). Qubit™ fluorometry has also demonstrated its capability of quantifying DNA extracted from histopathological samples as well as the associated NGS libraries (Bruijns *et al*., 2022; Hussing *et al*., 2015; Hussing *et al*., 2018; Nakayama *et al*., 2016; Simbolo *et al*., 2016) .

1.6 Validation

The Qubit™ Assay has been developmentally validated by the manufacturer (Thermo Fisher Scientific, 2018c), but has not been validated for forensic use. The manufacturer's validation focused on performance parameters of the Qubit™ Assay using control samples and not forensic samples. There are no published internal validation studies for the use of the Qubit™ Assay in forensic laboratories. Internal validation studies may have been done but the protocol and findings may not have been published and kept in-house.

Validation is an important part of a laboratory's quality management system (QMS). Laboratory QMS refers to the procedures, policies and practices that are implemented in a laboratory to guide its testing processes and yield results that are accurate, reliable and reproducible (Ross & Neuteboom, 2021). A laboratory QMS aims to maintain or improve the quality of the laboratory's practice as well as minimise errors. QMS in forensic laboratories is especially important as the conclusions that are drawn from analyses conducted in said laboratories have a medico-legal significance (Neuteboom *et al.*, 2023; Ross & Neuteboom, 2021). Forensic laboratory QMS is often established according to the International Organization for Standardization's standard 17025 (ISO 17025) guidelines (Ross & Neuteboom, 2021). ISO 17025 outlines the criteria for a laboratory to be able to competently perform accurate and reliable testing, including technical competence of personnel as well as laboratory operations and management (e.g. document control, proficiency testing, reporting of results, equipment calibrations, etc.) (Milošević, Bjelovuk & Kesić, 2009; Neuteboom *et al.*, 2023).

ISO 17025 requires forensic laboratories to carry out internal validation of methods or equipment that are new to a specific laboratory (International Organization for Standardization, 2017). Internal validation tests a method or kit within a specific laboratory environment and

compares the findings to those obtained by the manufacturer during developmental validation. Internally validating a workflow and its components demonstrates that a method or kit has been rigorously tested in the forensic laboratory and, should internal validation be passed, that it has proven to be accurate, reproducible and reliable. It is also tested if a method or component is fit for purpose in the workflow. Internally validated methods are important as casework findings may be presented in court and needs to withstand interrogation. Internal validation tests the accuracy, reliability and reproducibility of results obtained in a specific laboratory (Budowle *et al.*, 2008; Butler, 2007). During internal validation studies, control samples with known metrics (concentration, size, etc. established by manufacturer) are used and tested according to the new method or are measured on the new equipment and the findings are compared to the known metrics. If internal validation is passed by meeting the criteria of acceptance, the method or equipment can be reliably and accurately used in the forensic laboratory for casework.

1.7 Rationale, aim and objectives for the current study

Molecular autopsies are a useful tool in forensics as they assist with medico-legal investigations. The Molecular Forensics Research Group (MFRG) at the University of Cape Town (UCT) is establishing a workflow for molecular autopsies that will be utilised at the OFPI. The MFRG works closely together with the OFPI as the research group carries out research to directly address gaps in service delivery and occasionally assists with service delivery. Implementation of a validated molecular autopsy workflow will significantly contribute to the growing capacity of forensics in Africa, as well as possibly improving understanding of COD, and the implications for surviving relatives. Thus, improving public health outcomes.

The OFPI will house the first molecular autopsy service in Africa. Ahead of this, each component of the workflow needs to be developed, optimised, and validated (Heathfield, Martin & Ramesar, 2019; Heathfield, Bennet & Martin, 2021; Heathfield *et al*., 2020; Heathfield, 2019; Heathfield, Martin & Ramesar, 2018; Heathfield, Martin and Ramesar, 2019; Heathfield *et al*., 2021a; Heathfield *et al*., 2023). One such component is the quantification of extracted DNA from forensic samples and NGS libraries using the Qubit™ Assay. However, the Qubit™ Assay would need to be validated to assess if it would be fit for forensic purposes. The workflow also needs to be internally validated so that it may be incorporated into the molecular autopsy service delivery in accordance with ISO 17025 guidelines. As part of internal validation, the suitability of the assay for molecular autopsies needs to be evaluated and parameters for our laboratory need to be established.

Therefore, the aim of this study is to internally validate the Qubit™ Assay on the Fluorometer for incorporation into a molecular autopsy workflow. The aim will be achieved by the following research objectives:

- Optimising the Qubit™ Assay workflow.
- Determining the accuracy, precision (intra-assay and inter-assay), range and sensitivity of the assay.
- Comparing the measured concentrations of control DNA samples and forensic samples to other quantification techniques.
- Evaluating the suitability of the assay in our forensic laboratory by comparing our results to the manufacturer's criteria of acceptance and establishing acceptable performance parameters for the assay in our setting.

Chapter 2: Materials and Methodology

2.1Study design

A quantitative study design was used for this project. The project aimed to assess and verify the performance of the Qubit™ Assay on Fluorometer. Control DNA samples with known DNA concentrations and forensic samples were used. The forensic samples were used as they represented the samples which would typically be used during casework in the laboratory.

DNA concentrations of the control and forensic sample replicates were measured and analysed to determine the accuracy, precision (intra-assay and inter-assay precision) as well as the range and sensitivity of the Qubit™ Assay. These validation parameters were also evaluated for their suitability within our forensic laboratory.

This study received ethics approval from the Human Research Ethics Committee (HREC) (HREC: 687/2023) (Appendix 1).

2.2 System verification test

The Fluorometer that was used for this project was verified using the Qubit™ 4 Fluorometer System Verification Assay Kit (Thermo Fisher Scientific, Waltham, USA, Catalogue no. Q33237) as per the manufacturer's instructions (Thermo Fisher Scientific, 2018b) prior to samples being quantified. The System Verification Assay was carried out to ensure the fluorometer was working optimally.

2.3. Calibration of the Fluorometer with the Qubit™ Assay

Prior to samples being quantified on the Fluorometer, the instrument was calibrated using the Standard 1 and Standard 2 for the Qubit™ Assay as per the manufacturer's instructions (Thermo Fisher Scientific, 2020).. The successful calibration results in the generation of a *Fluorescence vs Concentration* graph.

2.4. Optimisation of the Qubit™ Assay on the Fluorometer

Prior to carrying out the internal validation experiments, the standard operating procedure (SOP) for quantifying DNA using the Qubit™ Assay was optimised. The manufacturer's protocol (Thermo Fisher Scientific, 2020) was used as a baseline, and three parameters were systematically tested to ensure an optimal workflow.

2.4.1. Incubation period

Upon adding a DNA sample to the Qubit™ Assay's Working Solution, an incubation period of 2 minutes was recommended by the manufacturer, to allow for the fluorescent dye to intercalate with the DNA sample (Thermo Fisher Scientific, 2020). Experiments were carried out to determine an effective and appropriate incubation period. To this end, stock Fermentas Lambda DNA (Thermo Fisher Scientific, Waltham, Cat no. SD0011) was diluted to 100 ng/µl, 50 ng/µl, 10 ng/µl, 1 ng/µl and 0.01 ng/µl using distilled water. The Working Solution was equilibrated to room temperature for 30 minutes and then the samples were added (the mixture of which will be referred to as a Qubit™ aliquot hereon). Table 2.1 shows the volumes of sample and

Working Solution that were used. The Qubit™ aliquots were covered with foil at room temperature and each Qubit™ aliquot was quantified at the following time intervals: 2 minutes, 10 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 150 minutes and 180 minutes. Each sample's concentration was measured in triplicate after each time interval. The accuracy and precision of each sample's measured concentration at each time interval was determined. The experimental procedure was repeated on two separate days.

Table 2.1 Volume of sample and working solution used in incubation time experiments.

Different sample volumes were added as the DNA samples that were quantified needed to be within the dynamic range of 0.2 ng – 100 ng of DNA.

Sample	Volume of sample (µl)	Volume of Working Solution (µl)
100 ng/µl	1	199
50 ng/µl	1	199
10 ng/µl	2	198
1 ng/µl	2	198
0.01 ng/µl	10	190

2.4.2 Assessment of temperature equilibration period

Based on findings from the initial incubation time experiments, additional tests were carried out to assess the impact of an increased time for the Working Solution to equilibrate to room temperature. The same experiment as described above in 2.4.1 was repeated using Lambda DNA diluted to 100 ng/µl and 50 ng/µl and the Working Solution equilibrated to room temperature for three hours instead of 30 minutes. The accuracy and precision of each sample's measured concentration from this experiment was compared to the previous experiments.

2.4.3. Dilution medium

Lastly, the medium in which samples were diluted was assessed. In addition to UltraPure DNAse/RNAse free Distilled Water (Thermo Fisher Scientific, Waltham, Cat no. Q33230) (hereon referred to as distilled water), low ethylenediaminetetraacetic acid (EDTA) Tris-EDTA (TE) buffer (Integrated DNA technologies, Iowa, Cat no. 10009855) and TE buffer (Thermo Fisher Scientific, Waltham, Cat no. 12090015) were assessed. Lambda DNA was diluted to 100 ng/µl, 10 ng/µl, 1 ng/µl and 0.1 ng/µl using TE buffer and low EDTA buffer. Using the optimised procedure emanating from the results above, the Qubit™ aliquots were prepared in triplicate and each quantified using the Qubit™ Assay. The concentration of the samples' replicates was measured in quadruplicate. The accuracy of the measured concentration for the samples diluted with distilled water (from previous optimisation experiments), TE buffer and low EDTA buffer were assessed using paired t-tests given that the data was normally distributed.

2.5 Samples used for validation experiments

2.5.1. Control samples

Lambda DNA was used as the control DNA sample. It was purchased as a 500-µg unit with a concentration of 300 ng/µl. The Lambda DNA was diluted to concentrations of 100 ng/µl, 10 ng/µl, 8 ng/µl, 6 ng/µl, 4 ng/µl, 2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.1 ng/µl, 0.03 ng/µl, 0.02 ng/µl and 0.01 ng/µl as carried out by the Qubit™ Assay manufacturer (Thermo Fisher Scientific, 2018c). The dilutions were prepared using distilled water. Figure 2.1 illustrates the dilution series that was followed. The dilution series was carried out in triplicate.

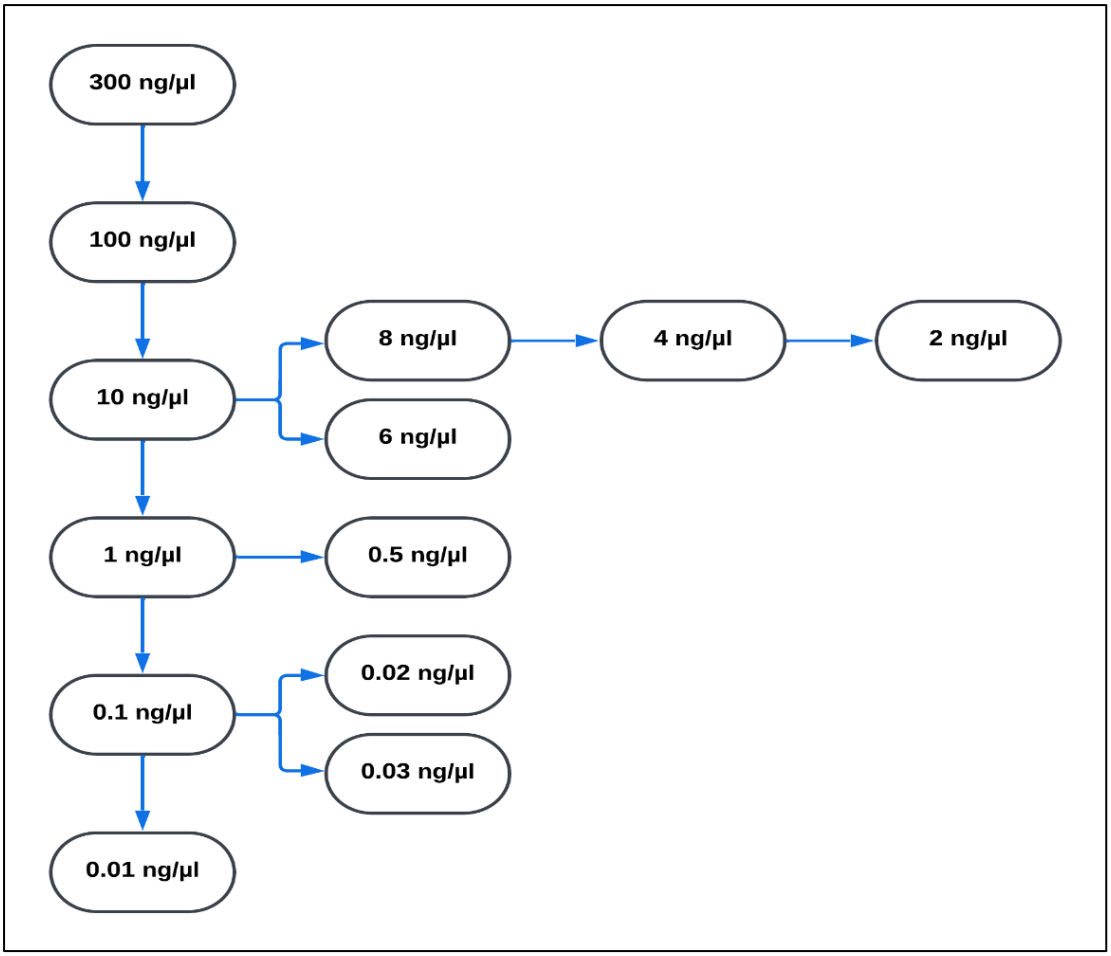

Figure 2.1. Lambda DNA dilution series.

Lambda DNA was diluted using distilled water to obtain samples at the required concentrations. The dilution series was prepared in triplicate.

2.5.2. Extracted DNA samples

DNA extracted from forensic samples including blood, a buccal swab, bone, teeth, nail and formalin fixed paraffin embedded (FFPE) tissue were used. DNA was extracted from the buccal swab, bone, tooth, nail and FFPE tissue samples using the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany, Catalogue no. 56504) according to the manufacturer's protocol (Qiagen, 2020). DNA was extracted from blood using a salting out method as previously described (Heathfield *et al.*, 2020). These sample types were used as they represented the

biological samples that would be available for collection at OFPI for molecular autopsies. The use of different sample types also accounted for biological replicates required for internal validation studies.

The extracted DNA from blood and nail samples underwent a 1:4 dilution using distilled water. The dilutions were prepared in triplicate. The extracted DNA from bone, the buccal swab, teeth and FFPE tissue were used undiluted.

2.5.3. Next generation sequencing (NGS) libraries

In another study in the MFRG at UCT, libraries were prepared using the TruSight One Sequencing Panel Series (Illumina™, California, USA, Catalogue no. 20042621) for molecular autopsies as described by Heathfield *et al.* (2023). The TruSight One Sequencing Panel Series (Illumina™, California, USA, Catalogue no. 20042621) targets regions in the exome which possess known pathogenic variants (Illumina, 2023). The prepared libraries had undergone sequencing and a summary of the quality reports for the sequencing runs were available (Appendix 2). The libraries from that study were quantified in this study using the Qubit™ Assay. One unpooled library sample and one pooled library sample were used. The unpooled library underwent a 1:1 dilution using distilled water. The dilution was prepared in triplicate. The pooled library was used undiluted.

2.6 Validation experiments - DNA quantification using the Qubit™ Assay

Prepared samples were quantified using the Qubit™ Assay on the fluorometer. Diluted samples had three replicates while undiluted samples had one replicate. From each replicate, three Qubit™ aliquots were used for quantification (Figure 2.2). The aliquots were prepared in Qubit™ assay tubes (Thermo Fisher Scientific, Waltham, Cat no. Q32856). The Qubit™ aliquots were prepared by adding a volume of the Working Solution and a volume of the sample ensuring that the final volume of the aliquots was 200 µl. Different volumes of the sample added for quantification were assessed as shown in Table 2.2. Each Qubit™ aliquot was measured four times.

Table 2.2. Volumes of sample and Working Solution added for aliquots that were quantified.

Different sample volumes were added as the DNA samples that were quantified needed to be within the dynamic range of 0.2 ng – 100 ng of DNA.

Sample		Volume of sample (µl)	Volume of Working Solution (µl)
Lambda DNA	100 ng/µl	1	199
	10 ng/µl	2	198
		10	190
	8 ng/µl	2	198
	6 ng/µl	2	198
	4 ng/µl	2	198
	2 ng/µl	2	198
	1 ng/µl	2	198
	0.5 ng/µl	2	198
	0.1 ng/µl	2	198
	0.03 ng/µl	2	198
		10	190
	0.02 ng/µl	2	198
		10	190
	0.01 ng/µl	2	198
		10	190
		20	180
Extracted DNA	Blood	2	198
	Buccal swabs	2	198
	Nail	2	198
	Teeth	2	198
	Bone	2	198
	FFPE tissue	2	198
NGS libraries	Unpooled library	1	199
	Pooled library	2	198

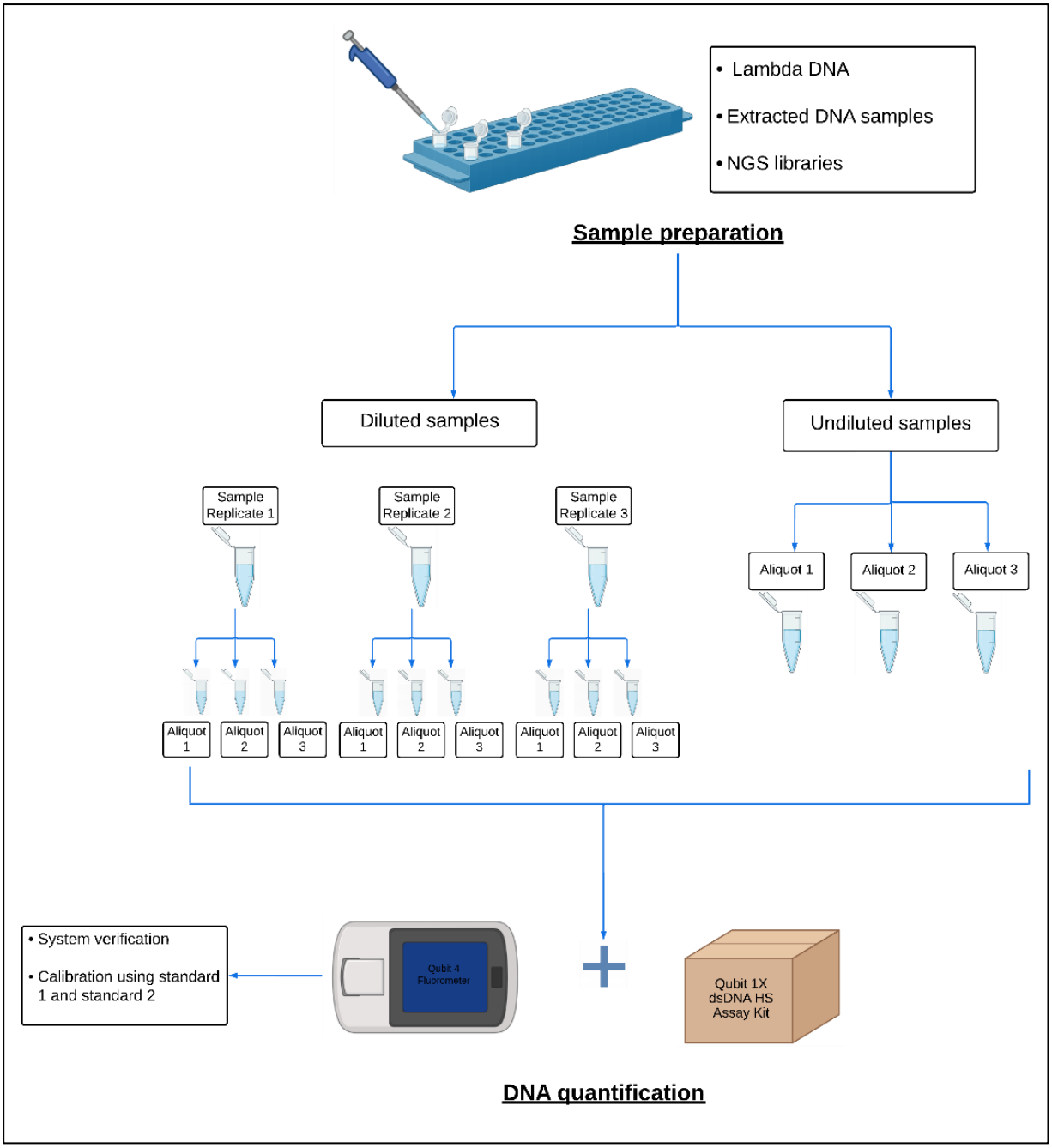

Figure 2.2. Process that was followed for quantification of samples using the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 Fluorometer.

Samples were prepared and had either one replicate (undiluted samples) or three replicates (diluted samples).

Each replicate had three Qubit™ aliquots quantified on the calibrated Qubit™ 4 Fluorometer.

2.7. Alternative DNA quantification using qPCR and TapeStation

The extracted DNA samples were also assessed using qPCR, which was carried out on the 7500 real-time PCR thermal cycler (Thermo Fisher Scientific, USA) using the Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific, USA, Catalogue no. 4482910). qPCR could not be applied to the Lambda DNA samples as the primers that were used were specific to human gene targets that were not present in Lambda DNA.

The Agilent 4200 TapeStation (Agilent, USA, Catalogue no. G2991BA) was used with the HS D1000 ScreenTape Assay (Agilent, USA) to quantify the prepared NGS library samples, while the Genomic ScreenTape Assay (Agilent, USA) was used to quantify the prepared Lambda DNA and extracted DNA samples.

Only one replicate of each sample was quantified using qPCR and TapeStation as these methods have previously undergone validation experiments in our laboratory and have known associated accuracy and precision rates. A single quantification result was sufficient for comparisons to the Qubit™ concentration, which was the focus of this study. Manufacturer's protocols which have previously been internally validated in our laboratory were followed for both quantification methods (Agilent Technologies, 2015a; Agilent Technologies, 2015b; Thermo Fisher Scientific, 2018a).

2.8. Data analysis

DNA concentration measurements were exported to Microsoft® Excel®. Statistical analyses and graphical representations were performed using Microsoft® Excel®, IBM SPSS Statistics version 28.0.1.1 (SPSS Inc., Chicago, USA) and GraphPad Prism version 10.1.2 (234)

(GraphPad Software, Chicago, USA). Statistical and data analyses were performed using either all four concentration measurements, the last three concentration measurements only or the first three measurements only (referred to as M1 – M4, M2 – M4 and M1 – M3 respectively from here on, where M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4).

2.8.1. Quantification method comparison

Concentration measurements obtained from the different methods were compared using Wilcoxon Signed-Ranks test given that the data was not normally distributed. Measurements were compared between the methods for the Lambda DNA, extracted DNA and NGS library samples separately.

Given the result of the t-test was not significant ($p = 0.125$), a Bland-Altman plot was constructed to compare the concentration measurements from Qubit™ and TapeStation.

2.8.2. Accuracy testing

The accuracy of the Qubit™ Assay was determined by comparing the sample's measured concentration to the sample's known concentration and assessing if they were in agreement (Figure 2.3). Accuracy was determined for each sample's replicates as well as the overall accuracy for the sample. Furthermore, accuracy was calculated using M1 – M4, M2 – M4 and M1 – M3. This was done as it was observed that the first measurement was sometimes different relative to the other measurements of the same sample. Lambda DNA samples were used to

assess accuracy as these samples had known concentrations. Accuracy was calculated as follows,

$$Accuracy = 100 - \left(\frac{Measured\ concentration - True\ concentration}{True\ concentration}\ x\ 100\right).$$

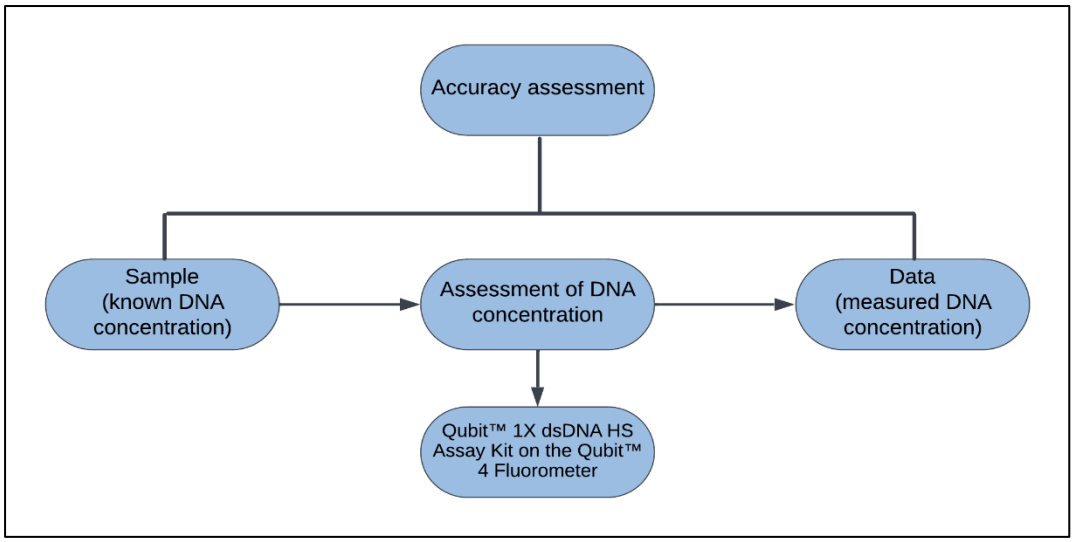

Figure 2.3. Process for testing accuracy of the Qubit™ 1X dsDNA HS Assay Kit.

Lambda DNA samples were prepared and had known DNA concentrations. The samples were then quantified using the Qubit™ 1X dsDNA HS Assay and the measured concentrations were compared to the known concentrations of the samples to determine the accuracy of the Qubit™ 1X dsDNA HS Assay.

Bland-Altman plots were used to assess the agreement between the average concentration of each Lambda DNA sample to the expected (known) concentration. Bland-Altman plots were constructed for M1 – M4, M2 – M4 and M1 – M3 accuracies given that the results of the t-tests were not significant ($p = 0.142$, $p = 0.221$ and $p = 0.069$ respectively).

2.8.3. Precision testing

The intra- and inter-assay precision of the Qubit™ Assay was determined by assessing the variability of the data produced (Figure 2.4).

Intra-assay precision was determined by comparing replicate measurements of the same aliquoted sample. Inter-assay precision was determined by comparing measurements between the aliquots of a sample. M1 – M4, M2 – M4 and M1 – M3 were used for calculations. Lambda DNA, extracted DNA samples and NGS library samples were used in this assessment. The percentage coefficient of variation (% CV) was used to assess the precision of measurements and was calculated as follows,

$$\% \, CV = \frac{standard \; deviation \; of \; measurements}{mean \; of \; measurements} \, x \; 100.$$

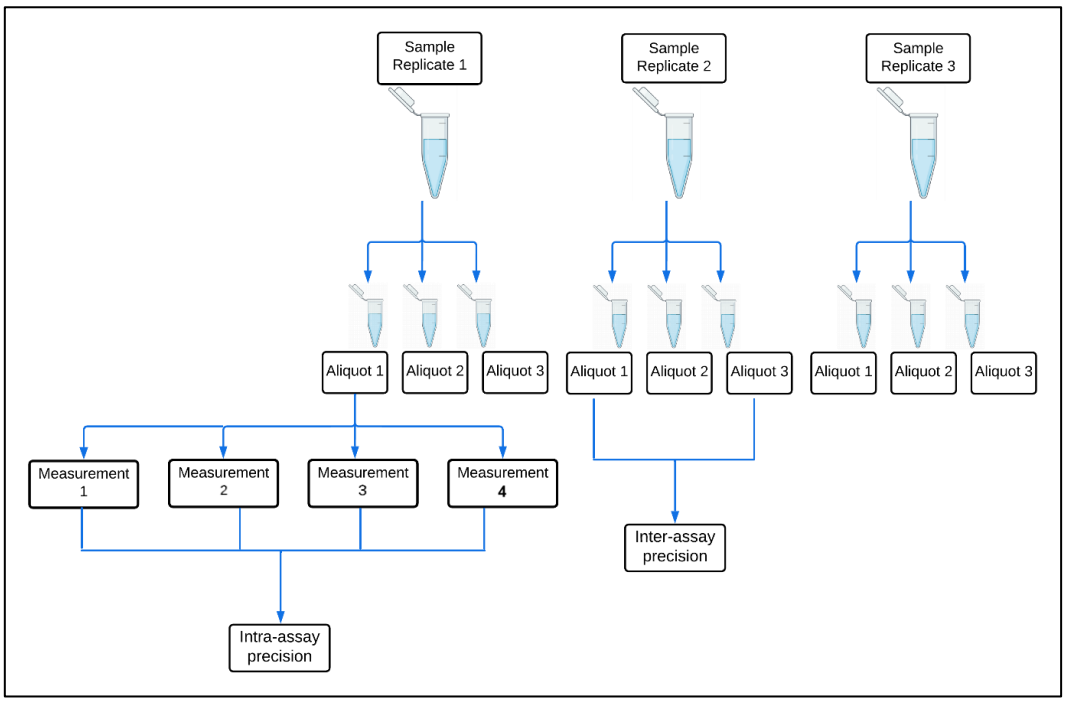

Figure 2.4. Intra- and inter-assay precision testing of the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 Fluorometer.

Intra-assay precision was determined by comparing replicate measurements of the same aliquoted sample. While the inter-assay precision was determined by comparing measurements between the aliquots of a sample.

The intra-class correlation coefficient (ICC) for each sample was also determined to assess the variability of the measurements. ICC was determined using all four measurements as well as only the first three measurements and only the last three measurements only based on an

absolute-agreement, 2-way mixed-effects model.

2.8.4. Range and sensitivity

The range of the Qubit™ Assay was determined by the highest and lowest value, for which any values in between could be taken as precise and accurate. The lowest value in the range specifically indicated the sensitivity of the assay. Lambda DNA at concentrations of 100 ng/µl, 0.02 ng/µl and 0.01 ng/µl were used to assess the range and sensitivity. A volume of 1 µl of the 100 ng/µl control Lambda DNA sample, 10 µl of the 0.02 ng/µl control Lambda DNA sample and 20 µl of the 0.02 ng/µl control Lambda DNA sample were used.

2.8.5. Criteria of acceptance

The overall accuracy, precision (intra- and inter-assay precision), range and sensitivity for the measured concentrations were calculated and compared to the manufacturer's criteria of acceptance (Table 2.3) (Thermo Fisher Scientific, 2018c). A comparison was made for all four measurements as well as the last three measurements only and first three measurements only.

Table 2.3 Criteria of acceptance for validation parameters

The criteria of acceptance for the validations as determined by the manufacturer are shown (Thermo Fisher Scientific, 2018c).

Parameters	Manufacturers obtained values
Accuracy	> 80 %
Intra-assay precision (samples < 0.5 ng/µl)	< 15 % CV
Intra-assay precision (samples ≥ 0.5 ng/µl)	< 1 % CV
Inter-assay precision (samples < 0.5 ng/µl)	< 15 % CV
Inter-assay precision (samples ≥ 0.5 ng/µl)	< 1 % CV
Range	0.01– 100 ng/µl (0.2 – 100 ng DNA)
Sensitivity	0.01 ng/µl (0.2 ng)

Chapter 3. Results

3.1 System verification test and calibration

The Fluorometer passed the system verification test and was successfully calibrated for use with the Qubit™ Assay, indicating that the instrument was functioning as intended as well as optimally.

3.2 Optimisation of the Qubit™ Assay on the Fluorometer

3.2.1 Incubation period

It was observed that the measured concentration for the 100 ng/µl and 50 ng/µl Lambda DNA samples decreased as the incubation period increased, while the concentrations of the remaining samples had negligible differences (Figure 3.1). It was also noted that the 0.01 ng/µl sample did not have any concentration measurements after 180 minutes of incubation. The samples' average concentration from both experiments (Appendix 3) were assessed and it was observed that none of the samples were most accurately quantified after the manufacturer's recommended 2-minute incubation period (Figure 3.2). The 0.01 ng/µl sample was most accurate after 10 minutes while the other samples were most accurate between 90 and 180 minutes of incubation. Accuracy was calculated for each DNA concentration at each time interval and the middle most accuracy was represented on the graph for each time interval as depicted by the median accuracy points in Figure 3.2.

The differences between the concentration measurements for the 0.01 ng/µl, 1 ng/µl and 10 ng/µl samples across the time intervals were negligible (< 1ng/µl). The determined accuracy

was more sensitive to deviations of the measured concentrations from the expected (known) concentrations for samples with lower concentrations. Therefore, concentration measurements for the 0.01 ng/µl, 1 ng/µl and 10 ng/µl samples at all time intervals were acceptable. The 100 ng/µl and 50 ng/µl samples, however, had differences of up to 15 ng/µl and 6 ng/µl respectively between the concentration measurements across the time intervals.

Furthermore, the intra-assay precision for all samples were within the manufacturer's threshold (< 1% CV for samples ≥ 0.5 ng/µl and < 15% CV for samples < 0.5 ng/µl) for all time intervals (Figure 3.3) even with observed increases for the 0.01 ng/µl and 1 ng/µl samples concentration measurements across the time intervals.

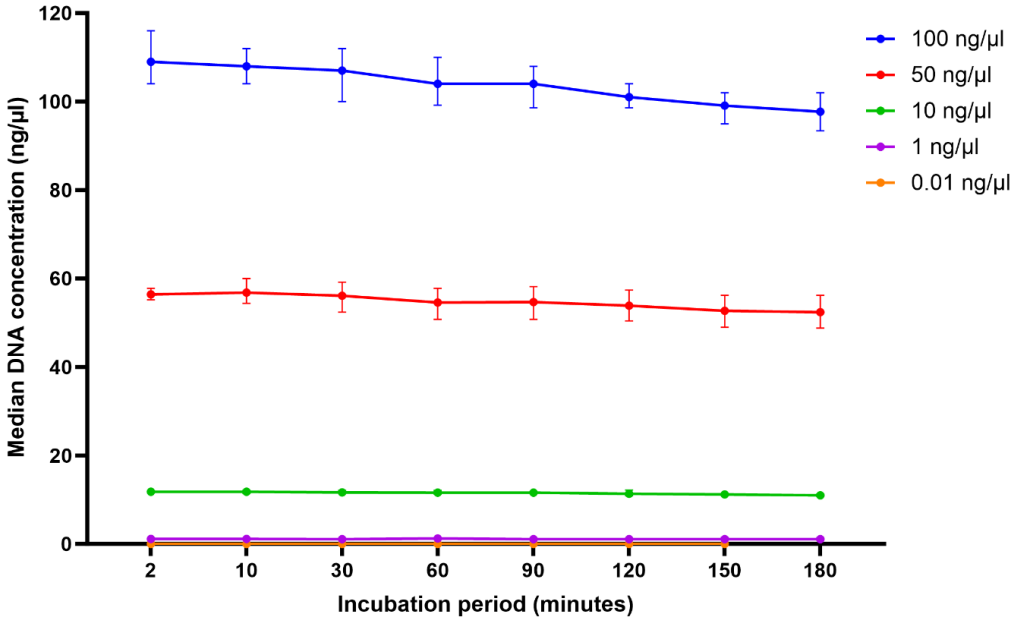

Figure 3.1 Median DNA concentrations of Lambda DNA samples measured during incubation period experiments.

The DNA concentration measurements for each Lambda DNA sample from both incubation period experiments were grouped and the median measurement as well as the range of the measurements were plotted (n = 8). The 100 ng/µl and 50 ng/µl samples' concentrations were observed to steadily decrease as the incubation period increased, while the concentrations of the 10 ng/µl, 1 ng/µl and 0.01 ng/µl samples had negligible differences between measurements taken at each time interval.

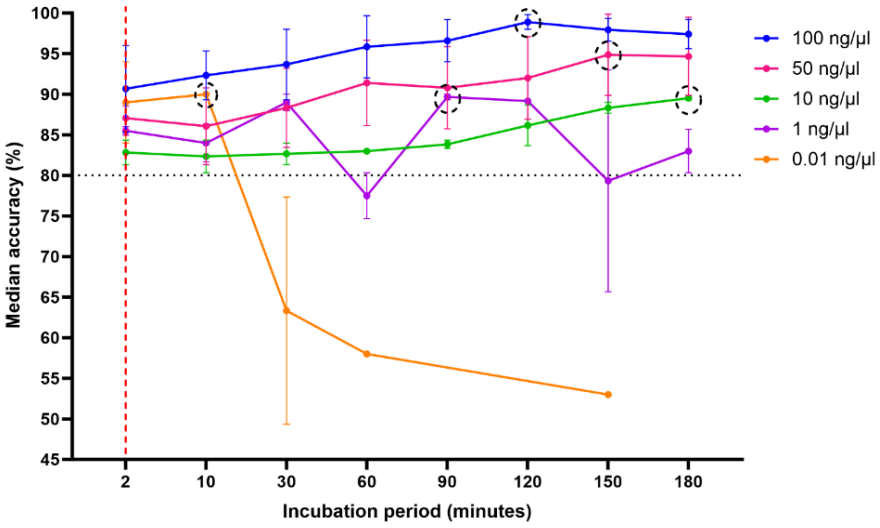

Figure 3.2 Median accuracy for measured concentrations of Lambda DNA samples during incubation period experiments.

The median accuracy of Lambda DNA samples' concentration measurements as well as the accuracy ranges are shown (n = 8). The horizontal dotted line indicates the manufacturer's criteria of acceptance for accuracy of 80%. It was observed that none of the samples were most accurate after the manufacturer's recommended 2-minute incubation period indicated by the red vertical dotted line. The most accurate points for each sample are circled.

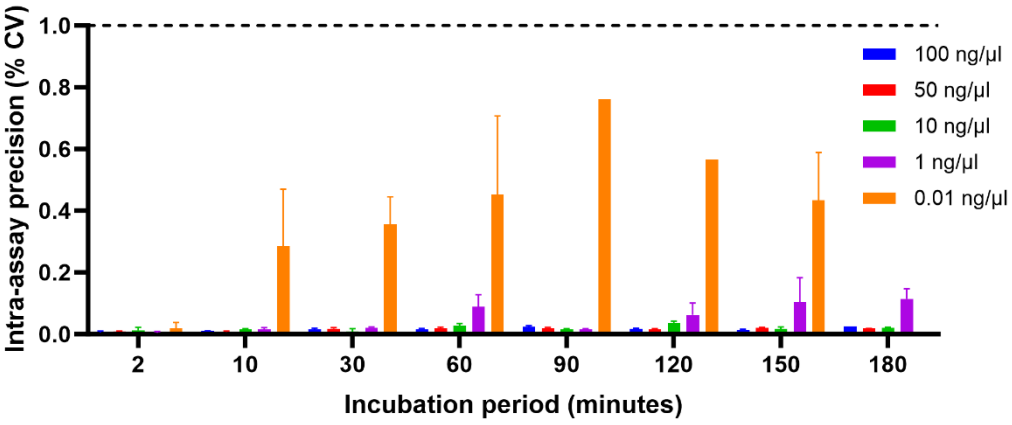

Figure 3.3 Intra-assay precision (% CV) for measured concentrations of Lambda DNA samples during incubation period experiments.

The intra-assay precision (% CV) for the Lambda DNA sample's measured concentrations remained below the manufacturer's threshold (< 1% CV for samples ≥ 0.5 ng/µl and < 15% CV for samples < 0.5 ng/µl) for all time

intervals. The lowest threshold of 1% CV is indicated by the horizontal dotted line. The 0.01ng/µl sample did not have any concentration measurements after 180 minutes of incubation.

3.2.2. Assessment of temperature equilibration period

Equilibrating the Working Solution for 3 hours resulted in the 100 ng/µl and 50 ng/µl Lambda DNA samples being most accurate after 30 minutes and 10 minutes of incubation instead of after 120 minutes and 150 minutes respectively (Figure 3.4). Accuracy was calculated for all samples with varying DNA concentrations at each time interval and the mean accuracies for each time interval were represented on the graph as depicted in Figure 3.4. The manufacturer's recommended 2-minute incubation period was still not appropriate for obtaining the most accurate concentration measurements. The intra-assay precision of the 100 ng/µl and 50 ng/µl samples' concentration measurements were < 0.1% CV when the Working Solution was equilibrated for 30 minutes and 3 hours (Figure 3.5). Changes between the intra-assay precisions across the time intervals were negligible.

Based on the findings from the incubation period and temperature equilibration period experiments, it was decided that the Working Solution should be equilibrated to room temperature for three hours and the Qubit™ aliquots should be incubated for at least 10 minutes but no longer than 30 minutes for optimal quantification. The incubation time was decided on due to there being consistently high accuracies between the 10-minute incubation and 30-minute incubation periods.

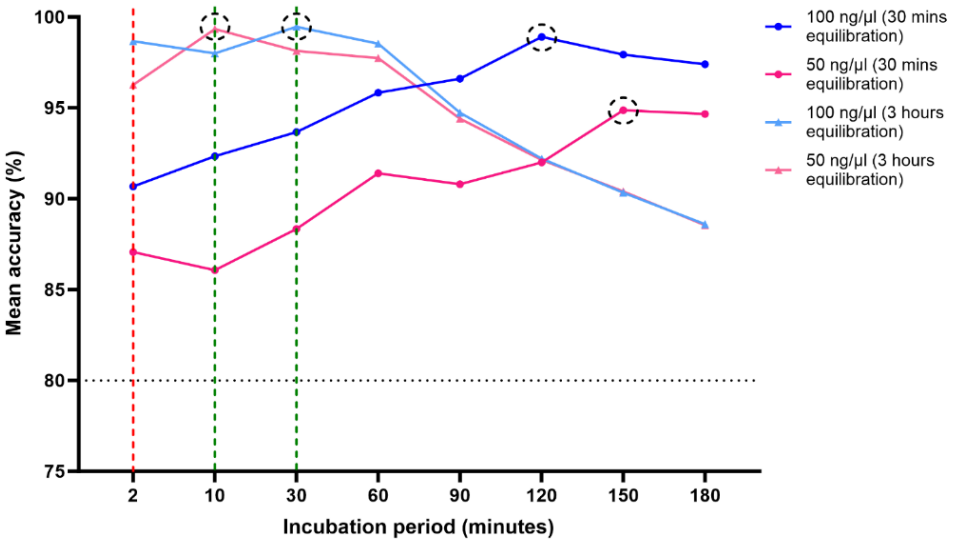

Figure 3.4 Mean accuracy of measured concentrations for 100 ng/µl and 50 ng/µl Lambda DNA samples at different time intervals and the Working Solution being equilibrated to room temperature for three hours prior to use.

The mean accuracy of Lambda DNA samples' concentration measurements is shown (n = 8). The horizontal dotted line indicates the manufacturer's criteria of acceptance for accuracy. It was observed that none of the samples were most accurate after the manufacturer's recommended 2 minutes of incubation (red dotted vertical lines), but instead were most accurate between 10 and 30 minutes (green dotted vertical lines) when the Working Solution was equilibrated to room temperature for 3 hours instead of 30 minutes. The most accurate points for each sample are circled.

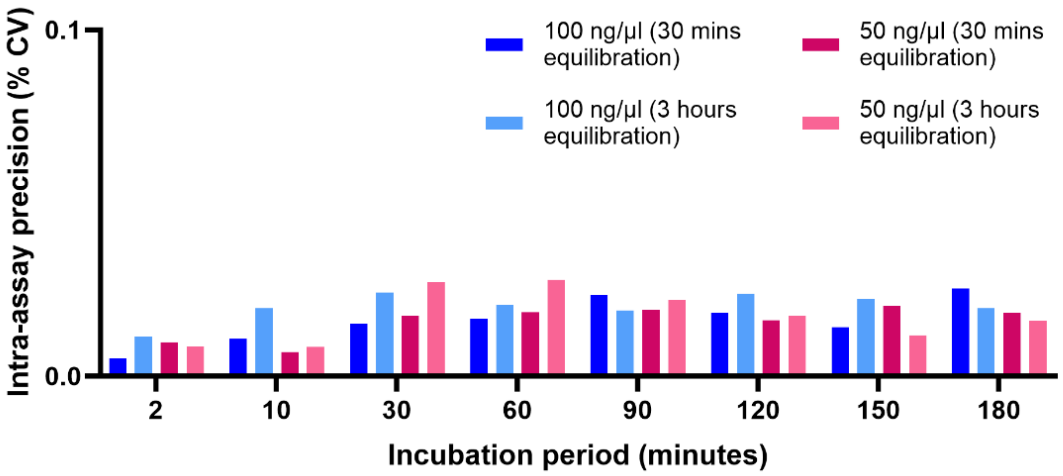

Figure 3.5 Intra-assay precision (% CV) for measured concentrations at different incubation periods. The Working Solution was equilibrated for 30 minutes vs 3 hours.

The intra-assay precision (% CV) of the 100 ng/µl and 50 ng/µl Lambda DNA samples' concentration measurements were < 0.1% CV when the Working Solution was equilibrated for 30 minutes and 3 hours. The manufacturer's threshold was < 1% CV for samples ≥ 0.5 ng/µl and < 15% CV for samples < 0.5 ng/µl.

3.2.3 Dilution medium

All samples diluted with all mediums had accuracies above the manufacturer's threshold (80%) (Figure 3.6). Given that the data was normally distributed ($p = 0.067$, distilled water data; $p = 0.678$, low EDTA TE buffer data; $p = 0.779$, TE buffer data), paired t-tests were carried out to compare the accuracy of measurements for samples diluted with the different mediums. It was observed that there was only a significant difference between the accuracy of measurements for samples diluted with TE buffer and samples diluted with low EDTA TE buffer (Table 3.1).

The intra-assay precision of measurements remained consistent for all dilution mediums for all samples except the 0.1 ng/µl sample (Figure 3.7), however, all precision estimates were still below the manufacturer's threshold (15% CV). Based on these findings, it was decided that

distilled water would be used as the dilution medium as it performed adequately and is the medium routinely used in our laboratory.

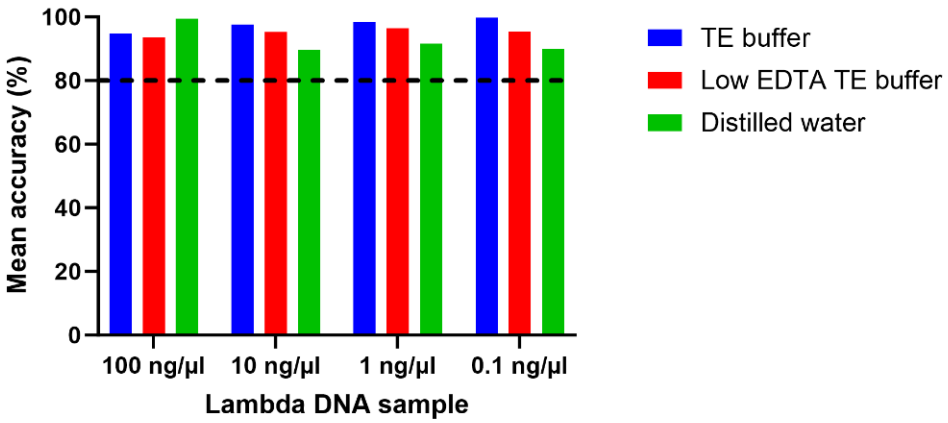

Figure 3.6 Mean accuracy of measured concentrations for Lambda DNA samples diluted with different mediums.

The mean accuracy of measurements for all Lambda DNA samples diluted with different mediums were above the accuracy threshold of 80% (horizontal dotted line) (n = 12 for TE buffer and low EDTA TE buffer; n = 9 for distilled water).

Table 3.1 Pairwise comparison of dilution buffers using paired sample t-tests.

Pairwise comparison of the samples' accuracy of concentration measurements when the samples were diluted with different mediums indicated that there was only a statistically significant difference between the accuracy of measurements when samples were diluted with TE buffer vs Low EDTA TE buffer.

Pairwise comparisons of sample's accuracies	**p - value**
Distilled water – TE buffer	0.223
Distilled water – Low EDTA TE buffer	0.429
TE buffer – Low EDTA TE buffer	**0.032***

*Statistically significant value

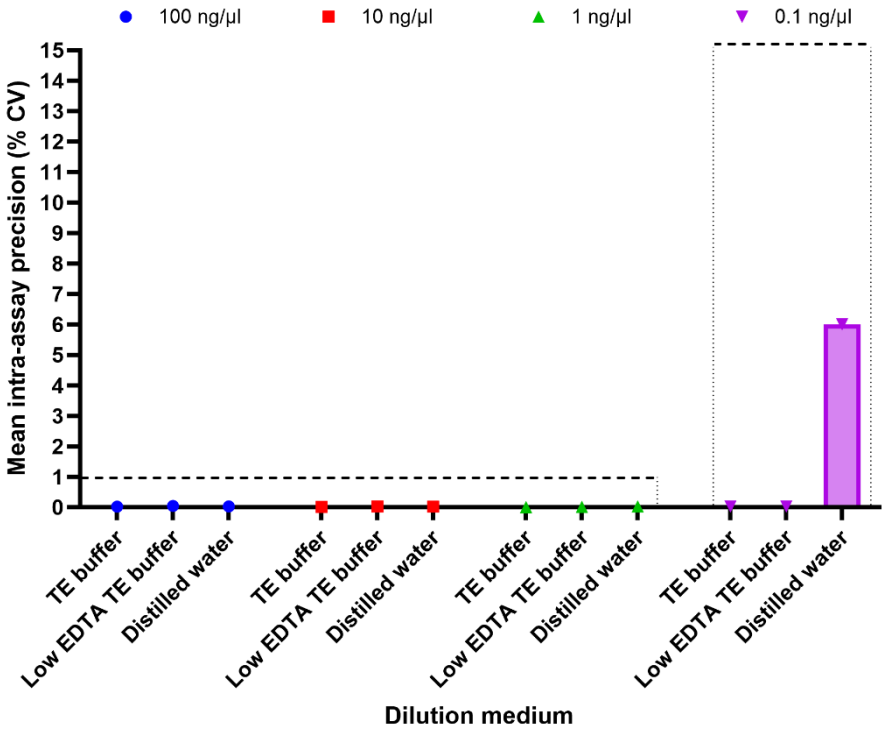

Figure 3.7 Mean intra-assay precision for Lambda DNA samples diluted with different mediums.

All samples had consistent precision for the different dilution mediums except the 0.1 ng/µl sample which spiked when diluted with distilled water. However, the mean intra-assay precision was still below the manufacturer's thresholds of 1% CV (concentration < 0.5 ng/µl) and 15% CV (concentration ≥ 0.5 ng/µl) as shown by the horizontal dotted lines (n = 12 for TE buffer and low EDTA TE buffer; n = 9 for distilled water).

3.3 DNA quantification using the Qubit™ Assay

Lambda DNA, extracted DNA and NGS library samples had either three or nine Qubit™ aliquots which were each measured four times (Appendix 6). Table 3.2 shows the average concentration, average accuracy, intra-assay precision and inter-assay precision for each sample when using M1 – M4 (n = 36), M2 – M4 (n = 27) and M1 – M3 (n = 27).

Table 3.2 Average concentration, accuracy, intra-assay precision and inter-assay precision for Lambda DNA, extracted DNA and NGS library samples for M1 – M4, M2 – M4 and M1 – M3.

Green values indicate those which met the manufacturer's criteria of acceptance and in-house established parameters. Green values underlined in red indicate those which did not meet the manufacturer's criteria of acceptance but did meet the in-house parameters established after validation experiments.

Sample		M1 - M4 (n = 36)				M2 - M4 (n = 27)				M1 – M3 (n = 27)			
		Average concentration (ng/µl)	Average accuracy (%)	Average intra-assay precision (% CV)	Average inter-assay precision (% CV)	Average concentration (ng/µl)	Average accuracy (%)	Average intra-assay precision (% CV)	Average inter-assay precision (% CV)	Average concentration (ng/µl)	Average accuracy (%)	Average intra-assay precision (% CV)	Average inter-assay precision (% CV)
Lambda DNA	100 ng/µl	98.82	98.41	2.75	3.77	98.41	97.87	2.46	3.84	99.36	98.81	2.44	3.84
	10 ng/µl	11.47	85.28	1.30	1.38	11.41	85.89	0.81	1.43	11.51	84.93	1.23	1.22
	8 ng/µl	9.06	86.70	1.83	1.55	9.01	87.39	1.54	1.86	9.10	86.19	1.58	1.35
	6 ng/µl	6.86	85.65	1.99	1.90	6.81	86.45	1.62	1.70	6.91	84.83	1.56	1.87
	4 ng/µl	4.41	89.79	1.55	2.31	4.39	90.34	1.38	2.47	4.43	89.23	1.20	2.21
	2 ng/µl	2.13	93.28	2.52	1.87	2.13	93.67	2.58	2.14	2.15	92.48	2.24	2.18
	1 ng/µl	0.85	83.88	1.40	1.41	0.85	83.64	1.50	0.85	0.86	84.00	1.25	0.90
	0.5 ng/µl	0.52	95.72	1.99	2.00	0.52	96.53	1.43	1.84	0.52	95.34	1.99	1.93
	0.1 ng/µl	0.11	90.33	6.01	7.42	0.11	89.41	5.45	8.43	0.11	90.04	6.43	8.02
	0.03 ng/µl	0.031	92.93	3.10	10.99	0.031	93.31	2.07	10.11	0.032	92.37	2.69	11.63
	0.02 ng/µl	0.022	91.31	3.53	3.45	0.022	91.41	3.86	4.00	0.022	90.93	2.90	3.00
	0.01 ng/µl	0.011	93.92	4.01	4.66	0.010	93.58	4.28	4.65	0.011	93.64	3.90	4.87
Extracted DNA	Blood	9.59	-	1.56	1.29	9.55	-	1.42	1.27	9.65	-	1.05	1.32
	FFPE tissue	56.92	-	1.87	0.25	56.56	-	1.58	0.34	57.22	-	1.35	0.89
	Tooth	0.21	-	7.54	7.78	0.21	-	5.96	7.45	0.22	-	6.56	8.24
	Bone	22.58	-	2.20	0.58	22.40	-	1.74	0.65	22.73	-	1.86	0.96
	Buccal swab	10.14	-	1.57	4.38	10.08	-	1.11	4.46	10.16	-	1.84	4.34
	Nail	0.57	-	4.43	6.01	0.56	-	3.54	6.14	0.58	-	3.42	6.13
NGS libraries	Pooled library	19.98	-	2.12	2.07	19.86	-	2.10	2.14	20.17	-	0.98	1.72
	Unpooled library	34.42	-	1.22	9.50	34.41	-	1.25	9.59	34.53	-	0.96	9.28

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

- Indicates parameter was not calculated

3.4 DNA quantification method comparisons

qPCR was used to quantify the Lambda DNA and extracted DNA samples and TapeStation was used to quantify all of the samples (Table 3.3).

Table 3.3 DNA concentrations for Lambda DNA, extracted DNA and NGS library samples as determined by Qubit™ qPCR and TapeStation.

Qubit™ concentrations are an average of M1 – M4, M2 – M4 and M1 – M3 concentrations (n = 36). qPCR was only able to quantify the extracted DNA samples (n = 1) while TapeStation was able to quantify all samples (n = 1).

Sample		Qubit™ concentration (ng/µl)	qPCR concentration (ng/µl)	TapeStation concentration (ng/µl)
Lambda DNA	**100 ng/µl**	98.86	-	45.9
	10 ng/µl	11.46	-	8.27
	8 ng/µl	9.06	-	7.72
	6 ng/µl	6.86	-	4.61
	4 ng/µl	4.41	-	3.39
	2 ng/µl	2.14	-	1.37
	1 ng/µl	0.85	-	0.613
	0.5 ng/µl	0.52	-	0.325
	0.1 ng/µl	0.11	-	0.0247
	0.03 ng/µl	0.031	-	0.122
	0.02 ng/µl	0.022	-	0.122
	0.01 ng/µl	0.011	-	0.142
Extracted DNA	**Blood**	9.60	9.31	5.72
	FFPE tissue	56.90	15.43	50.80
	Tooth	0.21	0.14	1.35
	Bone	22.57	42.25	17.70
	Buccal swab	10.13	5.54	6.59
	Nail	0.57	0.08	2.68
NGS libraries	**Pooled library**	19.99	NA	12.3
	Unpooled library	34.45	NA	23.85

- Indicates no measurement

NA Indicates that the sample was not included therefore no measurement

Pairwise comparisons of the concentration measurements obtained from the three quantification methods were carried out and it was observed that there was only a significant difference between the concentration measurements of Lambda DNA samples as measured by Qubit™ and TapeStation (Table 3.4).

Table 3.4 Pairwise comparison of concentration measurements of Lambda DNA, extracted DNA and NGS library samples as quantified by Qubit™, TapeStation and qPCR.

	p – value for pairwise comparisons		
Sample type	**Qubit™ vs qPCR**	**Qubit™ vs TapeStation**	**TapeStation vs qPCR**
Lambda DNA	-	**0.019***	-
Extracted DNA	0.249	0.753	0.463
NGS libraries	-	0.18	-

*Statistically significant value

Concentrations obtained using the Qubit™ Assay (n = 36) and TapeStation (n = 1) were also assessed using a Bland-Altman plot (Figure 3.8). The mean difference between the concentration measurements for the two methods was 4.096, indicating that Qubit™ consistently produced higher measurements than TapeStation. Furthermore, the broad range between the upper and lower limits of agreement (LOAs) show that there were large differences between the two methods' measurements (Table 3.5). The mean concentration measurement for the 100 ng/µl sample fell outside the upper LOA limit, suggesting the difference between the measurements for this sample was substantially higher than the difference between the measurements for the other samples.

Table 3.5 Parameters used to construct Bland-Altman plot comparing the concentration measurements for Lambda DNA samples measured by Qubit™ and TapeStation.

The mean difference refers to the mean of the differences between the two sets of concentration measurements. and lower and upper limit of agreement (LOA) for Bland-Altman plots depicting agreement between measured and expected Lambda DNA concentrations.

	Mean difference	Lower LOA	Upper LOA
Qubit™ - TapeStation	4.096	-19.16667	27.35855

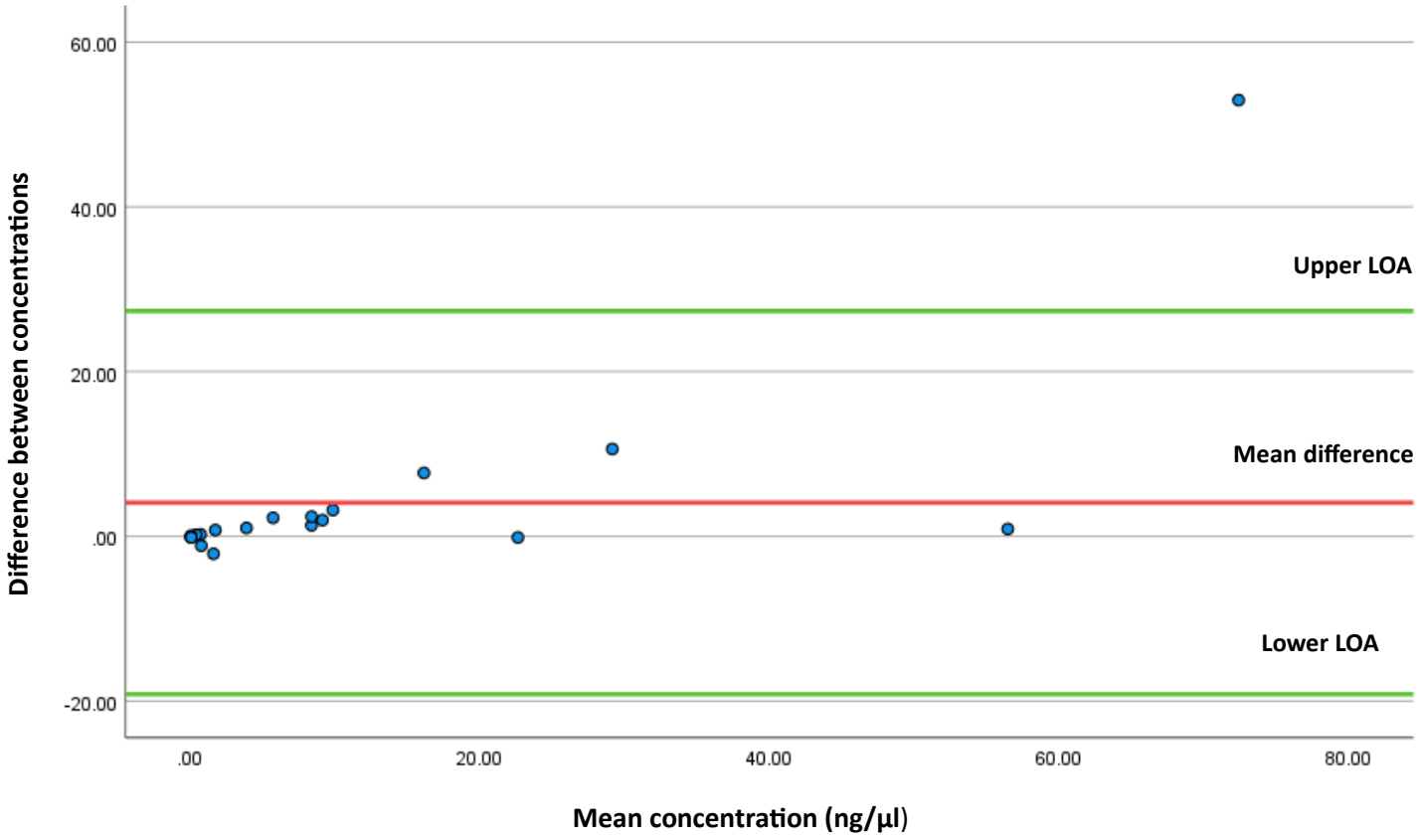

Figure 3.8. Bland-Altman plot depicting agreement between concentrations measured by Qubit™ and TapeStation.

The difference between the concentrations measured by Qubit™ (n = 36) and TapeStation (n = 1) are plotted against the mean of the concentrations from the two methods. The dispersion of the dots close around the mean (red horizontal line) indicates consistent variability between the measurements of the two methods. However, a mean difference of 4.096 demonstrates that Qubit™ constantly had higher measurements than TapeStation. The 100 ng/µl sample fell above the upper limit of agreement (LOA) and may be due to the difference between the measurements being substantially higher than the difference between the other measurements.

3.5 Accuracy of the Qubit™ Assay

The average accuracy for each sample was determined and ranged from 83.88% - 98.41% for M1 – M4, from 83. 64% - 97.87% for M2 – M4 and from 84% - 98.81% for M1 – M3 (Table 3.2). The accuracy for each sample's replicates is shown in Appendix 7. The 1 ng/µl sample was the least accurate while the 100 ng/µl sample was the most accurate for all measurement groupings (Figure 3.9). Statistical analysis revealed that the accuracies for M1 – M3 were significantly different from M1 – M4 accuracies and M2 - M4 accuracies (Table 3.6). However, the overall accuracy for all samples' measurements was 90.54% (M1 – M4), 90.79% (M2 – M4) and 90.23% (M1 – M3) (Table 3.11). Therefore, despite a statistical significance, the difference between the mean accuracies was negligible.

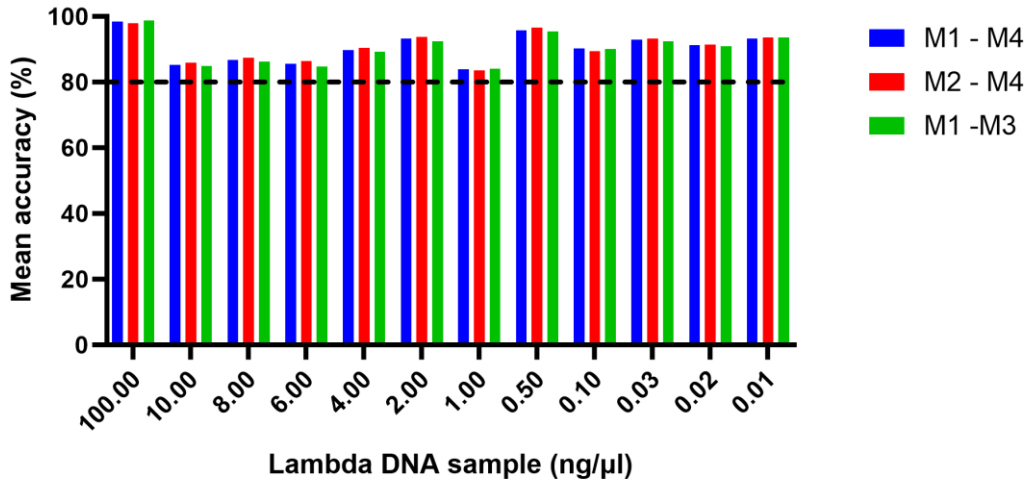

Figure 3.9. Mean accuracy of Lambda DNA sample quantified with the Qubit™ 1X dsDNA HS Assay Kit. M1 – M4 vs M2 – M4 vs M1 – M3.

The mean accuracy of Lambda DNA samples' measurements (M1 – M4, n =36 vs M2 – M4, n = 27 vs M1 – M3, n = 27) were all above the manufacturer's criteria of 80% (indicated by horizontal dotted line). The 100 ng/µl was the most accurate sample with the 1 ng/µl being the least accurate.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Table 3.6 Pairwise comparisons for accuracy of concentration measurements of Lambda DNA samples when using different measurements.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Pairwise comparisons	p – value
M1 – M4 vs M2 – M4	0.1452
M1 – M4 vs M1 – M3	**0.0261***
M2 – M4 vs M1 – M3	**0.0423***

* Statistically significant value

Bland-Altman plots (Appendix 8) indicated that the DNA concentration readings obtained using the Qubit™ Assay were similar and close in agreement with the expected concentrations for all measurement groupings. Thus, further supporting that the Qubit™ Assay had an acceptable degree of accuracy. Furthermore, negligible differences were observed between the measured and expected concentrations between the measurement groupings.

3.6. Precision of the Qubit™ Assay

3.6.1. Intra-assay precision

The intra-assay precision of measurements was significantly different for M1 – M4 vs M2 – M4 and M1 – M3 vs M1 – M4 (Table 3.7). Thus, on average the intra-assay precision for M2 – M4 and M1 – M3 samples was greater than that of M1 – M4 samples (Figure 3.10 and Table 3.2). The intra-assay precision of measurements for all samples with concentrations < 0.5 ng/µl was below the manufacturer's threshold of 15% CV (Figure 3.10). However, the precision of measurements for samples with concentrations ≥ 0.5 ng/µl were above the manufacturer's threshold (1% CV) for almost all samples across the measurement groupings. Interestingly, it was observed that the precision of measurements for samples with concentrations < 0.5 ng/µl

were the most variable across the measurement groupings. The samples that had the most precise measurements were the unpooled library sample (M1 – M4 and M1 – M3) and the 10 ng/µl Lambda DNA sample (M2 – M4).

Given the findings from the validation experiments, it was established that acceptable intra-assay precision thresholds for our setting were < 4% CV for all sample types with a concentration ≥ 0.5 ng/µl and < 15% CV for all sample types with a concentration < 0.5 ng/µl.

Table 3.7 Pairwise comparisons for the intra-assay precision of concentration measurements of Lambda DNA samples when using different measurements.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Pairwise comparisons	p - value
M1 – M4 vs M2 – M4	**0.004***
M1 – M4 vs M1 – M3	**0.002***
M2 – M4 vs M1 – M3	0.881

* Statistically significant value

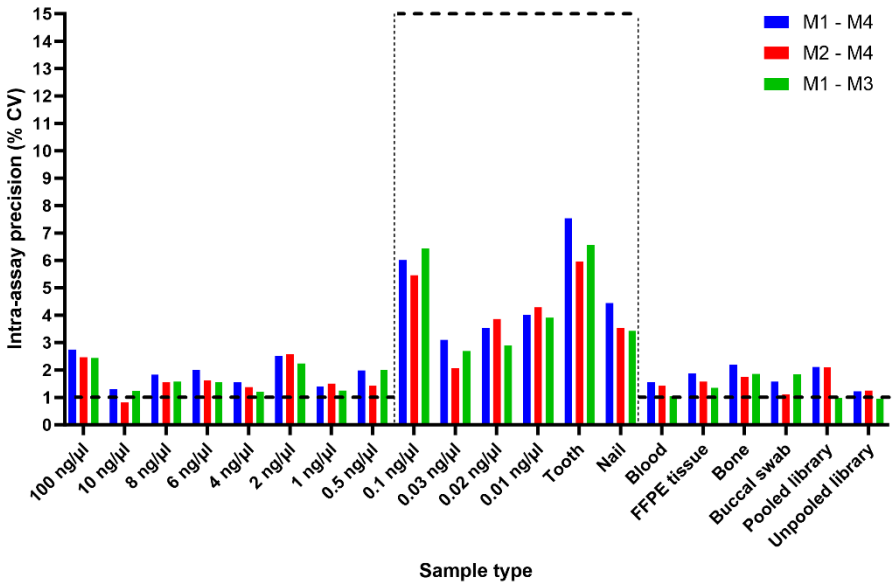

Figure 3.10. Mean intra-assay precision for Lambda DNA, extracted DNA and NGS library samples. M1 – M4 vs M2 – M4 vs M1 – M3.

Most samples with concentrations ≥ 0.5 ng/µl had measurement precision estimates above the manufacturer's threshold of 1% CV (horizontal dotted line). The precision of measurements for samples with concentrations < 0.5 ng/µl were all below the manufacturer's threshold (15% CV). However, the precision of measurements for samples with concentrations < 0.5 ng/µl were the most variable across the measurement groupings.

M1 = measurement 1. M2 = measurement 2. M3 = measurement 3 and M4 = measurement 4

3.6.2 Inter-assay precision

There were no statistically significant differences between the inter-assay precision of measurements for across the measurement groupings (Table 3.8). The inter-assay precision of measurements for all samples with concentrations < 0.5 ng/µl was below the manufacturer's threshold of 15% CV (Figure 3.11). However, the precision of measurements for samples with concentrations ≥ 0.5 ng/µl were above the manufacturer's threshold (1% CV) for almost all samples across the measurement groupings. The samples that had the most precise measurements were the extracted DNA samples from bone and FFPE tissues and the 1 ng/µl Lambda DNA sample. Despite high intra-assay precision of measurements, the unpooled NGS

library samples had one of the highest degrees of variability between sample replicate measurements.

Given the findings from the validation experiments, it was established that acceptable inter-assay precision thresholds for our setting were < 4% CV for Lambda DNA samples with a concentration ≥ 0.5 ng/µl, < 15% CV for extracted DNA and NGS library samples with a concentration ≥ 0.5 ng/µl and < 15% CV for all sample types with a concentration < 0.5 ng/µl.

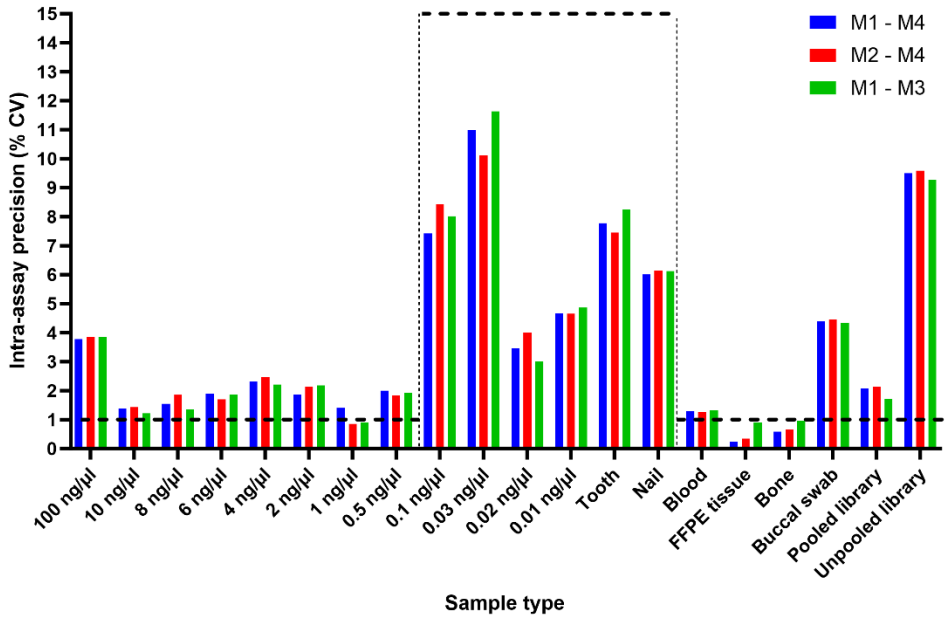

Figure 3.11. Average inter-assay precision for Lambda DNA, extracted DNA and NGS library samples.

The precision of measurements for samples with concentrations < 0.5 ng/µl were all below the manufacturer's threshold of 15% CV (horizontal dotted line). These samples, however, also had a lot of variability between the precision of measurements across the measurement groupings. Extracted DNA from bone and FFPE tissue as well as the 1 ng/µl Lambda DNA sample had the most precise measurements between sample replicates. The unpooled library had the lease precise measurements between sample replicates.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Table 3.8 Pairwise comparisons for the inter-assay precision of concentration measurements of Lambda DNA samples when using different measurements.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Pairwise comparisons	p - value
M1 – M4 vs M2 – M4	0.351
M1 – M4 vs M1 – M3	0.055
M2 – M4 vs M1 – M3	0.97

The intra-class correlation coefficient (ICC) for all samples was determined (Appendix 10). Poor reliability was observed in 90% of M1 – M4 samples, 95% of M2 – M4 samples and 85% of M1 – M3 samples. The 6 ng/µl Lambda DNA sample had moderate reliability (ICC 0.5 – 0.75) for all measurement groupings. The extracted DNA from bone samples also had moderate reliability for M1 – M4 and M1 – M3. Additionally, some samples were observed to have greater variability for the measurements between replicate than the measurements within replicates (indicated by negative ICC values).

3.7 Range and sensitivity

The Qubit™ 1X dsDNA HS Assay Kit was able to accurately quantify all replicates for the 100 ng/µl (1 µl), 0.02 ng/µl (10 µl) and 0.01 ng/µl (20 µl) Lambda DNA samples (Table 3.9). The intra- and inter-assay precision for the 0.01 ng/µl and 0.02 ng/µl samples met the manufacturer's criteria (< 15% CV) while the 100 ng/µl sample did not meet the manufacturer's threshold of 1% CV. However, after in-house parameters were established based on validation experiment findings, the 100 ng/µl was observed to be precise. Therefore, the quantification range for the Qubit™ Assay was accepted as 0.01 - 100 ng/µl.

Table 3.9. Accuracy and precision of Lambda DNA samples used to determine the range and sensitivity.

All samples were accurately and precisely quantified according to parameters established in-house after validation experiments. Therefore, the range of the Qubit™ Assay was accepted as 0.01 – 100 ng/µl.

			Precision	
Sample (sample volume)	**Replicate**	**Accuracy (%)**	**Intra-assay (% CV)**	**Inter-assay (% CV)**
100 ng/µl (1 µl)	**1**	96.32	3.24	2.88
	2	99.53	2.35	2.59
	3	99.38	2.65	5.82
0.02 ng/µl (10 µl)	**1**	86.92	4.64	4.11
	2	93.08	2.85	2.82
	3	93.92	3.11	3.42
0.01 ng/µl (20 µl)	**1**	98.5	4.81	3.99
	2	97.33	2.88	3.53
	3	97.67	2.83	5.74

Green values indicate those which met the in-house established parameters.

Green values underline in red indicate those which did not meet the manufacturer's criteria but did meet the in-house established parameters.

3.8 Criteria of acceptance

The overall accuracy, range and sensitivity of the Qubit™ Assay met the criteria of acceptance set by the manufacturer (Table 3.10). The intra- and inter-assay precision for M1 – M4, M2 – M4 and M1 – M32 samples that were < 0.5 ng/µl were also in agreement with the criteria of acceptance. However, the intra- and inter-assay precision for samples that were ≥ 0.5 ng/µl were higher than the accepted % CV set by the manufacturer. Therefore, in-house parameters were established for intra- and inter-assay precision based on the findings from the validation experiments (Table 3.11).

Table 3.10. Comparison of manufacturer's criteria of acceptance to obtained accuracy, precision, range and sensitivity.

Performance parameters obtained for M1 – M4, M2 – M4 and M1 – M3 compared to manufacturer's criteria.

M1 = measurement 1, M2 = measurement , M3 = measurement 3 and M4 = measurement 4

Parameters	Manufacturers obtained values	M1 – M4	M2 – M4	M1 – M3
Accuracy	**> 80 %**	90.54%	90.79%	90.23%
Intra-assay precision (samples < 0.5 ng/µl)	**≤ 15 % CV**	4.77% CV	4.19% CV	4.32% CV
Intra-assay precision (samples ≥ 0.5 ng/µl)	**≤ 1 % CV**	1.85% CV	1.61% CV	1.54% CV
Inter-assay precision (samples < 0.5 ng/µl)	**≤ 15 % CV**	6.72% CV	6.79% CV	6.98% CV
Inter-assay precision (samples ≥ 0.5 ng/µl)	**≤ 1 % CV**	2.45% CV	2.47% CV	2.43% CV
Range	**0.01 ng/µl – 100 ng/µl**	0.01 ng/µl – 100 ng/µl (0.2 ng – 100 ng)		
Sensitivity	**0.01 ng/ µl (0.2 ng)**	0.01 ng/ µl (0.2 ng)		

Green values indicate those which met the manufacturer's criteria

Red values indicate those which did not meet the manufacturer's criteria

Table 3.11. In-house established performance parameters

Performance parameters established in-house for M1 – M4, M2 – M4 and M1 – M3 based on validation experiment findings. Different criteria were established for the control Lambda DNA and forensic (extracted DNA and NGS library samples) samples. The criteria were also established based on the concentrations of the samples.

Parameters	In-house established parameters	M1 – M4	M2 – M4	M1 – M3
Accuracy	**> 80 %**	90.54%	90.79%	90.23%
Intra-assay precision (samples < 0.5 ng/µl)	**≤ 15 % CV**	4.77% CV	4.19% CV	4.32% CV
Intra-assay precision (samples ≥ 0.5 ng/µl)	**≤ 4 % CV**	1.85% CV	1.61% CV	1.54% CV
Inter-assay precision (samples < 0.5 ng/µl)	**≤ 15 % CV**	6.72% CV	6.79% CV	6.98% CV
Inter-assay precision (Lambda DNA samples ≥ 0.5 ng/µl)	**≤ 4 % CV**	2.02% CV	2.02% CV	1.94% CV
Inter-assay precision (Extracted DNA and NGS library samples ≥ 0.5 ng/µl)	**≤ 15% CV**	3.01% CV	3.07% CV	3.09% CV
Range	**0.01 ng/µl – 100 ng/µl**	0.01 ng/µl – 100 ng/µl (0.2 ng – 100 ng)		
Sensitivity	**0.01 ng/ µl (0.2 ng)**	0.01 ng/ µl (0.2 ng)		

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Green values indicate those which met the in-house established parameters

Chapter 4. Discussion

SUD cases are ever rising and pose a challenge to forensic pathologists when determining COD (Martínez-Barrios *et al.*, 2023). Molecular autopsies can overcome this challenge by looking to an individual's DNA for a genetic COD. Molecular autopsy workflows make use of NGS to identify potential pathogenic variants. Appropriate library concentrations are required for successful DNA sequencing (Young, Stewart & Dimalanta, 2017). Assessing the findings from this validation study, in the context of the literature as well as the manufacturer's findings, allows us to determine if the Qubit™ Assay is fit for purpose and can be incorporated into the molecular autopsy workflow to quantify extracted DNA samples and most importantly, NGS libraries.

4.1 Optimisation of the Qubit™ Assay workflow

Optimisation is the process whereby a method, process or reagent is tested and the best protocol and parameters for optimal results is selected. Optimisation may be carried out when a new instrument, method or reagent is introduced into a laboratory. In this validation study, the Qubit™ Assay was optimised on the Fluorometer. Optimisation involved assessing three parameters in the manufacturer's protocol: the incubation period of the aliquots to be quantified, the duration of the Qubit™ Assay's Working Solution to equilibrate to room temperature and the medium used to dilute samples.

4.1.1 Incubation and Working Solution temperature equilibration periods

The incubation period was important to assess as Qubit™ fluorometry makes use of a fluorescent dye that intercalates with the dsDNA and sufficient time needs to be allocated for that process to occur successfully, resulting in accurate quantification (Nakayama *et al.*, 2016; Thermo Fisher Scientific, 2020). The manufacturer's instructions advised a 2-minute incubation period (Thermo Fisher Scientific, 2022); however, it was observed that when following this timeframe, the Lambda DNA samples that were quantified initially gave high concentration measurements which steadily decreased with time (Figure 3.1 and Appendix 3). This phenomenon made it difficult to discern the true concentration of the sample. The manufacturer stated that an aliquot to be quantified is stable for up to three hours (Thermo Fisher Scientific, 2022), but during the incubation period experiments it was observed that the concentration measurements for the samples drastically changed between the first and second measurements which were taken after two and 10 minutes of incubation respectively (Appendix 3).

Lambda DNA samples were used for incubation period experiments as they had known concentrations, allowing for the accuracy of measured concentrations to be determined. The calculated accuracy was then used to evaluate the suitability of the incubation period of the samples. An increase in accuracy of the sample's concentration measurement was observed as the incubation period increased (Figure 3.2). As the accuracy increased after 10 minutes, as seen in Figure 3.2, it was hypothesised that this increase may be due to the temperature of the Qubit™ Assay's Working Solution further reaching equilibrium with room temperature as the aliquots were incubating. It is a recommendation by the manufacturer that reagents be equilibrated to room temperature before use, thus it was hypothesised that 30 minutes was

insufficient for the Working Solution to equilibrate to room temperature fully from a starting temperature of 4°C (Thermo Fisher Scientific, 2020).

Three hours was chosen for the Working Solution equilibration period experiments as the highest accuracies during incubation period experiments were observed between 120 and 150 minutes of incubation (Figure 3.2). Lambda DNA at 50 ng/µl and 100 ng/µl were only used for the temperature equilibration period experiments as the lower concentration samples that were used in the incubation period experiments were considered to have negligible differences in the concentration measurements at each time interval despite varied accuracies (Appendix 3). Allowing the Working Solution to equilibrate fully before use resulted in highly accurate concentration measurements for the 50 ng/µl and 100 ng/µl samples after shorter incubation periods (Figure 3.4). A decrease in accuracy was observed as time progressed but that was to be expected as the Qubit™ aliquot becomes unstable over time (Thermo Fisher Scientific, 2020). The intra-assay precision of the concentration measurements for all samples were below the manufacturer's threshold with negligible differences between the precision of measurements for the different temperature equilibration periods (Figure 3.3 and Figure 3.5).

Based on the findings from the incubation period and temperature equilibration period experiments, it was observed that the manufacturer's recommendations of a 2-minute incubation period of Qubit™ aliquots and 30 minutes for the Working Solution to equilibrate to room temperature were unsuitable for accurate concentration measurements (Figure 3.2). The Qubit™ aliquot incubation period was adjusted to 10 – 30 minutes as the most accurate concentration measurements were observed between these intervals, taking into account the negligible differences between concentration measurements of sample with concentrations < 50 ng/µl (Figure 3.2, Figure 3.4 and Appendix 3). Furthermore, it is more practical to equilibrate the reagents for 3 hours before conducting experiments as the Working Solution is

light sensitive and comes in a light protective bottle, thus protecting it from light during the equilibration period. Equilibrating this single bottle is easier than protecting multiple Qubit™ aliquots (in transparent Qubit™ assay tubes) from light for 3 hours in order to get accurate measurements. Therefore, the equilibration period was increased from 30 minutes to 3 hours.

4.1.2 Dilution medium

The medium used to dilute samples during the validation experiments was also assessed as the stability of DNA varies in different mediums, and changes to the stability may affect the interaction of the target sample (dsDNA) and the Working Solution in the Qubit™ aliquot (Nakayama *et al.*, 2016; Xiang *et al.*, 2012). The Lambda DNA sample was purchased as a DNA pellet suspended in TE buffer, motivating the assessment of TE buffer and low EDTA TE buffer as potential dilution mediums. Higher accuracies were observed for all samples except the 100 ng/µl when diluted with TE buffer and low EDTA TE buffer compared to those diluted with distilled water (Figure 3.6), although not statistically significant. A similar trend was observed by Nakayama *et al.* (2016) when DNA samples diluted with TE buffer had Qubit™ measurements closer to the expected Nanodrop measurement compared to samples diluted with distilled water. This may be due to the TE buffer's ability to protect DNA from degradation, improving stability of the DNA sample (Panda, Meher & Hazra, 2019). Furthermore, it was observed that DNA samples diluted with at least 1 mM NaCl improved the accuracy of Qubit™ measurements (Nakayama *et al.*, 2016). Therefore, dilutants containing salt (such as TE buffer and low EDA TE buffer) also contribute to DNA stability.

Nevertheless, the accuracy of measurements for all samples diluted with distilled water was above the manufacturer's threshold (Figure 3.6), with no observed significant differences

between the accuracy of measurements for samples diluted with distilled water compared to those diluted with TE buffer and low EDTA TE buffer (Table 3.1). Additionally, the intra-assay precision remained consistent for all samples and dilution mediums except the 0.1 ng/µl diluted with distilled water which had a substantial increase to 6% CV (Figure 3.7). Although, this level of precision was still within the manufacturer's and in-house threshold of < 15% CV for samples with concentrations < 0.5 ng/µl. The difference between the concentration measurements for the 0.1 ng/µl sample was negligible (Appendix 5), further demonstrating that a precision of 6% CV is inconsequential in the context of the molecular autopsy workflow. Taking all these findings into consideration, an informed decision was made to use distilled water as the dilution medium due to all samples having accurate and precise readings as well as distilled water routinely being used in the laboratory. Therefore, using distilled water during validation experiments allowed fair insight into anticipated outcomes from case work applications.

After all optimisation experiments were conducted, the most appropriate and efficient protocol was determined. The optimised factors that were chosen were: (i) using distilled water to dilute samples, (ii) equilibrating the Working Solution to room temperature for at least three hours prior to use, and (iii) incubating the Qubit™ aliquot for at least 10 minutes but no longer than 30 minutes. The optimised factors ensured that a reliable validation procedure was used for the study.

4.2 Evaluation of validation parameters

Using the optimised protocol, Lambda DNA, extracted DNA and NGS library samples were prepared and quantified using the Qubit™ Assay on the Fluorometer. Validation parameters

were determined (Table 3.2) and used to assess the performance of the Qubit™ Assay on the Fluorometer in our laboratory. These parameters are essential to validation studies as they evaluate the performance of the method and provide insight into the capability, reliability and error associated with the method (Raposo & Ibelli-Bianco, 2020). Method validation is especially important in forensic laboratories due to the medico-legal significance forensic test results carry. A report by Morgan (2023) assessed the factors associated with the wrongful conviction of 732 cases and attributed the cause in over 50% of cases to poor testimony standards, outdated technology and testing methods that did not follow scientifically sound, validated, standard practices (*Frye v. United States*, 1923; *Daubert v. Merrell Dow Pharmaceuticals, Inc*, 1993).

4.2.1 Accuracy

Comparison of the validation parameters for this study to the manufacturer's parameters indicated that the Qubit™ Assay met the criteria of acceptance and had an accuracy of over 90% for all measurement groupings (Table 3.10). The lowest accuracy observed was for the 1 ng/µl sample (83 – 84%) (Table 3.2 and Figure 3.9). However, assessment of the individual accuracy of each 1 ng/µl Lambda DNA replicate indicated that there was high variability between the replicates' accuracies (71 – 97%), decreasing the overall accuracy for the sample (Appendix 7). Therefore, low precision inevitably resulted in lower accuracy.

The Qubit™ Assay had an associated error rate of 9. 46% (M1 – M4), 9.21% (M2 – M4) and 9.77% (M1 – M3) which was less than half the error rate that was anticipated by the manufacturer (20%) (Thermo Fisher Scientific, 2018c). From the samples that were used to determine accuracy, 58.33% of samples had an error rate < 10% (Table 3.2). Statistical analyses

indicated that there were significant differences between the accuracy of concentration measurements for M1 – M4 vs M1 – M3 and M2 – M4 vs M1 – M3 (Table 3.6). However, this statistical significance was negligible in the context of the validation outcomes as all measurement groupings had overall accuracies > 90%. Furthermore, Bland-Altman plots demonstrated close agreement between measured concentrations and expected concentrations for all measurement groupings (Appendix 8).

The observed accuracy and error rate of the Qubit™ Assay on the fluorometer was deemed acceptable for our workflow.

4.2.2 Precision

Samples with concentrations < 0.5 ng/µl had acceptable average intra- and average inter-assay precision estimates which were within the manufacturer's criteria of acceptance (Table 3.2, Figure 3.10 and Figure 3.11) (Thermo Fisher Scientific, 2018c). The average intra- and average inter-assay precision did not fall below the manufacturer's threshold for samples with concentrations ≥ 0.5 ng/µl (Table 3.2, Figure 3.10 and Figure 3.11) (Thermo Fisher Scientific, 2018c). Nevertheless, samples with low precision still had overall accurate measurements (Table 3.2). The laboratory's SOP for Qubit™ fluorometry recommends multiple aliquots and measurements of a sample's concentration to be taken and the average of the measurements to be taken as the concentration of the sample, further making the variation in measurements negligible. However, in-house parameters that were acceptable for our laboratory and the molecular autopsy workflow were established based on the validation experiment findings (Table 3.11). These parameters were established as the Qubit™ Assay was not developmentally validated for forensic use and the samples did have accurate concentration measurements.

Therefore, the manufacturer's precision criteria were not suitable for our setting and applications.

The effect of using M1 – M4, M2 – M4 and M1 – M3 was important for the assessment of intra- and inter-assay precision. Statistical analyses indicated that there were significant differences between the intra-assay % CV of concentration measurements for M1 – M4 vs M2 – M4 and M1 – M3 vs M1 – M4 (Table 3.7), while none of the measurement groupings had significant differences between the inter-assay % CVs (Table 3.8). Therefore, M2 – M4 and M1 – M3 measurements had better intra-assay precision than M1 – M4 measurements (Table 3.11). This observation was interesting as it would be expected that taking more measurements (M1 – M4) would yield more precise results compared to taking fewer measurements (M2 – M4 and M1 – M3) (Illumina, 2010). However, the statistical significance was negligible as the observed differences between the mean intra-assay precision for the measurement groupings were minor (0.3 – 0.5%) (Table 3.10 and Table 3.11). Furthermore, assessment of the reliability between measurements of a single aliquot revealed that there were similarities in findings across the measurement groupings (Appendix 10).

Therefore, the intra- and inter- assay precision values for samples with concentrations < 0.5 ng/µl met the manufacturer's criteria which were kept as in-house parameters while the threshold for samples with higher concentrations was established in-house as the manufacturer's criteria was deemed too stringent for our laboratory and workflow.

4.2.3 Range and sensitivity

Given the validation experiment results, the Qubit™ Assay was observed to have a range of 0.01 - 100 ng/µl (0.2 – 100 ng), as recommended by the manufacturer (Table 3.9 and Table

3.10) (Thermo Fisher Scientific, 2018c). This range was appropriate as the molecular autopsy workflow typically requires input DNA ranging from 1 - 50 ng and 3 – 40 ng/µl of NGS library samples. Extracted DNA concentrations in our setting are seldomly above 50 ng/µl except for DNA extracted from blood. However, these extracted DNA samples are diluted for our workflow. A challenge that may arise, however, is when higher sample volumes are required for more accurate and precise measurements for samples with lower concentrations as there may be limited DNA available. Additionally, when a sample is quantified the concentration of that sample is initially unknown and it cannot be determined what sample volume is required for the quantification aliquot. If too much or too little sample is used researchers may not be able to obtain a concentration measurement for that aliquot and limited sample may be wasted. However, the broad quantification range of the Qubit™ 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA, Catalogue no. Q33230) does allow for most samples to be quantified using this kit. The sensitivity of the kit was determined to be 0.01 ng/µl as all measurements were accurate and precise at that concentration (Table 3.9 and Table 3.11).

4.3 Evaluation of suitability of the assay in our forensic laboratory

4.3.1 Quantification of DNA extracted from forensic samples

Samples commonly used for molecular autopsies are blood, buccal swabs and FFPE tissue or frozen tissue. The amount of DNA that is extracted is variable between different sample types, and between samples of the same biological origin. Typically, these sample types have high DNA yields (Table 4.1) which may exceed the dynamic range of the Qubit™ Assay, however, this can be easily overcome by diluting the sample prior to quantification. The issue arises when samples have too low a concentration and a higher sample volume is required.

Table 4.1 DNA yield ranges for different forensic samples as seen in the literature.

Sample type	DNA yield	Reference
Bone	0.006 - 135.85 ng/µl 0.8 - 900 ng	Hasap *et al.* (2020); Kulstein, Hadrys and Wiegand (2018); Marshall *et al.* (2014); Mundorff and Davoren (2014)
Teeth	29.3 – 2988.1 ng/µl	Carrasco *et al.* (2020); Mundorff and Davoren (2014)
Blood	0.087 – 100.96 ng/µl	Carrasco *et al.* (2020); Ghatak, Muthukumaran and Nachimuthu (2013); Livy *et al.* (2012)
Buccal swabs	18.95 - 296.86 ng/µl	Ghatak, Muthukumaran and Nachimuthu (2013); Livy *et al.* (2012)
FFPE tissue	0.31 – 1147 ng	Ghatak, Muthukumaran and Nachimuthu (2013); Livy *et al.* (2012); (Mundorff & Davoren, 2014)
Nail	1.7 – 1500 ng	Ghatak, Muthukumaran and Nachimuthu (2013); (2012); Mundorff and Davoren (2014)

Extracted DNA from nail and tooth samples met the manufacturer's criteria for intra- and inter-assay precision while only the inter-assay precision for FFPE tissue and bone met the criteria (Table 3.2, Figure 3.10 and Figure 3.11). The observed variability between measurements of the same aliquot (intra-assay precision) or between replicates of the same sample (inter-assay precision) may be due to the heterogeneous nature of forensic samples (Heathfield *et al.*, 2021). Thus, different concentrations of DNA may be extracted from different samples collected from the same biological origin. Therefore, a precision threshold of < 1% CV for samples with concentrations ≥ 0.5 ng/µl is too stringent for quantification of such samples, especially considering the Qubit™ Assay was not developmentally validated for forensic applications (Thermo Fisher Scientific, 2023). However, similar deviations from the threshold were observed for the Lambda DNA samples (Table 3.2, Figure 3.10 and Figure 3.11) which were expected to be homogenous. Although, all Lambda DNA samples had accurate measurements

despite precision estimates above the threshold (Table 3.2, Figure 3.10 and Figure 3.11). Measurements that may not meet the criteria in terms of precision can still be fit for purpose in terms of the larger workflow.

This was demonstrated in this study as extracted DNA from forensic samples were successfully used in downstream analyses in our laboratory after being quantified using the Qubit™ Assay (Heathfield *et al.*, 2023). Therefore, the observed variability in the concentration measurements was negligible in the context of the laboratory's purposes and downstream applications. The observed variability also emphasises the importance of replicate measurements in casework to account for the heterogenous nature of forensic samples.

Assessment of the affect different measurement groupings had on the precision of measurements indicated that the greatest difference between the intra-assay precision was 0.51% CV (samples with a concentration ≥ 0.5 ng/µl) and 1.88% CV (samples with a concentration < 0.5 ng/µl) across the groupings (Appendix 9). However, the precision for samples with a concentration < 0.5 ng/µl was below the manufacturer's threshold for all groupings. Similarly, the greatest difference between the inter-assay precision across the measurement grouping was 0.79% CV (Appendix 10). Therefore, for our workflow, differences in precision for the different measurement groupings was negligible. Furthermore, the observations and findings from this study lead to the establishment of appropriate in-house precision parameters for extracted DNA samples (Table 3.11).

4.3.2 Quantification of NGS libraries

The suitability of the Qubit™ Assay was also evaluated by reviewing the quality data for the sequencing run for the NGS libraries that were quantified (Appendix 2). Assessing the quality

or success of the NGS run can indicate whether an appropriate concentration of the NGS library was used for sequencing. Low library concentrations result in lower sequencing coverage and depth as there are insufficient DNA fragments available for the sequencing (Hess *et al.*, 2020). High library concentrations negatively impact the efficiency of the sequencing run while also causing less uniform coverage. Additionally, low concentration libraries do not fully utilise the flow-cell surface area, wasting resources and the sequencing capacity (Young, Stewart & Dimalanta, 2017). On the other hand, libraries at too high concentrations crowd the flow cell resulting in difficulty interpreting the sequencing data (Young, Stewart & Dimalanta, 2017). The quality data for the pooled library sample showed that the %Q30 for the libraries that were pooled ranged from 85.9 - 87.7%, while the %Q30 for the unpooled library was 95.7% (Appendix 2). Therefore, the quantified libraries had successful sequencing runs and appropriate library concentrations were used.

The intra- and inter-assay precision for the quantified NGS libraries were above the manufacturer's threshold of 1% CV (Table 3.2, Figure 3.10 and Figure 3.11. However, the variability of the concentration measurements was negligible, especially considering the library samples were successfully used in sequencing runs as demonstrated above. Minor differences were observed between measurement groupings for inter-assay precision of pooled and unpooled library measurements as well as intra-assay precision of the unpooled library measurements (0.3 – 0.4% CV) (Appendix 9 and Appendix 10). However, a difference of 1.14% CV was observed between measurement groupings for the intra-assay precision of pooled library measurements with M1 – M3 yielding the most precise results that were also below the 1% CV threshold (Table 3.2).

4.4 Evaluation of number of measurements taken

Once validation experiments had commenced, it was observed that at times the first concentration measurement for a sample was higher than subsequent measurements (Appendix 6). Therefore, it was decided to investigate if there was a difference in the accuracy and precision of a sample's measured concentration when using all four measurements, the last three measurements only and the first three measurements only.

After this investigation, it was observed that there were negligible differences between the measurement groupings in the context of the laboratory's purpose. However, based on the findings we are confident that the initial recommendation to take three measurements (M1 – M3) is appropriate.

4.5 Quantification method comparison

TapeStation and/or qPCR were also used to measure the samples' concentrations to compare how Qubit™ quantification compares to other quantification methods used in our laboratory. qPCR was only able to quantify the extracted DNA samples as Lambda DNA does not contain the human gene targets that are used to quantify the samples; hence no results were obtained for those samples. Despite a lack of statistical significance for the differences between the concentration measurements for extracted DNA and NGS library samples from each quantification method (Table 3.3), the differences for some samples were sizable enough to be relevant in the context of our workflow (Table 3.3). This is due to the molecular autopsy workflow requiring specific DNA and sequencing library concentrations.

Statistically significant differences were observed between the Qubit™ Assay and TapeStation measurements for Lambda DNA samples (Table 3.4). This is in agreement with previous studies where noticeable discrepancies between Qubit™ and TapeStation concentration measurements were reported (Hussing *et al.*, 2018). Kemp, Beaty and Monroe (2020) also observed differences between Qubit™ and Agilent Fragment Analyzer. The Fragment Analyzer and TapeStation are both automated capillary electrophoresis quantification systems, and are therefore comparable (Hussing *et al.*, 2018). This is an interesting observation as both methods make use of the same quantification approach whereby an intercalating dye binds with the dsDNA target, giving off a fluorescent signal, therefore, it is expected that both methods should yield similar results (Nakayama *et al.*, 2016).

The main difference between the methods is that Qubit™ involves the intercalation of the fluorescent dye to the target dsDNA molecules, excitation of the fluorescently tagged dsDNA molecules and emission of a fluorescent signal used to quantify the sample (Thermo Fisher Scientific, 2020). While TapeStation involves the migration of the intercalating dye bound to the target dsDNA through a capillary, resulting in an electropherogram (Agilent Technologies, 2016). The DNA concentration of the sample is determined by the area under the peak on the electropherogram. Factors affecting the migration of the fluorescently tagged dsDNA through the capillary may impact the measured concentration and subsequently the accuracy of the quantification (Kemp, Beaty & Monroe, 2020). Contaminants in the DNA sample such as salts or DNA extraction reagents (e.g. phenol. chloroform or ethanol) can affect the mobility of the target molecule during TapeStation quantification, resulting in decreased concentration measurements (Smith & Nelson, 2004). Qubit™, on the other hand, has been shown to be less susceptible to these potential contaminants (Thermo Fisher Scientific, 2022).

Another factor to consider is that forensic samples are generally not the purest samples as they may have naturally occurring substances present that can inhibit PCR, affect intercalation of dyes as well as migration through the capillary (Vajpayee *et al*., 2023). Furthermore, due to the nature of forensic samples, DNA samples may not be from a single source meaning there may be DNA from difference species present (e.g. bacterial DNA contamination) or DNA from more than one biological donor (e.g. two donors from the same species). This is an important consideration when quantifying extracted DNA from forensic samples as concentration measurements may reflect contaminant DNA as well. Within the context of cross-species contamination, qPCR may overcome this, as primers targeting regions specific to the human genome are used, therefore, reducing the chance of contaminant DNA quantification. This factor may not affect quantification of NGS libraries though, unless WES or WGS is carried out, as primers or probes used to enrich target sequencing regions would be more specific to the species of interest.

Quantification of DNA extracted from forensic samples using TapeStation was also challenging as the range of the Genomic ScreenTape Assay was 10 - 100 ng/µl, while the DNA concentration for forensic samples is often lower than 10 ng/µl (Table 4.1). TapeStation was able to quantify all samples, however, concentrations for the 0.1 ng/µl, 0.03 ng/µl, 0.02 ng/µl and 0.01 ng/µl Lambda DNA samples and extracted DNA from the tooth and nail were substantially different from Qubit™ and qPCR measurements (Table 3.3). Qubit™ concentration measurements may be considered more reliable than TapeStation's measurements as Qubit™ was shown to be accurate through the validation experiments (Table 3.2 and Figure 3.9) while previous internal validation of TapeStation demonstrated that it was only accurate for fragment sizing and not DNA concentration quantification (Heathfield, Bennett and Martin, 2021).

Conclusion

The Qubit™ Assay quantification workflow was optimised to determine the protocol that would produce the most accurate and precise concentration measurements. The optimised Qubit™ Assay workflow has been proven to quantify samples accurately and precisely within the manufacturer's dynamic range of 0.01 – 100 ng/µl in our laboratory, within criteria that are deemed appropriate for a successful NGS workflow typical in a molecular autopsy. Although, different intra- and inter-assay precision thresholds for samples with concentrations ≥ 0.5 ng/µl were established in-house for the molecular autopsy workflow as the manufacturer's threshold of 1% CV was too stringent for forensic applications. However, the higher than anticipated variability of measurements was deemed acceptable for our laboratory, as downstream DNA sequencing results presented %Q30 scores consistently above 85%. Qubit™ is more reliable than TapeStation for quantification of extracted DNA and NGS library samples as Qubit™ has been validated for accurate quantification while TapeStation has only been validated for accurate fragment sizing. qPCR was comparable to Qubit™ for some of the extracted DNA samples.

After thorough internal validation, the Qubit™ Assay on the Fluorometer was deemed fit for purpose, thus making it appropriate for quantification of extracted DNA and NGS library samples in the molecular autopsy workflow. This study has enabled the use of this Qubit™ Assay in a forensic setting for the first time and has advanced our progression towards implementing a molecular autopsy workflow for the first time not only at the OFPI, but in South Africa as well. It is hoped that utilising molecular autopsies will assist with determining COD in SUD cases, which may provide answers for grieving family members. Moreover, it is intended that such results will guide genetic testing in relatives to identify family members carrying the same gene variants and link them with appropriate clinical intervention

(Ackerman, Tester & Driscoll, 2001). Such translation of post-mortem results for the benefit of the living is the epitome of an integrated and world-class health service, and this study has enabled one small cog to turn in the overall mechanism of our intended integrated medico-legal death investigation.

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Appendices

Appendix 1: Ethics approval letter

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

Room 45 E-52-E-Floor- Old Main Building
Groote Schuur Hospital
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20 September 2023

HREC REF: 687/2023

A/Prof L Heathfield
Department of Pathology
Falmouth Building- FHS
Email: Laura.heathfield@uct.ac.za
Student: ndxade004@myuct.ac.za

Dear A/Prof Heathfield

PROJECT TITLE: TOWARDS MOLECULAR AUTOPSIES: INTERNAL VALIDATION OF THE QUBITTM 1X DSDNA HS ASSAY KIT- LINKED TO R014/2018 (MPHIL CANDIDATE-MISS ADELE NAIDOO)

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 September 2024.

Please submit a progress report, using the standardised Annual Progress Report Forms (FHS016) **or** (FHS 017) if the study continues beyond the approval period. Please submit a Standard Closure form (FHS 010) when the study has been completed, this includes after publication or thesis submission and final completion.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: Miss Adele Naidoo also be involved in this study.

Please quote HREC REF 687/2023 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

Signed by candidate

PROFESSOR M BLOCKMAN
CHAIRPERSON, FACULTY OF HEALTH SCIENCES 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

HREC/ref 687.2023

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 2020), 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.

Appendix 2: NGS libraries sequencing run quality report summary

Table A. Summary of the quality reports for the pooled and unpooled library sample's sequencing run

Sample	Percent Q30
Unpooled library	95.7
Library 1 in pooled library	87.5
Library 2 in pooled library	87.7
Library 3 in pooled library	85.9

Appendix 3: Incubation period experiments full results

Table B. Concentration measurements, accuracy and precision for 100 ng/µl, 50 ng/µl, 10 ng/µl, 1 ng/µl and 0.01 ng/µl Lambda DNA samples from incubation experiments.

The concentration measurements, mean concentration, accuracy and intra-assay precision for the Lambda DNA samples at each time period is given. The average concentration, average accuracy and average intra-assay precision for both incubation period experiments is also given.

		Experiment 1						Experiment 2						Grand average concentration (ng/µl)	Grand average accuracy (%)	Grand average intra-assay precision (% CV)
Sample	**Time (mins)**	**Measurement 1 (ng/µl)**	**Measurement 2 (ng/µl)**	**Measurement 3 (ng/µl)**	**Mean (ng/µl)**	**Accuracy (%)**	**Intra-assay precision (% CV)**	**Measurement 1 (ng/µl)**	**Measurement 2 (ng/µl)**	**Measurement 3 (ng/µl)**	**Mean (ng/µl)**	**Accuracy (%)**	**Intra-assay precision (% CV)**			
100 ng/µl	**2**	116	114	114	114.67	85.33	0.0101	104	104	104	104	96	0.0000	109.33	90.67	0.0050
	10	112	110	110	110.67	89.33	0.0104	106	104	104	104.67	95.33	0.0110	107.67	92.33	0.0107
	30	112	110	110	110.67	89.33	0.0104	104	102	100	102	98	0.0196	106.33	93.67	0.0150
	60	110	108	106	108	92	0.0185	102	99.8	99.2	100.33	99.67	0.0147	104.17	95.83	0.0166
	90	108	106	104	106	94	0.0189	104	99.8	98.6	100.8	99.2	0.0281	103.40	96.60	0.0235
	120	104	102	100	102	98	0.0196	102	100	98.6	100.2	99.8	0.0171	101.10	98.90	0.0183
	150	102	100	100	100.67	99.33	0.0115	98.2	96.4	95	96.53	96.53	0.0166	98.60	97.93	0.0140
	180	102	98.4	97.2	99.2	99.2	0.0252	98.2	95.2	93.4	95.6	95.6	0.0254	97.40	97.40	0.0253
50 ng/µl	**2**	56.2	55.8	55.2	55.73	88.53	0.0090	56.6	57.2	57.8	57.2	85.6	0.0105	56.47	87.07	0.0098
	10	54.8	54.4	54.6	54.6	90.8	0.0037	60	59.2	58.8	59.33	81.33	0.0103	56.97	86.07	0.0070
	30	54.6	53.2	52.4	53.4	93.2	0.0209	59.2	58	57.6	58.27	83.47	0.0143	55.83	88.33	0.0176
	60	53	51.2	50.8	51.67	96.67	0.0227	57.8	56.8	56.2	56.93	86.13	0.0142	54.30	91.40	0.0184
	90	53	52.4	50.8	52.07	95.87	0.0218	58.2	56.8	56.4	57.13	85.73	0.0165	54.60	90.80	0.0192
	120	52	50.4	52	51.47	97.07	0.0179	57.4	56.4	55.8	56.53	86.93	0.0143	54.00	92.00	0.0161
	150	51.2	50	49	50.07	99.87	0.0220	56.2	54.8	54.2	55.07	89.87	0.0186	52.57	94.87	0.0203
	180	50.6	48.8	49.8	49.73	99.47	0.0181	56.2	54.8	54.2	55.07	89.87	0.0186	52.40	94.67	0.0184
10 ng/µl	**2**	11.9	11.9	11.8	11.87	81.33	0.0049	11.3	11.6	11.8	11.57	84.33	0.0218	11.72	82.83	0.0133
	10	12.1	12	11.8	11.97	80.33	0.0128	11.4	11.5	11.8	11.57	84.33	0.0180	11.77	82.33	0.0154
	30	12.1	11.8	11.7	11.87	81.33	0.0175	11.6	11.6	11.6	11.6	84	0.0000	11.73	82.67	0.0088
	60	12	11.6	11.6	11.73	82.67	0.0197	11.3	12.1	11.6	11.67	83.33	0.0346	11.70	83.00	0.0272
	90	11.8	11.5	11.4	11.57	84.33	0.0180	11.5	11.7	11.8	11.67	83.33	0.0131	11.62	83.83	0.0155
	120	11.5	11	10.9	11.13	88.67	0.0289	11.4	11.3	12.2	11.63	83.67	0.0424	11.38	86.17	0.0356
	150	11.4	11	10.9	11.1	89	0.0238	11.3	11.3	11.1	11.23	87.67	0.0103	11.17	88.33	0.0171
	180	11.3	11	10.8	11.03	89.67	0.0228	11.3	11	10.9	11.07	89.33	0.0188	11.05	89.50	0.0208
1 ng/µl	**2**	1.16	1.15	1.14	1.15	85	0.0087	1.14	1.14	1.14	1.14	86	0.0000	1.15	85.50	0.0043
	10	1.21	1.18	1.16	1.18	81.67	0.0213	1.15	1.13	1.13	1.14	86.33	0.0102	1.16	84.00	0.0157
	30	1.14	1.12	1.1	1.12	88	0.0179	1.13	1.09	1.08	1.1	90	0.0241	1.11	89.00	0.0210
	60	1.22	1.24	1.13	1.20	80.33	0.0490	1.26	1.09	1.41	1.25	74.67	0.1277	1.23	77.50	0.0884

	90	1.12	1.1	1.08	1.1	90	0.0182	1.12	1.11	1.09	1.11	89.33	0.0138	1.10	89.67	0.0160
	120	1.11	1.07	1.06	1.08	92	0.0245	1.27	1.07	1.07	1.14	86.33	0.1016	1.11	89.17	0.0630
	150	1.1	1.06	1.05	1.07	93	0.0247	1.48	1.06	1.49	1.34	65.67	0.1827	1.21	79.33	0.1037
	180	1.4	1.1	1.09	1.20	80.33	0.1472	1.07	1.11	1.25	1.14	85.67	0.0827	1.17	83.00	0.1149
	2	0.012	0.012	0.012	0.012	84	0.0000	0.011	0.010	0.011	0.011	94	0.0377	0.01	89.00	0.0189
	10	0.011	0.012	0.010	0.011	90	0.1012	0.034	0.023	0.012	0.023	-34	0.4701	0.02	28.00	0.2857
	30	0.016	0.011	0.010	0.012	77.33	0.2656	0.011	0.023	0.012	0.015	49.33	0.4453	0.01	63.33	0.3554
0.01	**60**	0.016	-	0.012	0.014	58	0.1992	0.011	-	0.034	0.023	-28	0.7071	0.02	15.00	0.4531
ng/µl	**90**	-	-	-	-	-		0.019	0.054	0.014	0.029	-87.33	0.7609	0.03	-87.33	0.7609
	120	-	-	-	-	-		0.026	0.011	0.041	0.026	-61.33	0.5664	0.03	-61.33	0.5664
	150	0.018	0.012	-	0.015	53	0.2790	0.012	0.053	0.040	0.035	-148.67	0.5883	0.02	-47.83	0.4337
	180	-	-	-	-	-		-	-	-	-	-		-	-	-

- Indicates no measurement

Green values indicate those which meet the manufacturer's criteria and in-house established parameters

Red values indicate those which do not meet the manufacturer's criteria and in-house established parameters

Appendix 4: Temperature equilibration period experiments full results

Table C. Concentration measurements for 100 ng/µl and 50 ng/µl Lambda DNA samples from temperature equilibration period experiments.

Concentration measurements, accuracy, intra-assay precision for the samples at each time period is given.

Sample	Time (mins)	Measurement 1 (ng/µl)	Measurement 2 (ng/µl)	Measurement 3 (ng/µl)	Average concentration (ng/µl)	Accuracy (%)	Intra – assay precision (% CV)
100 ng/µl	**2**	102	102	100	101.33	98.67	0.0114
	10	104	102	100	102.00	98.00	0.0196
	30	102	99.2	97.2	99.47	99.47	0.0242
	60	100	99.4	96.2	98.53	98.53	0.0207
	90	96.6	94.6	93	94.73	94.73	0.0190
	120	94.4	92.2	90	92.20	92.20	0.0239
	150	92.2	90.6	88.2	90.33	90.33	0.0223
	180	90.6	87.4	87.8	88.60	88.60	0.0197
50 ng/µl	**2**	48	47.8	48.6	48.13	96.27	0.0086
	10	50	49.2	49.8	49.67	99.33	0.0084
	30	50.6	48.4	48.2	49.07	98.13	0.0271
	60	50.4	48.4	47.8	48.87	97.73	0.0279
	90	47.8	47.8	46	47.20	94.40	0.0220
	120	46.8	46.2	45.2	46.07	92.13	0.0175
	150	45.6	45.4	44.6	45.20	90.40	0.0117
	180	45	44.2	43.6	44.27	88.53	0.0159

Appendix 5: Dilution medium experiments

Table D. Concentration measurements for Lambda DNA samples used for dilution buffer experiments.

Concentration measurements, accuracy and intra-assay precision for Lambda DNA samples diluted with TE buffer, low EDTA TE buffer and distilled water are given.

Dilution buffer	Sample	Replicate	Measurement 1 (ng/µl)	Measurement 2 (ng/µl)	Measurement 3 (ng/µl)	Measurement 4 (ng/µl)	Average (ng/µl)	Accuracy	Intra-assay precision (%CV)
TE buffer	100ng/µl	1	96	96	97.6	96.4	94.82	94.82	0.0179
		2	90.8	94	94.6	93			
		3	95	94.8	95.6	94			
	10ng/µl	1	10.1	10.3	10.2	9.83	10.25	97.51	0.0143
		2	10.4	10.3	10.4	10.5			
		3	10.2	10.5	10.3	9.96			
	1ng/µl	1	1.01	0.972	0.952	0.982	0.985	98.48	0.0051
		2	0.998	0.995	0.979	0.978			
		3	0.996	0.996	0.98	0.979			
	0.1ng/µl	1	0.1	0.097	0.101	0.097	0.1003	99.75	0.0303
		2	0.097	0.099	0.099	0.098			
		3	0.102	0.102	0.114	0.097			
Low EDTA TE buffer	100ng/µl	1	96.6	95.4	96.6	96.6	93.58	93.58	0.0427
		2	89.6	88.6	89	88.8			
		3	95	96.2	95.8	94.8			
	10ng/µl	1	10.4	10.3	10.4	10.2	10.48	95.25	0.0248

		2	10.8	10.8	10.5	11			
		3	10.4	10.4	10.4	10.1			
	1ng/µl	**1**	1.03	1.15	1.02	1.01			
		2	1.03	1	1.02	1.04	1.04	96.42	0.0147
		3	1.03	1.03	1.05	1.02			
	0.1ng/µl	**1**	0.108	0.111	0.101	0.106			
		2	0.105	0.101	0.099	0.101	0.1045	95.45	0.0263
		3	0.109	0.106	0.103	-			
Distilled water	**100ng/µl**	**1**	102	99.2	97.2	NA	99.47	99.47	0.0242
	10ng/µl	**1**	11.3	11	10.8	NA	11.05	89.5	0.0208
		2	11.3	11	10.9	NA			
	1ng/µl	**1**	1.1	1.06	1.05	NA	1.085	91.5	0.0244
		2	1.13	1.09	1.08	NA			
	0.1ng/µl	**1**	0.0955	0.0918	0.1053	NA			
		2	0.0943	0.0983	0.1068	NA	0.1078	92.17	6.01
		3	0.1345	0.1138	0.1305	NA			

- Indicates no measurement obtained

NA indicates that measurement was not taken

Appendix 6: Qubit™ 1X dsDNA HS Assay Kit DNA quantification measurement results

Table E. Concentration measurements for Lambda DNA, extracted DNA and NGA library samples quantified using the Qubit™ 1X dsDNA HS Assay Kit.

Concentration measurements for each sample's aliquots and replicates.

				Aliquot 1 (ng/µl)				Aliquot 2 (ng/µl)				Aliquot 3 (ng/µl)			
Sample		**Sample volume (µl)**	**Replicate**	**M1**	**M2**	**M3**	**M4**	**M1**	**M2**	**M3**	**M4**	**M1**	**M2**	**M3**	**M4**
Lambda DNA	**100 ng/µl**	**1**	**1**	100	98.2	93.4	93.2	96.4	96.4	90.8	90.8	100	102	99	95.6
			2	102	96.8	96.2	96.2	100	100	98	95.2	102	104	100	104
			3	106	102	104	104	104	106	106	100	90	97.4	92	96
	10 ng/µl	**2**	**1**	11.5	11.3	11	10.7	11.4	11.4	11.4	11.3	11.7	11.2	11.4	11.4
			2	11.4	11.2	11.2	11.1	11.8	11.6	11.6	11.5	11.7	11.3	11.3	11.4
			3	11.8	11.6	11.7	11.6	11.6	11.5	11.6	11.6	12	11.7	11.8	11.7
		10	**1**	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH
			2	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH
			3	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH	TH
	8 ng/µl	**2**	**1**	9.41	9.18	9.24	8.89	9.2	8.96	9.07	8.79	9.55	9.1	9.22	9.45
			2	9.1	8.86	8.94	8.74	9.06	8.96	9.04	8.8	8.77	8.77	8.7	8.56
			3	9.4	9.27	8.93	9.01	9.26	9.43	9.17	9.28	9.32	9.07	8.85	8.96
	6 ng/µl	**2**	**1**	6.87	6.75	6.49	6.51	7.01	6.79	6.7	6.58	7.26	7	6.85	6.84
			2	6.79	6.81	6.78	6.65	7.02	7.02	6.87	6.75	7.04	6.99	6.91	6.79
			3	6.89	6.87	6.78	6.61	7.08	6.96	6.82	6.76	7.08	7.02	7.12	6.93
	4 ng/µl	**2**	**1**	4.39	4.28	4.33	4.26	4.48	4.39	4.28	4.26	4.41	4.33	4.38	4.41
			2	4.6	4.61	4.47	4.43	4.58	4.56	4.55	4.45	4.35	4.36	4.33	4.24
			3	4.42	4.37	4.43	4.34	4.62	4.57	4.6	4.51	4.42	4.33	4.19	4.17

2 ng/µl	**2**	**1**	2.08	2.46	2.02	1.97	2.11	2.11	2.09	2.04	2.07	2.04	2.04	2
		2	2.02	2	2	1.96	2.14	2.1	2.07	2.07	2.14	2.15	2.09	2.09
		3	2.27	2.26	2.26	2.2	2.3	2.27	2.19	2.22	2.29	2.23	2.26	2.23
1 ng/µl	**2**	**1**	1.01	1.03	1.03	1.01	1.01	1.02	1.04	0.996	1.02	1.02	1.05	1.03
		2	0.801	0.815	0.812	0.796	0.845	0.841	0.808	0.82	0.832	0.825	0.835	0.814
		3	0.735	0.715	0.735	0.715	0.718	0.716	0.719	0.703	0.723	0.725	0.709	0.707
0.5 ng/µl	**2**	**1**	0.54	0.527	0.514	0.512	0.552	0.54	0.546	0.534	0.551	0.537	0.531	0.53
		2	0.564	0.542	0.535	0.535	0.521	0.514	0.51	0.502	0.533	0.532	0.509	0.509
		3	0.509	0.509	0.499	0.498	0.505	0.495	0.495	0.504	0.527	0.502	0.49	0.517
0.1 ng/µl	**2**	**1**	0.104	0.093	0.093	0.092	0.088	0.094	0.094	0.091	0.1	0.108	0.108	0.105
		2	0.094	0.095	0.089	0.099	0.099	0.1	0.098	0.096	0.108	0.105	0.108	0.106
		3	0.124	0.149	0.125	0.14	0.116	0.113	0.11	0.116	0.115	0.121	0.174	0.112
0.03 ng/µl	**2**	**1**	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
		2	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
		3	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
	10	**1**	0.0358	0.0342	0.0338	0.0334	0.0302	0.0296	0.0292	0.0294	0.0334	0.0338	0.0348	0.0312
		2	0.031	0.0318	0.031	0.0308	0.0354	0.0342	0.034	0.0328	0.034	0.0334	0.0328	0.0318
		3	0.0336	0.0314	0.0312	0.0306	0.0204	0.0222	0.0226	0.0224	0.0336	0.0334	0.0322	0.0318
0.02 ng/µl	**2**	**1**	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
		2	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
		3	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
	10	**1**	0.0222	0.0236	0.0202	0.0202	0.0226	0.023	0.0246	0.0228	0.0226	0.0238	0.0228	0.023
		2	0.0224	0.022	0.0222	0.0212	0.0208	0.0206	0.0202	0.0214	0.0214	0.0208	0.0226	0.021
		3	0.0218	0.0214	0.0208	0.0208	0.021	0.0204	0.0208	0.0198	0.0214	0.0216	0.0214	0.0234

	0.01 ng/µl	**2**	**1**	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
			2	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
			3	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
		10	**1**	0.0126	0.0124	0.0108	0.0114	0.0112	0.0116	0.0104	0.0108	0.011	0.0106	0.0102	0.0106
			2	TL	TL	TL	TL	0.0104	0.0106	TL	TL	TL	TL	TL	TL
			3	0.0108	0.011	0.01	TL	0.011	0.0114	0.011	0.0116	0.0118	0.0122	0.0114	0.011
		20	**1**	0.01	0.0095	0.0105	0.0101	0.0112	0.0098	0.0096	0.0099	0.0095	0.0094	0.009	0.0097
			2	0.0107	0.0103	0.0101	0.01	0.0108	0.0111	0.0102	0.0104	0.0101	0.0098	0.01	0.0097
			3	0.0107	0.0109	0.0106	0.0111	0.0099	0.0098	0.0097	0.0092	0.01	0.0106	0.0104	0.0099
Extracted DNA	**Blood**	**2**	**1**	9.49	9.68	9.47	9.19	9.83	9.74	9.71	9.5	9.53	9.53	9.49	9.39
			2	9.66	9.48	9.57	9.48	9.72	9.8	9.55	9.21	9.91	9.91	9.67	9.44
			3	9.88	9.68	9.79	9.72	9.73	9.47	9.44	9.32	9.74	9.58	9.57	9.48
	FFPE tissue	**2**	**1**	58	58	57	55	58	57	57	56	58	56	56	57
	Tooth	**2**	**1**	0.207	0.197	0.188	0.181	0.246	0.241	0.208	0.192	0.238	0.22	0.212	0.218
	Bone	**2**	**1**	23.1	22.4	22.6	22.5	23.2	23.2	22.5	21.7	23	22.6	22	22.1
	Buccal swab	**2**	**1**	10.8	10.8	10.4	10.6	9.96	9.88	9.68	9.76	10.2	9.92	9.84	9.88
	Nail	**2**	**1**	0.593	0.601	0.546	0.511	0.496	0.461	0.446	0.434	0.582	0.56	0.563	0.554
			2	0.624	0.603	0.582	0.56	0.596	0.574	0.552	0.523	0.589	0.568	0.553	0.53
			3	0.61	0.586	0.574	0.569	0.651	0.633	0.611	0.59	0.589	0.588	0.602	0.568
NGS library	**Pooled library**	**2**	**1**	19.9	19.8	19.6	18.7	20.5	20.5	20.1	19.6	20.6	20.2	20.3	19.9
	Unpooled library	**2**	**1**	33	33	32.6	31.8	34.6	35.8	35.4	34.8	35.6	35.6	36	35.6
			2	37.4	36.8	37.4	37.4	32.6	33	33.4	33.2	36.4	36.4	36.8	35.8
			3	35.4	35	35.4	35	27.2	27.2	26.6	25.8	38	37.6	38.2	37.4

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

TH = “Too High”

TL = “Too Low”

Appendix 7: Accuracy full results

Table F. Accuracy for quantified Lambda DNA samples.

Accuracy given for each sample's replicates are given. The accuracy for samples after using different sample volumes is also given. The accuracy of measurements is compared across the measurement groupings.

		M1 - M4				M2 - M4				M1 – M3			
Sample	**Sample volume (µl)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Replicate 3 (%)**	**Sample average (%)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Replicate 3 (%)**	**Sample average (%)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Replicate 3 (%)**	**Sample average (%)**
100 ng/µl	**1**	96.32	99.53	99.38	98.41	95.49	98.93	99.18	97.87	97.36	99.89	99.18	98.81
10 ng/µl	**2**	86.92	85.75	83.17	85.28	87.67	86.44	83.56	85.89	86.33	85.33	83	84.93
	10	-	-	-	-	-	-	-	-	-	-	-	-
8 ng/µl	**2**	85.35	89.27	85.47	86.70	86.25	89.76	86.15	87.39	84.82	88.61	85.14	86.19
6 ng/µl	**2**	86.60	85.53	84.83	85.65	87.94	85.98	85.43	86.45	85.7	84.76	84.04	84.83
4 ng/µl	**2**	91.25	88.48	89.65	89.79	91.89	88.89	90.25	90.34	90.92	87.75	89.03	89.23
2 ng/µl	**2**	95.71	96.54	87.58	93.28	95.72	97.06	88.22	93.67	94.33	96.06	87.06	92.48
1 ng/µl	**2**	97.78	82.03	71.83	83.88	97.49	81.84	71.60	83.64	97.44	82.38	72.17	84
0.5 ng/µl	**2**	93.10	94.90	99.17	95.72	93.98	95.82	99.80	96.53	92.49	94.22	99.31	95.34
0.1 ng/µl	**2**	97.50	99.75	73.75	90.33	97.56	99.56	71.11	89.41	98	99.56	72.56	90.04
0.03 ng/µl	**2**	-	-	-	-	-	-	-	-	-	-	-	-
	10	92.00	90.83	95.94	92.93	92.81	91.63	95.48	93.31	90.81	89.78	96.52	92.37
0.02 ng/µl	**2**	-	-	-	-	-	-	-	-	-	-	-	-
	10	86.92	93.08	93.92	91.31	86.67	93.33	94.22	91.41	85.89	92.78	94.11	90.93
0.01 ng/µl	**2**	-	-	-	-	-	-	-	-	-	-	-	-

	10	86.92	-	93.08	90.00	90.22	-	88.78	89.50	88	-	88.22	88.11
	20	98.50	97.33	97.67	97.83	97.22	98.22	97.56	97.67	98.33	96.56	97.11	97.33

- Indicates no measurement

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

Red values indicate those which did not meet the manufacturer's criteria of acceptance or in-house established parameters.

Green values indicate those which met the manufacturer's criteria of acceptance and in-house established parameters.

Appendix 8: Bland - Altman plots for measurement grouping comparison

Bland-Altman plots indicated that the DNA concentration readings obtained using the Qubit™ Assay were similar and close in agreement with the expected concentrations for all measurement groupings. The scatter pattern for all plots also demonstrated that there was better agreement between the measured and expected concentrations for samples with a lower mean concentration. The range between the upper and lower LOA for all plots was not broad, indicating that the differences between the expected and measured concentrations were small. Thus, Bland-Altman plots further support that the Qubit™ Assay has an acceptable degree of accuracy. Furthermore, negligible differences were observed between the measured and expected concentrations between the measurement groupings.

Table G. Mean differences and lower and upper limit of agreements (LOAs) for Bland-Altman plots depicting agreement between measured and expected Lambda DNA concentrations.

	Mean difference	Lower LOA	Upper LOA
M1 - M4	- 0.220719	-1.548774	1.107336
M2 - M4	- 0.170266	-1.620489	1.279957
M1 – M3	- 0.279291	-1.465562	0.9069799

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

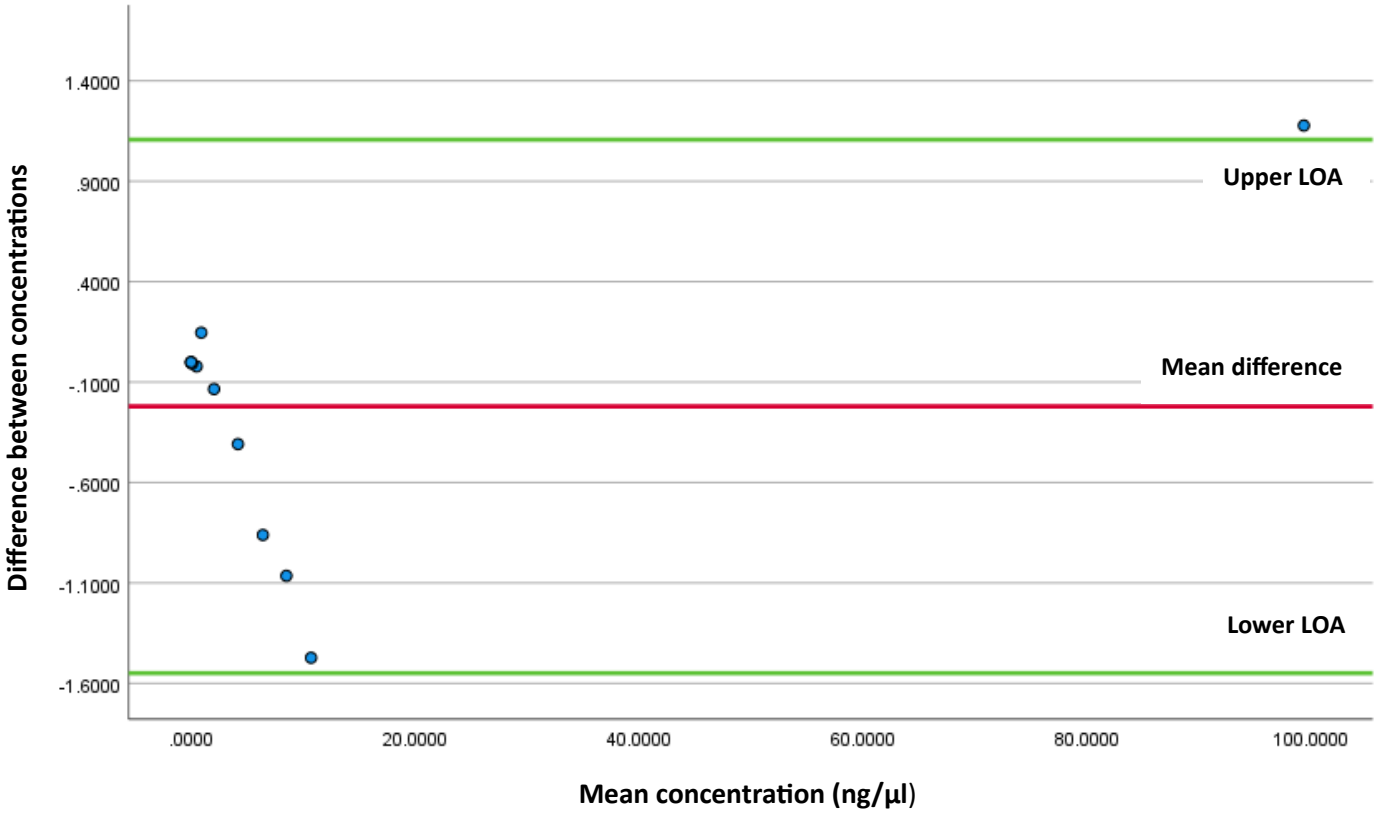

Figure A. Bland-Altman plot depicting agreement between measured and expected Lambda DNA sample concentrations for M1 – M4.

The difference between the measured and expected concentrations are plotted against the mean of the measured and expected concentrations. The mean difference between the concentrations was -0.220719, indicating close agreement between the expected and measured concentrations. The 100 ng/µl sample had the poorest agreement. Samples with a lower mean concentration had better agreement.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

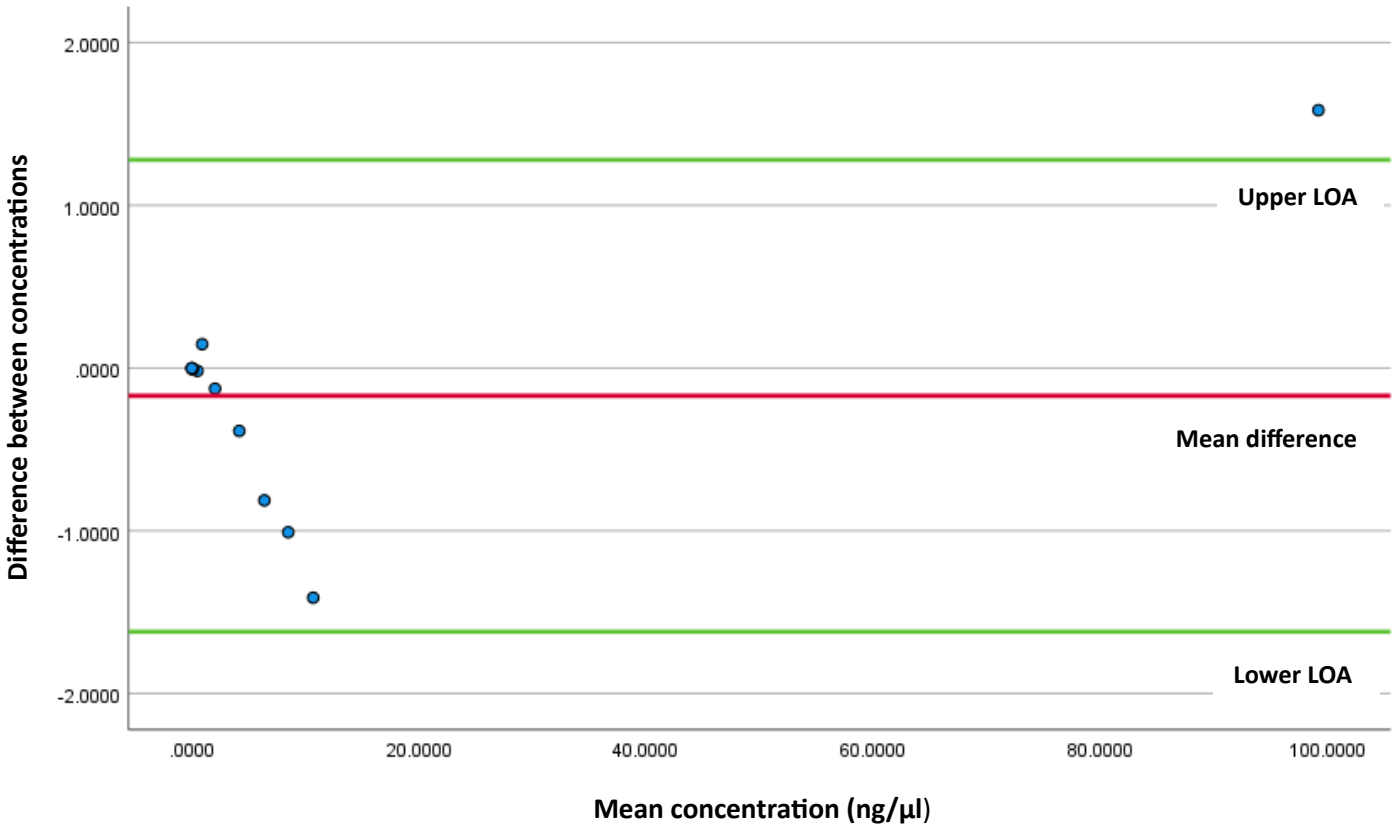

Figure B. Bland-Altman plot depicting agreement between measured and expected Lambda DNA sample concentrations for M2 – M4.

The difference between the measured and expected concentrations are plotted against the mean of the measured and expected concentrations. The mean difference between the concentrations was -0.279291, indicating close agreement between the expected and measured concentrations. The 100 ng/µl sample had the poorest agreement. Samples with a lower mean concentration had better agreement.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

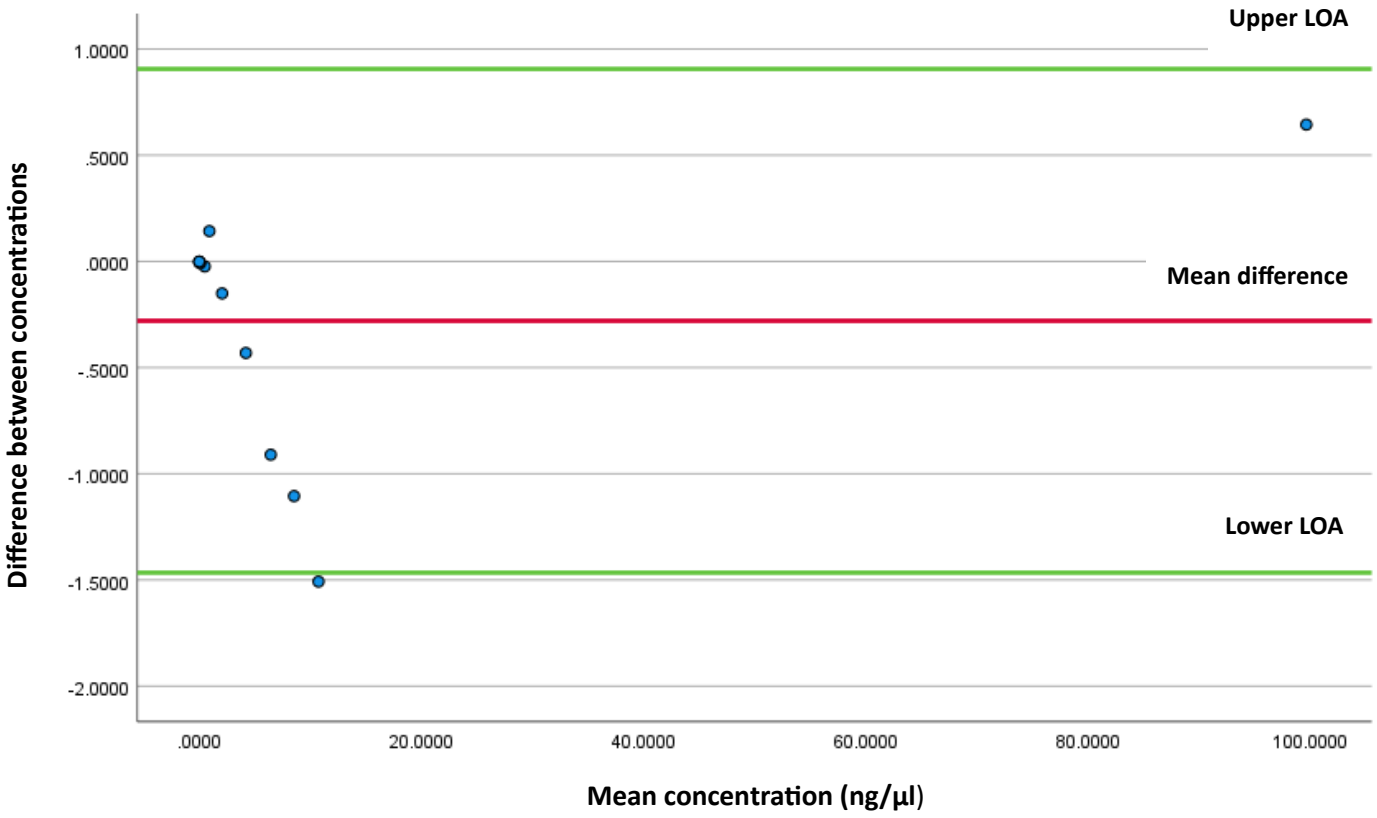

Figure C. Bland-Altman plot depicting agreement between measured and expected Lambda DNA sample concentrations for M1 – M3.

The difference between the measured and expected concentrations are plotted against the mean of the measured and expected concentrations. The mean difference between the concentrations was -0.220719, indicating close agreement between the expected and measured concentrations. Samples with a lower mean concentration had better agreement.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement

Appendix 9: Intra-assay precision full results

Table H. Intra-assay precision for quantified Lambda DNA, extracted DNA and NGS library samples.

Intra-assay precision given for each sample's aliquots and replicates. The intra-assay precision of measurements after using different sample volumes is also given. The intra-assay precision of measurements is compared across the measurement groupings.

				M1 - M4					M2 - M4					M1 - M3				
Sample		**Sample volume (µl)**	**Replicate**	**Aliquot 1 (% CV)**	**Aliquot 2 (% CV)**	**Aliquot 3 (% CV)**	**Replicate average (% CV)**	**Sample average (% CV)**	**Aliquot 1 (% CV)**	**Aliquot 2 (% CV)**	**Aliquot 3 (% CV)**	**Replicate average (% CV)**	**Sample average (% CV)**	**Aliquot 1 (% CV)**	**Aliquot 2 (% CV)**	**Aliquot 3 (% CV)**	**Replicate average (% CV)**	**Sample average (% CV)**
Lambda DNA	**100 ng/µl**	**1**	**1**	3.56	3.45	2.7	3.24		2.98	3.49	3.24	3.24		3.51	3.42	1.52	2.82	
			2	2.88	2.31	1.87	2.35	2.75	0.36	2.47	2.25	1.69	2.46	3.24	1.16	1.96	2.12	2.44
			3	1.57	2.72	3.66	2.65		1.12	3.33	2.95	2.46		1.92	1.1	4.11	2.38	
	10 ng/µl	**2**	**1**	3.15	0.44	1.8	1.8		2.73	0.51	1.02	1.42		2.23	0	2.2	1.48	
			2	1.12	1.08	1.66	1.29	1.3	0.52	0.5	0.51	0.51	0.81	1.02	0.99	2.02	1.34	1.23
			3	0.82	0.43	1.2	0.82		0.5	0.5	0.49	0.5		0.85	0.5	1.29	0.88	
		10	**1**	-	-	-	-		-	-	-	-		-	-	-	-	
			2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			3	-	-	-	-		-	-	-	-		-	-	-	-	
	8 ng/µl	**2**	**1**	2.36	1.93	2.21	2.17		2.06	1.58	1.92	1.85		1.29	1.32	2.51	1.71	
			2	1.69	1.32	1.14	1.38	1.83	1.14	1.37	1.23	1.25	1.54	1.36	0.59	0.46	0.8	1.58
			3	2.4	1.16	2.22	1.93		1.96	1.4	1.23	1.53		2.64	1.42	2.59	2.22	
	6 ng/µl	**2**	**1**	2.79	2.68	2.8	2.76		2.2	1.57	1.3	1.69		2.9	2.33	2.95	2.73	
			2	1.08	1.89	1.57	1.51	1.99	1.26	1.97	1.46	1.56	1.62	0.22	1.24	0.94	0.8	1.56
			3	1.88	2.08	1.17	1.71		1.96	1.5	1.35	1.6		0.86	1.87	0.71	1.15	
	4 ng/µl	**2**	**1**	1.34	2.35	0.86	1.52		0.84	1.62	0.92	1.13		1.27	2.29	0.92	1.49	
			2	2.01	1.28	1.27	1.52	1.55	2.1	1.35	1.45	1.63	1.38	1.71	0.33	0.35	0.8	1.2
			3	0.97	1.05	2.78	1.6		1.05	1	2.06	1.37		0.73	0.55	2.69	1.32	
	2 ng/µl	**2**	**1**	10.45	1.58	1.41	4.48		12.54	1.73	1.14	5.14		10.91	0.55	0.84	4.1	
			2	1.26	1.58	1.51	1.45	2.52	1.16	0.83	1.64	1.21	2.58	0.58	1.67	1.51	1.25	2.24
			3	1.42	2.2	1.28	1.63		1.55	1.82	0.77	1.38		0.26	2.52	1.33	1.37	

1 ng/µl	**2**	**1**	1.13	1.82	1.37	1.44		1.13	2.16	1.48	1.59		1.13	1.49	1.68	1.43	
		2	1.11	2.11	1.13	1.45	1.4	1.26	2.03	1.27	1.52	1.5	0.91	2.44	0.62	1.32	1.25
		3	1.59	1.04	1.3	1.31		1.6	1.19	1.38	1.39		1.59	0.21	1.21	1	
0.5 ng/µl	**2**	**1**	2.48	1.43	1.8	1.9		1.57	1.11	0.71	1.13		2.47	1.1	1.9	1.82	
		2	2.52	1.55	2.61	2.23	1.99	0.75	1.2	2.57	1.51	1.43	2.77	1.08	2.59	2.15	1.99
		3	1.21	1.1	3.2	1.84		1.21	1.04	2.69	1.65		1.14	1.16	3.73	2.01	
0.1 ng/µl	**2**	**1**	5.95	3.13	3.59	4.22		0.62	1.86	1.62	1.37		6.57	3.77	4.38	4.91	
		2	4.36	1.74	1.41	2.5	6.01	5.34	2.04	1.44	2.94	5.45	3.47	1.01	1.62	2.03	6.43
		3	9.01	2.53	22.41	11.32		8.79	2.65	24.69	12.04		10.67	2.65	23.76	12.36	
0.03 ng/µl	**2**	**1**	-	-	-	-		-	-	-	-		-	-	-	-	
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-		-	-	-	-		-	-	-	-	
	10	**1**	3.07	1.46	4.56	3.03		1.18	0.68	5.59	2.48		3.06	1.7	2.12	2.29	
		2	1.42	3.12	2.84	2.46	3.1	1.7	2.25	2.47	2.14	2.07	1.48	2.19	1.8	1.82	2.69
		3	4.14	4.63	2.7	3.82		1.34	0.89	2.56	1.6		4.15	5.39	2.29	3.94	
0.02 ng/µl	**2**	**1**	-	-	-	-		-	-	-	-		-	-	-	-	
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-		-	-	-	-		-	-	-	-	
	10	**1**	7.7	3.93	2.28	4.64		9.2	4.2	2.28	5.23		7.77	4.52	2.79	5.03	
		2	2.4	2.41	3.76	2.85	3.53	2.43	2.95	4.6	3.32	3.86	0.9	1.49	4.24	2.21	2.9
		3	2.31	2.58	4.42	3.11		1.65	2.48	4.98	3.03		2.36	1.47	0.54	1.46	
0.01 ng/µl	**2**	**1**	-	-	-	-		-	-	-	-		-	-	-	-	
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-		-	-	-	-		-	-	-	-	
	10	**1**	7.19	4.69	3.08	4.99		7.01	5.59	2.21	4.93		8.27	5.52	3.77	5.85	
		2	-	-	-	-	4.51	-	-	-	-	4.92	-	-	-	-	4.67
		3	4.99	2.67	4.45	4.04		6.73	2.7	5.3	4.91		4.99	2.07	3.39	3.49	
	20	**1**	4.1	7.18	3.13	4.81		5.02	1.56	3.75	3.44		5	8.55	2.84	5.46	
		2	3.01	3.79	1.84	2.88	3.51	1.51	4.47	1.55	2.51	3	2.95	4.28	1.53	2.92	3.39
		3	2.05	3.22	3.23	2.83		2.32	3.36	3.5	3.06		1.42	1.02	2.96	1.8	

Extracted DNA	Blood	2	1	2.13	1.44	0.7	1.42		2.6	1.36	0.76	1.57		1.21	0.64	0.24	0.7	
			2	0.9	2.73	2.32	1.98	1.56	0.55	3.11	2.43	2.03	1.42	0.94	1.32	1.41	1.22	1.05
			3	0.9	1.82	1.13	1.28		0.57	0.84	0.58	0.66		1.02	1.67	0.99	1.23	
	FFPE	2	1	2.48	1.43	1.69	1.87	1.87	2.7	1.02	1.02	1.58	1.58	1	1.01	2.04	1.35	1.35
	Tooth	2	1	5.83	11.74	5.04	7.54	7.54	4.25	11.69	1.92	5.96	5.96	4.82	8.91	5.96	6.56	6.56
	Bone	2	1	1.37	3.15	2.07	2.2	2.2	0.44	3.34	1.45	1.74	1.74	1.59	1.76	2.23	1.86	1.86
	Buccal swab	2	1	1.8	1.27	1.64	1.57	1.57	1.89	1.03	0.4	1.11	1.11	2.17	1.47	1.89	1.84	1.84
	Nail	2	1	7.49	5.85	2.14	5.16		8.21	3.03	0.82	4.02		5.12	5.49	2.1	4.24	
			2	4.64	5.56	4.44	4.88	4.43	3.7	4.65	3.48	3.94	3.54	3.48	3.83	3.17	3.5	3.42
			1	3.13	4.26	2.39	3.26		1.52	3.52	2.92	2.65		3.11	3.17	1.32	2.53	
NGS library	Pooled library	2	1	2.81	2.12	1.43	2.12	2.12	3.03	2.25	1.03	2.1	2.1	0.77	1.13	1.02	0.98	0.98
	Unpooled library	2	1	1.74	1.57	0.56	1.29		1.88	1.42	0.65	1.32		0.7	1.73	0.65	1.03	
			2	0.81	1.03	1.13	0.99	1.22	0.93	0.6	1.39	0.97	1.25	0.93	1.21	0.63	0.93	0.96
			3	0.66	2.48	0.97	1.37		0.66	2.65	1.1	1.47		0.65	1.28	0.81	0.91	

- Indicates no measurement

Red values indicate those which did not meet the manufacturer's criteria of acceptance or in-house established parameters.

Green values indicate those which met the manufacturer's criteria of acceptance and in-house established parameters.

Green values underlined in red indicate those which did not meet the manufacturer's criteria of acceptance but did meet the in-house parameters established after validation experiments.

Appendix 10: Inter-assay precision

Table I. Inter-assay precision for quantified Lambda DNA, extracted DNA and NGS library samples.

Inter-assay precision given for each sample's replicates. The inter-assay precision of measurements after using different sample volumes is also given. The inter-assay precision of measurements is compared across the measurement groupings.

				M1 - M4		M2 - M4		M1 - M3	
Sample		**Sample volume (µl)**	**Replicate**	**% CV**	**Sample average (% CV)**	**% CV**	**Sample average (% CV)**	**% CV**	**Sample average (% CV)**
Lambda DNA	**100 ng/µl**	**1**	**1**	2.88		3.29		2.98	
			2	2.59	3.77	3.34	3.84	1.90	3.84
			3	5.82		4.90		6.64	
	10 ng/µl	**2**	**1**	1.42		1.80		0.78	
			2	1.75	1.38	1.77	1.43	1.75	1.22
			3	0.96		0.72		1.14	
		10	**1**	-		-		-	
			2	-	-	-	-	-	-
			3	-		-		-	
	8 ng/µl	**2**	**1**	1.77		1.74		0.78	
			2	1.58	1.55	1.48	1.70	1.75	1.22
			3	1.29		1.86		1.14	
	6 ng/µl	**2**	**1**	2.48		2.37		0.78	
			2	1.40	1.90	1.20	1.86	1.75	1.22
			3	1.81		1.99		1.14	
	4 ng/µl	**2**	**1**	0.78		1.01		0.61	
			2	2.74	2.31	2.63	2.47	2.76	2.21
			3	3.40		3.76		3.25	
	2 ng/µl	**2**	**1**	2.28		2.97		3.26	
			2	3.15	1.87	3.12	2.14	3.06	2.18
			3	0.17		0.34		0.23	
	1 ng/µl	**2**	**1**	0.69		0.73		3.26	
			2	1.52	1.01	1.14	0.85	3.06	2.18
			3	0.82		0.69		0.23	

	0.5 ng/µl	2	1	1.90		2.15		1.80	
			2	3.17	2.00	2.84	1.84	3.10	1.93
			3	0.92		0.53		0.88	
	0.1 ng/µl	2	1	7.15		8.39		1.80	
			2	6.40	7.42	6.18	8.43	3.10	1.93
			3	8.72		10.71		0.88	
	0.03 ng/µl	2	1	-		-		-	
			2	-	-	-	-	-	-
			3	-		-		-	
		10	1	7.64		7.47		8.22	
			2	4.55	10.99	3.82	10.11	5.02	11.63
			3	20.79		19.04		21.67	
	0.02 ng/µl	2	1	-		-		-	
			2	-	-	-	-	-	-
			3	-		-		-	
		10	1	4.11		5.13		3.20	
			2	2.82	3.45	2.56	4.00	3.94	3.00
			3	3.42		4.30		1.84	
	0.01 ng/µl	2	1	-		-		-	
			2	-	-	-	-	-	-
			3	-		-		-	
		10	1	5.49		4.87		6.04	
			2		5.02		4.90		5.71
			3	4.55		4.93		5.38	
		20	1	3.99		3.45		4.81	
			2	3.53	4.42	3.62	4.48	3.55	4.30
			3	5.74		6.36		4.55	
Extracted DNA	Blood	2	1	1.36	1.29	1.17	1.27	1.38	1.32
			2	1.05		0.96		1.34	

			3	1.46		1.68		1.24	
	FFPE tissue	2	1	0.25		0.34		0.89	
	Tooth	2	1	7.78		7.45		8.24	
	Bone	2	1	0.58		0.65		0.96	
	Buccal swab	2	1	4.38		4.46		4.34	
	Nail	2	1	11.41		12.11		11.47	
			2	3.20	6.01	3.26	6.14	3.09	6.13
			3	3.43		3.06		3.84	
NGS library	Pooled library	2	1	2.07		2.14		1.72	
	Unpooled library	2	1	4.80		5.16		4.44	
			2	6.22	9.50	5.91	9.59	6.34	9.28
			3	17.47		17.69		17.07	

- Indicates no measurement

Red values indicate those which did not meet the manufacturer's criteria of acceptance or in-house established parameters.

Green values indicate those which met the manufacturer's criteria of acceptance and in-house established parameters.

Green values underlined in red indicate those which did not meet the manufacturer's criteria of acceptance but did meet the in-house parameters established after validation experiments.

Table J. The intra-class correlation coefficient (ICC) values for Lambda DNA, extracted DNA and NGS library samples.

The ICC for all samples was determined for M1 – M4, M2 – M4 and M1 – M3. The ICC indicated if the reliability between measurements of a sample was poor (ICC < 0.5), moderate (0.5 < ICC ≤ 0.75), good (ICC (0.75 < ICC < 0.9) or excellent (ICC > 0.9). Negative values indicate greater variability for the measurements between replicate than the measurements within replicates.

M1 = measurement 1, M2 = measurement 2, M3 = measurement 3 and M4 = measurement 4

		M1 – M4		M2 – M4		M1 – M3	
Sample		**ICC**	**p - value**	**ICC**	**p - value**	**ICC**	**p - value**
Lambda DNA	**100 ng/µl**	-0.13	0.808	-0.157	0.848	- 0.2	0.849
	10 ng/µl	0.246	0.007	0.116	0.101	0.219	0.035
	8 ng/µl	0.067	0.243	- 0.107	0.809	0.088	0.209
	6 ng/µl	0.707	< 0.001	0.625	< 0.001	0.613	< 0.001
	4 ng/µl	0.255	0.049	0.145	0.198	0.252	0.066
	2 ng/µl	0.015	0.402	0.476	0.017	-0.59	0.711
	1 ng/µl	0	0.557	0.001	0.297	-0.002	0.969
	0.5 ng/µl	0.049	0.262	- 0.096	0.887	0.08	0.158
	0.1 ng/µl	0.015	0.414	0.03	0.371	0.089	0.141
	0.03 ng/µl	0.084	0.249	0.116	0.21	0.078	0.294
	0.02 ng/µl	- 0.038	0.604	- 0.043	0.591	- 0.083	0.758
	0.01 ng/µl	0.023	0.419	- 0.063	0.621	0.02	0.428
DNA extracted from forensic samples	**Blood**	0.203	0.127	0.107	0.298	-0.198	0.824
	FFPE tissue	0.432	0.133	- 0.167	0.549	0.467	0.077
	Tooth	0.353	0.029	0.201	0.183	0.329	0.026
	Bone	0.57	0.056	0.211	0.304	0.536	0.069
	Buccal swab	0.092	0.006	0.049	0.065	0.123	0.024
	Nail	0.084	0.207	0.024	0.296	- 0.017	0.527
NGS libraries	**Pooled library**	0.439	0.004	0.404	0.03	0.141	0.093
	Unpooled library	0.192	0.122	0.163	0.196	0.218	0.136

Appendix 11: Data Management Plan

Towards molecular autopsies: Internal validation of the QubitTM 1X dsDNA HS Assay Kit - Student Full DMP

Project Details

PROJECT NAME - Replicate the title of your project, dissertation or thesis exactly as it appears in your proposal document.

Towards molecular autopsies: Internal validation of the QubitTM 1X dsDNA HS Assay Kit

PERSONAL DETAILS - Indicate the name(s) and student number(s) of the student(s) who will be involved in this project, dissertation or thesis.

Adele Naidoo (ndxade004)

SUPERVISOR(S) DETAILS - Indicate who will supervise this project, dissertation or thesis. If you do not yet have a supervisor, leave this section blank.

Dr Laura Heathfield

Miss Donna – Lee Martin

Project Summary

RESEARCH SUMMARY - Briefly summarise your study. Include the study's objectives, design, and methods.

Molecular autopsies are a useful tool in forensics as they assist with medico-legal investigations. The Molecular Forensics Research Group is establishing a workflow for molecular autopsies that make use of the Qubit™ 1X dsDNA HS Assay Kit, however, Qubit has not been validated for forensic use. It also needs to be internally validated so that it may be incorporated into the molecular autopsy workflow in accordance with ISO 17025. As part of internal validation, we will evaluate the suitability of the kit for our laboratory as well as establish acceptable parameters for our laboratory. The findings from this study will provide guidelines, standard operating procedures and a validation report for future internal validation studies on the Qubit™ 1X dsDNA HS Assay Kit. The aim of this study is to internally validate the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 fluorometer. The aim will be achieved by determining the accuracy, precision, range and sensitivity of the assay. The suitability of the assay in our forensic laboratory as well as evaluation of assay parameters will also be evaluated. This project will follow a validation study design. This project will assess and verify the performance of the Qubit™ 1X ds DNA HS Assay Kit on the Qubit™ 4 Fluorometer. Control DNA samples and authentic forensic samples with known DNA concentrations will be used. The authentic forensic samples will represent the samples which are typically used in the laboratory for forensic applications. DNA concentrations will be measured and analysed to determine the accuracy, precision (intra-assay and inter-assay precision) as well as range and sensitivity of the assay. Comparisons will be made to measurements acquired by the manufacturer as well as other quantification methods such as qPCR and TapeStation.

Description of the Data

DATA REUSE DESCRIPTION - If you re-used data from third-party sources in your study, record pertinent details here such as the source of the data, the extent of the data, usage rights or restrictions pertaining to the data, and how it was incorporated into your study.

- I have used existing data in my study.

I will compare my validation parameters (accuracy, precision, range and sensitivity) to the open access data in the manufacturer's technical note.

DATA DESCRIPTION - Describe the data you have gathered for your study. Briefly describe the nature, scope and scale of the data you have produced.

I will be producing my own quantitative data by quantifying the control and authentic forensic samples using the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 fluorometer, qPCR and TapeStation. I will then be determining the accuracy, precision, range and sensitivity of the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 fluorometer. Measurements taken using the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ 4 fluorometer will be compared to measurements taken using qPCR and TapeStation to evaluate the different quantification techniques. All quantitative data will be recorded in a Microsoft Excel spreadsheet. The size of my dataset will be minimal at approximately 10s or 100s of megabytes.

Formats and Quality Control

QUALITY CONTROL - Describe what measures you took to ensure the data you collected were of high-quality.

I will adhere to protocols to ensure that my experiments and subsequent data are reliable. I will also carry out my experiments in replicates to ensure accurate and reliable results. I will also enforce double entry when recording my data to ensure there are no errors.

FILE FORMATS - Indicate the formats in which your data will be collected and processed. Clarify whether these formats require specialised proprietary software to access or if they will be produced in or converted to more open, accessible formats for long-term accessibility and preservation. In the case of physical data objects (such as artworks or models) indicate whether these will be digitised or otherwise preserved for accessibility.

My data will be collected in XLS and CSV format.

Data Management, Documentation and Curation

CURATION (MANAGING AND STORING) DATA - Describe how you organise and manage your data. Specify any file-naming conventions or community data standards you have adopted.

Numeric data will be collated in Excel spreadsheets. The spreadsheets will be named according to the data, type of data captured and any version numbers if necessary.

BACKUP AND STORAGE - Describe how your data is being stored and backed-up. If you are using a data service provider, provide details on for how long they will retain the data.

Data will be stored on my personal laptop and will be backed on my UCT GoogleDrive account. Data will also be backed up on a password protected flash drive. Backups will be done each month of data collection and analysis, or more frequently depending on data capturing.

METADATA STANDARDS AND DATA DOCUMENTATION - Articulate what metadata and documentation you have produced about the data you have generated, collected or re-used.

A metadata list will be kept in the form of an Excel spreadsheet, detailing the data which has been collected. The methods section of my thesis will outline the assays and statistical analyses which will be carried out to produce the data that is collected.

Data Security and Confidentiality of Potentially Disclosive Information

SECURITY - Indicate to what extent your data can be considered sensitive or at-risk. Describe how you will control access to your data. Indicate whether you anticipate a need for encryption or password-controlled access, and if so, how you will enforce that access.

My data is low risk and is not sensitive as it does not contain any identifiable or personal information. I will record my data on Excel spreadsheets which will be stored on my password protected laptop which only I will have access to. Back-ups of the data will be stored on my UCT Google Drive account, and a password protected flash drive. Only my supervisor and I will have access to the data.

ETHICS AND PRIVACY - Describe, as per your Ethics Clearance form or other similar documentation, any ethical or privacy issues that your data are subject to (if any). Summarise the main risks to the confidentiality and security of information related to human

participants, the level of risk, and how this risk will be managed. If your project did not require ethical clearance, you may ignore this section.

This study does not have human participants as the authentic forensic samples that will be used have already been collected and are already available within the Forensic DNA Repository (HREC: R014/2014) for validation studies. The authentic forensic samples have been anonymized so there are no privacy issues. Therefore, there are no ethical issues.

Data Sharing and Open Access

DATA OWNERSHIP - If you have used existing datasets, note down any restrictions the data providers have indicated regarding data sharing. Otherwise, leave blank.

- I have used existing data in my study, and I have noted down the relevant restrictions as pertains to data sharing(details below).

I will use open access data provided in the manufacturer's technical note. There are no data sharing restrictions.

DATA LICENCE - Indicate under which licence you intend to share your research data. If you are not sharing your data, provide the appropriate justification as per the UCT Research Data Management guidelines.

- CC BY

I will share the de-identified data from my study under a CC BY licence.

DATA PUBLICATION - Indicate where you intend to publish your research data at the end of your project.

I will share my de-identified quantitative data on ZivaHub at the end of my project.

Relevant Institutional or Study Policies

Indicate the relevant departmental, unit, or institutional policies that influence your data management activities.

As I am funded by the NRF, the following policies apply: the UCT Intellectual Property Policy; the UCT Open Access Policy; the UCT Research Data Management Policy; and the NRF Open Access statement.