Development and application of a PCR multiplex to assess the quality and quantity of forensic DNA extracts

By

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A thesis submitted in partial fulfilment for the requirements for the degree of PhD at the University of Central Lancashire

November 2014

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ABSTRACT

Isolation of DNA from skeletonised human remains can be problematic. In addition to DNA degradation, enhanced by high temperature and humidity, there are often potent polymerase chain reaction (PCR) inhibitors present within the samples. It is therefore important to extract the maximum amount of available DNA whilst removing any amplification inhibitors that may be present.

Whilst real-time PCR methods are available for quantification and detection of PCR inhibitors the information received is limited as real-time PCR targets amplicons that are much smaller than those typically targeted in forensic analysis. To gain more information on the quality of extracted DNA a new multiplex PCR assay comprising a 4-plex targeting amplicons of 70 base pairs (bp), 194 bp, 305 bp and 384 bp along with two Internal Amplification Contols (IACs) of 90 bp and 410 bp was developed. This multiplex was optimised so that it worked with template amounts ranging between 0.10 ng and 200 ng; partial profiles were obtained with as little as 0.02 ng. The IACs were effective in detecting PCR inhibitors.

The multiplex also assessed as a quantification tool. Plotting peak height compared to input DNA of a standard dilution series produced a coefficient of determination (R^2) of 0.8308. The multiplex was found to provided reasonable estimates of DNA concentration, when the sample concentration was between 12.5 – 100 ng; relative standard deviations were all below 10% in this range for 30% of tested samples. However, real-time PCR proved to be more precise and was used in the rest of the study for the purposes of quantification.

In forensic cases bones and teeth often provide some of the most challenging samples to extract good quality DNA. Using the optimised multiplex to assess the quality of DNA

extracts five extraction methods: ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and phenol-chloroform-isoamyl alcohol extaction methods were assessed for their capability for extracting clean DNA from bone samples. Prior to the main experimentation several evaluation studies were carried out to optimise the methods being used. Based on the results, decalcification was not used for any of the extractions as non-decalcified extracts contained higher amounts of DNA. For the phenol-chloroform-isoamyl alcohol extraction it was determined that whilst ethanol precipitation provided higher amounts of DNA, the extracts using Amicon 30kDa filters (Amicon ultra-0.5 centrifugal filter unit with ultracel-30 membrane) were cleaner. Based on poor results with degraded bone samples a pre-process technique was developed; these extractions started with 250 mg of pulverised bone sample which was then concentrated and cleaned up using Amicon 30kDa filters (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration) before carrying out the standard extraction procedures.

After optimisation of the extraction methods the comparison study showed that the phenol-chloroform-isoamyl alcohol extraction method produced the highest DNA yields with both fresh and degraded bone samples, followed by DNeasy® Blood & Tissue Kit, ChargeSwitch® gDNA Plant Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit. However, all produced DNA that could be amplified and did not contain any inhibition.

Another application of the multiplex was to assess the effectiveness of different DNA preservation methods by examining the amount and quality of DNA recovered after preservation. Five methods: cell lysis solution (with 1% sodium azide), dehydration / freeze drying, ethanol (96%), freezing and room temperature storage were used to study the effectiveness of preservation methods on fresh and three-month old decomposed pig bone samples which were preserved for 6 weeks, 6 months and 1 year. The results showed that freezing is the best preservation method for both fresh and degraded bone samples for long-term storage followed by ethanol (96%), dehydration / freeze drying and room temperature storage. However, full profiles were obtained from both fresh and

degraded bone samples from all methods, except cell lysis solution (with 1% sodium azide). Cell lysis solution (with 1% sodium azide) preservation method tended to be good for short-term storage but with the long-term preservation, less DNA yield was obtained and also the electropherograms showed higher levels of DNA degradation.

Finally, using the optimised DNA extraction methods, the multiplex was tested using forensic samples comprising of 30 bone samples from casework in Malaysia and simulated body fluid evidences subjected to environmental insult in the United Arab Emirates. The application illustrated the effectiveness of the multiplex to identify PCR inhibitors and identify DNA degradation, providing supplementary information to real-time PCR.

TABLE OF CONTENTS

CHAPTER 2 : GENERAL METHODS AND MATERIALS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENT

I would love to acknowledge with deep reverence, sincerity and heartiest gratitude to the people for their endless guidance and enthusiastic encouragement to make this thesis possible.

Firstly, I would like to thank my supervisor, Dr. William Goodwin and co-supervisor Dr. Sibte Hadi for their excellent advices and constructive suggestions during this entire PhD project. I would also like to thank Dr. Arati Iyengar and Dr. Judith Smith for their critical reviews and guidance to improve my project. Many thanks go to Professor Jaipaul Singh for his timely helps and moral supports.

Many thanks are due to the staff members of Forensic Anthropology, especially Peter Cross for providing samples from TRACES for my project and Dr. Colin Moffatt for his advice and assistance in statistical calculations.

I would also like to thank fellow friends from UCLan for providing me an excellent support and encouragement during my project especially Sharizah Alimat, Muhammad Shahid Nazir, Rashed Alghafri and Nur Haliza Hassan. Many thanks also go to others for keeping me updated with help and support throughout my entire staying in Preston.

Finally, I wish to express my deep and sincere thanks to my family especially my beloved parents for their love and affection. Big thanks go to my brothers for providing me love, inspiration and encouragement.

CHAPTER 1

INTRODUCTION

1.1 DNA PROFILING

Since Alex Jeffreys first developed deoxyribonucleic acid (DNA) profiling in 1985 (Jeffreys et al. 1985) it has played a central role in many forensic investigations; the polymerase chain reaction (PCR) based techniques (Mullis et al. 1986) further increased the scope of forensic DNA profiling allowing smaller degraded template to be analysed. In addition to crime scene analysis DNA profiling is widely used for human identification purposes after various kinds of tragic events such as mass disaster, war and other situations of armed conflict and terrorist attacks. The processing of these samples presents specific challenges (Taroni et al. 2013, Mundorff et al. 2009, Allouche et al. 2008, Gojanovic & Sutlovic 2007).

1.1.1 The processes of DNA profiling

Once in the laboratory, the biological samples go through several processes before DNA profiles are produced. These processes are extraction, quantification, amplification and electrophoresis (Lee et al. 2010). In the extraction stage, DNA is separated from cellular materials and other biological compounds. Extraction is an important process because it will determine the outcome of any downstream processes. Following extraction of forensic samples, the amount of DNA is typically estimated by quantification and an appropriate amount is added to a PCR for amplification. Using capillary electrophoresis, DNA is then separated according to the base pair sizes and a DNA profile generated

(Hopwood et al. 2010, Jobling & Gill 2004). Both the crime and reference samples will undergo these processes separately to avoid cross contamination.

1.1.2 DNA profiling of challenging samples

Since the reference samples are fresh and typically contain high amounts of DNA, it is relatively easy to develop the DNA profiles from them. Because of the nature of crime samples, where samples are typically degraded, inhibited or contain little DNA, it can be very difficult to obtain a full DNA profile (Ballantyne et al. 2011). Thus research has been done and is on-going to improve the outcome of the crime samples analysis. This includes improvements not only in extraction methods, but also amplification techniques and electrophoresis enhancement (Liu et al. 2011, Vuichard et al. 2011, Hua Zhang et al. 2010, Kishore et al. 2006, Gill et al. 2005). One of the most challenging DNA samples to process is old bone (Alaeddini & Ahmadi 2011, Lee et al. 2010).

1.2 DNA DEGRADATION

1.2.1 Introduction

DNA degradation becomes rapid after the death. The common pathways for post-mortem DNA degradation is enzymatic degradation (Paabo et al. 2004). This is caused by the nuclease, namely DNase for DNA degradation. Nuclease causes DNA to degrade into smaller fragments. When the cell membranes rupture and release nutrient rich fluid, environmental microorganisms such as bacteria and fungi accelerate their growth. Most of the microorganisms also contain nuclease enzymes and are able to degrade nucleic acid (Hofreiter et al. 2001, Ogata et al. 1990, Antheunisse 1972).

Environmental conditions such as temperature and humidity also have influence on the DNA degradation. Hot and high temperature tends to degrade the DNA faster (Robins and

Furey 2001) while more chilly and low temperature preserve DNA for longer time (Willerslev et al. 2004, Lindahl 1993). However, longer-term, DNA will continue to degrade through spontaneous degradation routes including depurination followed by breakage at apurinic sites by alkaline hydrolysis, destruction of deoxyribose residues, and hydrolytic cleavage of pyrimidine-glycosyl bonds (Dixon et al. 2006, Lindahl 1993). Conditions such as dry environment may help to reduces DNA degradation, however high humidity provides the substrate for hydrolytic enzymes to degrade DNA (Bender et al. 2004). Phenolic acid such as from soil humus provides low pH which reduces the DNA stability thus causes DNA degradation (Bender et al. 2004, Haglund 1996). Therefore, proper preservation methods could prevent the DNA degradation. More details of the DNA preservation to prevent the DNA degradation are explained in Section 1.6.

1.2.2 DNA persistence in bone

Compared to other biological materials, bone provides protection against DNA degradation (Brundin et al. 2013, Lindahl 1993). Bones are composed of two fundamental structural elements. Cortical or compact bone found primarily on the walls of bone surface and shafts, is made up of solid and dense bone. Trabecular or cancellous bone, also known as spongy bone, is lightweight with honeycomb like, porous structure located at inner surface as shown in Figure 1.1 (Bao et al. 2013).

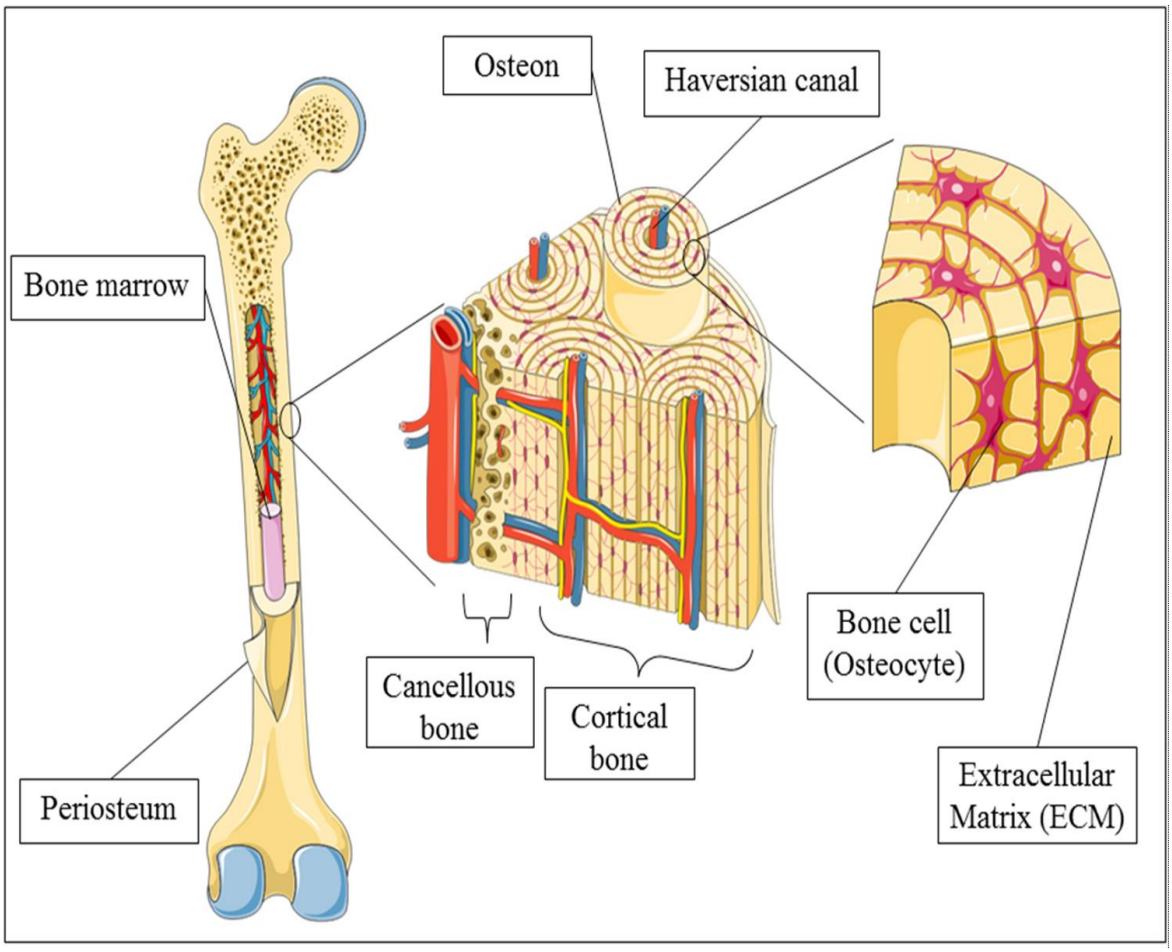


Figure 1.1: Schematic overview of bone, depicting gross overview and cellular distribution. [Taken from (Bao et al. 2013)]

The molecular structure of bones is made up largely of mineralised Type I collagen. This collagen is a large organic protein molecule comprising of insoluble protein, giving a slightly elastic and flexible fibrous property (Collins et al. 2002). An inorganic type of bioapatite mineral called hydroxyapatite component reinforces it, giving the bone its rigidity and hardness (Campos et al. 2012). Despite wide study of ancient DNA using bone, relatively little known about the degradation of DNA in bone. At structural level the absorption of DNA to the hydroxyapatite and/or binding of DNA to Type 1 collagen may stabilise the DNA but the relative contribution of each and what other factor may be relevant are unclear (Campos et al. 2012). Brundin et al. (2013) said that the presence of hydroxyapatite, which forms the framework of bone, binds DNA and prevents it from degradation. Environmental conditions such as humidity and heat also may affect the DNA survival in the bones (Lee et al. 2010). However, cooler temperature may preserve the DNA longer. Hoss et al. (1996) found that nucleotide bases decomposition in bone decreased about 10 – 25 fold with a 20 °C reduction in temperature. The commonly used bones in DNA analysis are rib (Kemp, Smith 2005, Perry et al. 1988) and femur (Kitayama et al. 2010, Kaiser et al. 2008).

1.2.3 Degraded DNA sample analysis

Degraded DNA samples will produce either partial DNA profiles or negative results (Marjanovic et al. 2009). Usage of size specific clean-up columns such as Microcon® (Merck Millipore) and Amicon® (Merck Millipore) can assist in capturing bigger fragments and eliminate degraded smaller fragments which could inhibit the PCR process by competing with the target DNA (Noren et al. 2013, Seo et al. 2012, Kim et al. 2000). But this will still produce partial DNA profiles since the bigger fragments were degraded. Even though it is not possible to do much in the extraction process, degraded DNA samples can either be enhanced or applied to different amplification processes to produce better DNA profiles. There are many amplification kits in market now that improve the results from degraded DNA samples. A kit such as MiniFiler™ PCR Amplification Kit (Life Technologies™) is more robust with poor quality DNA because of the improved primer

design, buffer composition and amplification conditions (Mulero et al. 2008, Loreille et al. 2007). This kit which has improved primers will bind to the primer binding sides which are closer to the target side thus even in degraded DNA samples, full DNA profiles could be obtained if the target side and the primer binding side are not degraded.

1.3 PCR INHIBITION

1.3.1 Introduction

PCR inhibition occurs when some samples are contaminated by chemicals that inhibit the amplification process, thereby preventing the generation of a DNA profile. PCR inhibitors generally have effects through direct interaction with DNA or interference with DNA polymerases (Mulero et al. 2008, Larkin & Harbison 1999, Katcher & Schwartz 1994). PCR inhibitors which interact with DNA can be co-purified together during extraction process and prevent the amplification of DNA (Alaeddini & Ahmadi 2011, Hudlow et al. 2011, Kim et al. 2000, Akane et al. 1994). Magnesium is a critical cofactor which is required during amplification process. PCR inhibitors which can reduce the Mg^{2+} availability or interfere the binding of Mg^{2+} to the DNA polymerase can inhibit the PCR. Common inhibitions are heme from blood (Hudlow et al. 2011, Akane et al. 1994), indigo from denim in jeans materials (Mulero et al. 2008, Larkin & Harbison 1999), humic acid from soil samples (Seo et al. 2013, Watson & Blackwell 2000), collagen from bones (Alaeddini & Ahmadi 2011, Kim et al. 2000) and phenol (Opel et al. 2010, Katcher & Schwartz 1994). The commonly known PCR inhibitors and their mechanism of inhibition are as shown in the Table 1.1.

Table 1.1: Table below shows the commonly encountered PCR inhibitors, the inhibited samples and their mechanism of inhibition.

Inhibitor	Effect of inhibition	Inhibited sample	References
Calcium	Calcium ions compete with the magnesium ions which are required for the polymerase activity in the PCR reaction	Bone and teeth	Alaeddini & Ahmadi 2011, Opel et al. 2010, Kim et al. 2000, Abu Al-Soud & Radstrom 1998
Collagen	Interrupt the magnesium ions and polymerase interaction in the PCR reaction	Tissue, bone and teeth	Opel et al. 2010, Kim et al. 2001, Makino et al. 1995
Tannic acid	Chelate with magnesium ions needed for the polymerase	Leather	Schrader et al. 2012, Opel et al. 2010, Abbaszadegan et al. 1993
Humic acid	Chelate with magnesium ions needed for the polymerase and also limiting the available DNA template for amplification through sequence specific binding of DNA	Soil and plant material contaminated samples	Seo et al. 2013, Hudlow et al. 2011, Sutlovic et al. 2005, Watson & Blackwell 2000, Kreader 1996, Akane et al. 1994
Dye	Intercalates with DNA	Textile dyes (e.g. indigo from denim)	Schrader et al. 2012, Opel et al. 2010, Mulero et al. 2008, Larkin & Harbison 1999, Shutler et al. 1999
Hematin	Ferric ions from heme unbalance the ions in the PCR reaction and inhibit the magnesium ions and polymerase interaction	Blood	Schrader et al. 2012, Al-Soud & Radstrom 2001, Al-Soud & Radstrom 2000
Phenol, ethanol, isopropanol	Degrade the polymerase	DNA extraction chemical	Schrader et al. 2012, Opel et al. 2010, Wiedbrauk et al. 1995, Katcher & Schwartz 1994
EDTA (Ethylene Diamine Tetraacetic Acid)	Chelating the magnesium ions which are required for the activity of polymerase	DNA extraction chemical (e.g. TE buffer)	Schrader et al. 2012, Rossen et al. 1992

1.3.2 Detection of PCR inhibition

PCR inhibition can result in reduced product yield or complete failure. In a partial profile caused by PCR inhibitors, the smaller loci are preferentially amplified. But the same pattern also can be obtained from highly degraded DNA sample, thus inhibited sample are very often misidentified as degraded (Butler et al. 2003, Takahashi et al. 1997).

Even though it is difficult to differentiate inhibited DNA from degraded DNA, real-time quantification helps to detect the presence of inhibitors (Seo et al. 2012, Kontanis & Reed 2006). The use of multiplex real-time PCR system such as Quantifiler® uses internal positive control (IPC) to identify the presence of PCR inhibitors. It detects the PCR inhibitors by analyzing target amplification efficiency (Kontanis & Reed 2006). Together with autosomal targets of different sizes, it can simultaneously identify inhibition and degradation in a sample (Swango et al. 2006). This additional information can be very useful in processing the sample with better choices and produce better amplification success rates. Also the usage of Internal Amplification Controls (IACs) / Internal PCR Control (IPC) can indicate the presence of PCR inhibition in the samples from the generated DNA profiles (Nazir et al. 2013).

1.3.3 Prevention of PCR inhibition

The best way of avoiding PCR inhibitors is preventing them from being processed together with the DNA template. For the inhibitors which present within the samples such as blood and certain tissues, this is highly impossible (Hudlow et al. 2011, Akane et al. 1994). For samples present on materials containing the PCR inhibitors such as denim, leather and soil, it will be suitable to swab the samples rather than processing them together with the materials (Seo et al. 2013, Mulero et al. 2008, Watson & Blackwell 2000, Larkin & Harbison 1999,). PCR inhibition can be reduced during the extraction phase (Alaeddini 2012, Alaeddini & Ahmadi 2011), however, to do this without loss of DNA is a big challenge. Thus, DNA extraction methods which favour the inhibitions

elimination should be preferred. Adding a separate clean-up also helps, for example, the use of the clean-up columns such as Microcon® (Merck Millipore), Amicon® (Merck Millipore), MinElute™ (Qiagen) or NucleoSpin® (Clontech) all aim to remove the inhibitors prior to amplification process (Noren et al. 2013, Seo et al. 2012, Kim et al. 2000).

For the extracted samples where the inhibitors are not eliminated, there are several options to overcome their effects during amplification process. The type of DNA polymerase plays big impact on inhibition resistance (Schrader et al. 2012, Wiedbrauk et al. 1995, Katcher & Schwartz 1994). Amplitaq Gold® DNA polymerase which is widely used in the commercial multiplex STR kits is among the most sensitive to inhibition (Schrader et al. 2012, Abu Al-Soud & Radstrom 1998). Increasing the amount of DNA polymerase or adding substance such as bovine serum albumin (BSA) will provide more resistance against PCR inhibitors (Schrader et al. 2012, Comey et al. 1994). Also adding less amount of DNA template into PCR reaction will also assist in reducing inhibitors and emphasize STR kit sensitivity as a key advantage in generating better profiles from inhibited samples (Alaeddini 2012).

1.4 MOLECULAR GENETIC TECHNIQUES FOR FORENSIC DNA ANALYSIS

1.4.1 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) developed by Kary Mullis and co-workers in the mid-nineteen eighties is an enzymatic amplification of specific regions of DNA to yield several million copies of a particular sequence (Mullis et al. 1986, Saiki et al. 1985). It is a well-known technique in forensic DNA profiling (Weusten & Herbergs 2012). The PCR is ideally suited for the analysis of forensic samples containing degraded DNA, due to its sensitivity, speed and ability to produce multiple copies of target sequences of DNA (Kline et al. 2005, Schneider et al. 2004).

1.4.2 Multiplex PCR Reaction

Multiplex PCR is a system where simultaneous amplification of multiple regions of template DNA occur (Guo et al. 2014, Chamberlain et al. 1988). Compared to single primer set PCR reaction, multiplex PCR faces great challenges. The optimisation and design of multiplex PCR primers are important because the annealing events in multiplex primer pairs need to occur without interfering with one another and to obtain a good balance between the amplicons (Ruitberg et al. 2001). Thus, multiplex PCR primer design and optimisation of primer annealing temperature, concentration of reaction buffer, polymerase and $MgCl_2$ need to be carried out. The formation of primer-dimers which occurs because of self-complementarities between primers should be avoided for a successful multiplex PCR reaction (Rachlin et al. 2005, Henegariu et al. 1997).

1.4.3 Internal Amplification Controls (IACs)

Internal Amplification Controls (IACs) are the DNA fragments co-amplified with the target DNA template in a PCR reaction. The sizes of the IACs DNA fragments usually differ from the size of the target DNA template. IACs are usually used to identify false results which may be caused by the PCR inhibitors (Nazir et al. 2013, Zahra et al. 2011, Sachadyn & Kur 1998). It is also useful to differentiate degraded DNA samples from inhibited samples. The presence of the IACs in the absence of the target PCR product demonstrates that the amplification conditions were appropriate, but the target DNA was absent. In competitive PCR reaction, the IACs and the target DNA are amplified with the same pairs of primers; while in non-competitive PCR, different pairs of primers are applied for IACs and target DNA (Huang et al. 2013, Zahra et al. 2011).

1.5 DNA EXTRACTION

The main and ultimate aim of the extraction process is to obtain maximum amount of DNA from the samples submitted. At the same time, it is also important to get a pure DNA extraction by eliminating inhibitors which can reduce the efficiency of the amplification process, thus selection of the extraction technique is very important for samples containing PCR inhibitors.

1.5.1 Phenol-chloroform-isoamyl alcohol

Phenol-chloroform-isoamyl alcohol has been in use for the longest period of time and for many years was most widely used method for DNA extraction (Caputo et al. 2013, Kitayama et al. 2010, Gornik et al. 2002, Hochmeister et al. 1991). Phenol-chloroform-isoamyl alcohol extraction which sometimes is referred as organic extraction involves serial addition of several chemicals. First the cellular material is placed in a tube and proteinase K and detergent such as sodium dodecyl sulphate (SDS) are added to break open the cell membrane and break down the proteins that protect the DNA molecules. The phenol-chloroform mixture is added to separate the proteins from the DNA. The tube is then centrifuged to separate the organic phenol-chloroform phase and the aqueous phase because the DNA more soluble in the aqueous phase. The denatured proteins form a pellicle at the interface of these phases. Then using ethanol precipitation or clean-up columns, the DNA in the aqueous phase can be purified (Hudlow & Buoncristiani 2012, Jakubowska et al. 2012). Even though it is a preferred DNA extraction method for high molecular weight DNA, phenol potentially causes health problems because of its toxic nature (Wang et al. 2011).

1.5.2 Silica-based extraction

Silica-based extraction can be divided into two types: silica column and silica resin (Phillips et al. 2012, Wang et al. 2011, Castella et al. 2006, Nagy et al. 2005, Greenspoon et al. 1998). In both the techniques, cellular material undergoes digestion and DNA binds to the silica surfaces. Silica column captures the DNA and the washing processes are carried out by centrifugal technique while for silica resin, a magnetic stand has to be used and the washing processes are carried out using pipetting. These washing steps only leave behind the clean DNA which is attached to them. Then the elution buffer will be placed to release the DNA into the buffer. Since the introduction of automated systems for DNA extraction, silica resin techniques are more preferred (Fregeau et al. 2010, Brevnov et al. 2009, Nagy et al. 2005, Greenspoon et al. 2004).

1.6 DNA PRESERVATION

Following mass fatality incidents, DNA profiling is essential for identification and re-association of fragmented, burnt or decomposed corpses. Since environmental conditions have more influence on DNA degradation compared to time elapsed since deposition of the biological samples, collection and preservation of biological material obtained from deceased individuals are very important (Graham et al. 2008). The constituents of DNA preservatives should play a role in arresting DNA degradation. Inefficient preservation methods can cause destruction of intact DNA and make it difficult for victim identification. There are number of physical and chemical treatments for successful preservation of biological samples (Graham et al. 2008, Dawson et al. 1998).

1.6.1 Dehydration / freeze drying

Dehydration is based on the assumption that DNA is more stable when dry. By freeze drying process, the moisture in the biological samples will be frozen by reducing the surrounding pressure and then make the water sublimate directly from solid phase to the gas phase, thus drying them for storage (Anchordoquy & Molina 2007). Dehydration also can be carried out using other techniques. Sodium chloride is among one of the common preservatives which has been used for centuries. In solid form, it desiccates the sample which inactivates nucleases and slows microbial growth (Nagy 2010). When in an aqueous solution, sodium chloride denatures proteins including nucleases (Nagy 2010). Silica beads also have a similar effect as sodium chloride in desiccating the biological samples (Nagy 2010, Grassberger et al. 2005). The growth of bacteria occurs in the presence of water as moisture, which also provides a substrate for hydrolytic enzymes (Nagy 2010, Grassberger et al. 2005).

1.6.2 Ethanol

Ethanol works as preservative by removing water from the sample and denaturing proteins and nucleases (Flournoy et al. 1996; Seutin et al. 1991). Ethanol also acts as antimicrobial agent by protecting the biological samples against bacterial degradation (Seutin et al. 1991) and inhibits cellular enzymes (Penna et al. 2001).

1.6.3 Freezing

A preferred method of DNA preservation is freezing. It is done by freezing the biological samples over dry ice (-78 °C) or in liquid nitrogen (-196 °C) (Seutin et al. 1991). Biological sample storage at -20 °C also enhances DNA preservation but ultra-low temperature freezing is recommended for long-term storage (Nagy 2010). Freezing reduces bacterial

activities, thus reduces the DNA degradation (Hosokawa-Muto et al. 2013). Freezing preservation method is the most common method of tissue preservation (Nagy 2010).

1.6.4 Cell lysis solution

Cell lysis buffer is another method for DNA preservation. Cell lysis solution readily permeates tissues, lyses the cells and binds the divalent cations which are required for nuclease activity (Caputo et al. 2011, Graham et al. 2008). Sodium azide can be added to inhibit any bacterial growth (Sadiq 1995). Nuclease activity also can be reduced by adding chelating agents like EDTA which bind to metal ions such as Mg^{2+} and Ca^{2+}, which are required for normal function of nuclease activity on DNA (Giannakis et al. 1991, Seutin et al. 1991).

1.6.5 Room temperature storage

Room temperature storage is the easiest way to store the biological samples. Dried stains and skeletal remains can be stored at room temperature with low humidity environment (Budowle et al. 2005; Lee et al. 2012). Normally brief storage at room temperature for biological samples is preferred for DNA analysis (Burger et al. 1999). For longer storage adding some chemical such as glycerol will be essential to inhibit the bacterial and fungal growth (Giovani et al. 2006) and to keep the biological samples at dry stage (Lee et al. 2012).

1.7 AIMS AND OBJECTIVES OF THE STUDY

The overall aims of this research were to develop a tool to provide more information than real-time PCR for the assessment of DNA extracts and to test on different types of challenging samples.

The specific objectives were:

1. Develop a new PCR assay by combining Internal Amplification Controls (IACs) with a 4-plex PCR assay to differentiate degraded and inhibited samples.
   Attempts were taken to combine an existing 4-plex PCR assay with two previously designed Internal Amplification Controls (IACs) and thus develop a new multiplex PCR assay. This new multiplex was validated to study its sensitivity and capability in amplifying DNA of various concentrations.

2. Use the new PCR assay for quantification of DNA.
   The PCR assay was used to amplify various concentrations of control DNA and by their peaks heights, plotted into a correlation graph for the purpose of quantification.

3. Assess the capability of five different extraction methods to extract DNA from fresh and decomposed bone samples.
   Phenol-chloroform is the common technique to extract DNA from bone samples. This technique was compared with four other silica-based extraction methods to identify the best extraction method for fresh and decomposed bone samples, which gives a better DNA extract with less inhibition.

4. Assess the efficiency of five different preservation methods to preserve fresh and decomposed bone samples.

   A lot of research has been done on preservation of tissue but very limited studies have been undertaken on bone preservation. Therefore, this study attempted to establish a model for the effectiveness of preservation methods on fresh and decomposed bone samples.

5. Assess the new PCR assay on different types of forensic biological samples.

   The optimised assay was assessed for it efficiency in identifying the degraded and inhibited biological samples.

CHAPTER 2

GENERAL METHODS AND MATERIALS

2.1 OVERVIEW

The methods and materials described in this chapter have been applied in all the studies detailed in this thesis. Any specific methods which are applied to certain analyses have been described in the relevant chapters.

Good laboratory practices were adopted while doing this research. Clean lab coats and disposable gloves were worn during any laboratory work to reduce contamination. Each batch of extractions was carried out together with a reagent blank to identify any contamination during extraction process. Prior to any experiment, the bench top surface and the equipment were cleaned with ethanol. Sample preparation for PCR was carried out using dedicated pipettes in a PCR hood together with positive and negative controls. All samples in each experiment were prepared in triplicate and the final volume of the extracted samples was fixed at 100 µl unless stated otherwise. This allowed for easier comparisons between methods.

2.2 HEALTH, SAFETY AND ETHICS

Risk assessments and COSHH training have been completed before the use of instruments and chemicals in any experiment (*Appendix 1*). Ethical approval was obtained from the University of Central Lancashire's Health, Safety, and Ethics Committee prior to the experimental work (*Appendix 2*).

The permission to collect forensic samples from Malaysia was obtained from the Forensic Division, Department of Chemistry Malaysia (*Appendix 3*).

2.3 SAMPLES

2.3.1 Sample collection

Pig bones were used for all evaluation and preservation experiments. Fresh pig bones were purchased from a local butcher while decomposed bones were provided by UCLan's experimental field site called TRACES (Taphonomic Research in Anthropology: Centre for Experimental Studies) in Burnley (Lancashire, UK).

Forensic samples involving human bones for casework study were collected from the Forensic Division, Department of Chemistry Malaysia and Department of Forensic Medicine, Penang General Hospital, Malaysia through permission and arrangement by Department of Chemistry Malaysia.

2.4 BONE PRESERVATION

2.4.1 Sample preparation

Prior to preservation of bone samples, any soft tissue on the bone was removed. The bone was soaked in commercial bleach (5% sodium hypochloride) for 15 min, rinsed with water and then dried. Bone samples were cut on different days (fresh bones on 1st June 2012 and decomposed bones on 14th June 2012) to avoid cross contamination. Both fresh and decomposed bones were cut into small pieces approximately 1 cm^3 using a bone saw. A total of 180 bone pieces (90 fresh and 90 decomposed) were prepared. Each piece was placed into a labelled 15 ml polypropylene tube. Each sample was prepared in triplicate.

2.4.2 Time frame

Samples were prepared to be extracted after 6 weeks, 6 months and 1 year of preservation.

2.4.3 Preservation methods

A total of 5 preservation methods were tested; cell lysis solution (with 1% sodium azide), dehydration/freeze drying, ethanol (96%), freezing and room temperature storage. Thirty-six bone pieces (9 fresh ribs, 9 fresh femurs and 18 decomposed bones (9 ribs and 9 femurs) taken from pigs that had been exposed to the environment at TRACES for three months) were used in each preservation method.

2.4.3.1 Cell lysis solution (with 1% sodium azide)

Each bone piece was placed in a labelled 15 ml polypropylene tube and covered with approximately 3 ml of cell lysis solution (with 1% sodium azide) and stored in an incubator at 25 °C. This solution was prepared by dissolving 1 g of sodium azide (Sigma-Aldrich®, UK) into 100 ml of cell lysis solution (Qiagen, UK).

2.4.3.2 Dehydration / freeze drying

The bone pieces were freeze dried using Micro Modulyo® Freeze Dryer (Thermo Electron). Each bone piece was placed in a 15 ml polypropylene tube which was placed on a plastic tray with the top removed. A dome covering was placed over the tubes and once the machine cooled down, the vacuum was turned on. The tubes were left in the machine for 24 h. Once removed from the machine, the tubes were recapped and stored in the incubator at 25 °C.

2.4.3.3 Ethanol (96%)

Each bone piece was placed in a labelled 15 ml polypropylene tube and covered with approximately 3 ml of ethanol (96%) (Sigma-Aldrich®, UK) and stored in an incubator at 25 °C.

2.4.3.4 Freezing

Each bone piece was placed in a labelled 15 ml polypropylene tube and stored in the freezer at -20 ^{o}C.

2.4.3.5 Room temperature

Each bone piece was placed in a labelled 15 ml polypropylene tube and stored in the incubator at 25 °C without any preservation.

2.5 BONE SAMPLE PULVERISATION

Prior to the extraction, all the bone samples were pulverised using a freezer mill (Kitmondo) under liquid nitrogen. Fresh and degraded bone samples were pulverised on different days to avoid cross contamination. Once pulverised, each sample was placed in a new labelled 15 ml polypropylene tube and was stored in the freezer at -20 °C until DNA extraction was carried out next day.

2.6 DNA EXTRACTION

A total of five extraction methods were used; phenol-chloroform-isoamyl alcohol (Sigma-Aldrich®, UK), Chargeswitch® gDNA Plant Kit (Life Technologies™, UK), DNeasy® Blood and Tissue Kit (Qiagen, UK), Prepfiler® BTA Forensic DNA Extraction Kit (Life Technologies™, UK) and DNA IQ™ System Extraction Kit (Promega, UK).

2.6.1 Phenol-chloroform-isoamyl alcohol

For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube (ELKay®, UK). A volume of 400 µl of digestion buffer (10 mM Tris-HCl, 10 mM EDTA pH 8, 50 mM NaCl and 2% (w/v) SDS) and 15 µl of proteinase K (20 mg/ml) (Qiagen, UK) was added to the powdered bone sample and placed in a rotary incubator at 56 °C overnight. After incubation, each sample was briefly vortexed and centrifuged at maximum speed (16,000 x g) for 1 min. The cleared lysate was transferred

to a new labelled 1.5 ml microcentrifuge tube, leaving behind the bone debris. A volume of 400 µl phenol-chloroform-isoamyl alcohol was added to the tube and vortexed for approximately 2 min until an emulsion formed. The tube was centrifuged at maximum speed (16,000 x g) for 5 min and then the aqueous phase was transferred to a new labelled 1.5 ml microcentrifuge tube. These steps were repeated 2 to 3 times until the aqueous layer was clear. Then the aqueous phase was transferred to an Amicon 30kDa filter (Amicon ultra-0.5 centrifugal filter unit with ultracel-30 membrane, Merck Millipore) with the provided microcentrifuge tube attached to it and labelled. The tube was capped and was centrifuged at 14,000 x g for 10 min. Then the Amicon 30kDa filter was removed from the tube and placed upside down into a new labelled microcentrifuge tube. The tube was centrifuged at 1,000 x g for 2 min to transfer the concentrated sample from the Amicon 30kDa filter. Since the final volume was approximately 23 µl, a volume of 77 µl 1 X TE buffer (10 mM Tris-HCl and 0.1 M EDTA pH 8) was added to make the final volume to 100 µl. The tube was stored at -20 °C for further use.

2.6.2 Chargeswitch® gDNA Plant Kit

DNA extraction from bone samples using Chargeswitch® gDNA Plant Kit was carried out according to the manufacturer's instructions. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. A volume of 1 ml ChargeSwitch® Lysis Buffer and 100 µl 10% SDS was added to the powdered bone sample and placed in a rotary incubator at 37 °C overnight. After incubation each sample was left to cool to room temperature and 5 µl ChargeSwitch® RNase was added and the sample was incubated at room temperature for 1 min. Then the tube was centrifuged at maximum speed (16,000 x g) for 5 min to remove the bone debris. The cleared lysate was transferred to a new labelled 1.5 ml microcentrifuge tube. A volume of 400 µl pre-chilled ChargeSwitch® Precipitation Buffer was added to the tube and mixed by inversion. The tube was centrifuged at maximum speed (16,000 x g) for 5 min to precipitate the SDS / proteins and any remaining bone debris. The cleared lysate was transferred to a new labelled 1.5 ml microcentrifuge tube. A volume of 100 µl ChargeSwitch® 10% Detergent

was added to the tube. Then 20 µl fully resuspended ChargeSwitch® Magnetic Beads were added to the tube and mixed for 5 times with the pipette until the beads were evenly suspended. The tube was then incubated at room temperature for 1 min to allow the DNA to bind to the beads. The tube was then placed in a magnetic separator for 1 min or until the beads formed a tight pellet. The supernatant was removed and discarded. The tube was removed from the magnetic separator and the bead pellet was fully resuspended in 1 ml of ChargeSwitch® Wash Buffer by mixing with the pipette. The tube was placed in the magnetic separator for approximately 5 min or until the beads formed a tight pellet. The supernatant was removed and discarded. These washing steps were repeated once again. After the second washing, the tube was removed from the magnetic separator and the bead pellet was resuspended in 50 µl ChargeSwitch® Elution Buffer. The tube was incubated at room temperature for 1 min. Then the tube was placed in the magnetic separator for 1 min or until the beads formed a tight pellet. The eluate was transferred to a new labelled 1.5 ml microcentrifuge tube. These elution steps were repeated once again. Both the eluates were pooled together to give a total volume of 100 µl. The tube was stored at -20 °C for further use.

2.6.3 DNeasy® Blood and Tissue Kit

DNA extraction from bone samples using DNeasy® Blood and Tissue Kit was carried out according to the manufacturer's user-developed protocol. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. A volume of 360 µl Buffer ATL and 40 µl proteinase K (20 mg/ml) were added to the powdered bone sample and placed in a rotary incubator at 56 °C until the bone powder completely lysed. After incubation each tube was briefly vortexed. A volume of 400 µl Buffer AL was added and mixed thoroughly by vortexing. Then 400 µl ethanol (96%) was added and mixed thoroughly by vortexing. A volume of 650 µl mixture was pipetted into a labelled DNeasy® Mini Spin Columns placed in a 2 ml collection tube and centrifuged at 6,000 x g for 1 min. Flow-through was discarded and the collection tube was reused to centrifuge the remaining mixture. The DNeasy® Mini Spin Column

was placed in a new 2 ml collection tube. A volume of 500 µl Buffer AW1 was added and centrifuged at 6,000 x g for 1 min. Flow-through and the collection tube was discarded. The DNeasy® Mini Spin Column was placed in a new 2 ml collection tube. A volume of 500 µl Buffer AW2 was added and the tube centrifuged at maximum speed (16,000 x g) for 3 min. Flow-through and the collection tube was discarded. The DNeasy® Mini Spin Column was placed in a new labelled 1.5 ml microcentrifuge tube. A volume of 50 µl Buffer AE was directly pipetted onto the DNeasy® membrane. The tube was incubated at room temperature for 1 min and then centrifuged at 6,000 x g for 1 min. These elution steps were repeated once again. Both the eluates were pooled together to give a total volume of 100 µl. The tube was stored at -20 °C for further use.

2.6.4 Prepfiler® BTA Forensic DNA Kit

Prepfiler® BTA Forensic DNA Kit extraction was carried out according to the manufacturer's instructions. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. A volume of 220 µl PrepFiler® BTA Lysis Buffer, 3 µl 1.0 M DTT and 7 µl proteinase K (20 mg/ml) were added to the powdered bone sample and placed in a rotary incubator at 56 °C for 2 h. After incubation, sample was left to cool to room temperature and centrifuged at 9,000 x g for 1 min. The cleared lysate was transferred to a new labelled 1.5 ml microcentrifuge tube. A volume of 300 µl PrepFiler® Lysis Buffer was added to the tube and briefly vortexed and centrifuged. Then 15 µl of thoroughly resuspended magnetic particles were added to the tube. The tube was vortexed for 10 s and then briefly centrifuged to collect any residual from the sides and cap of the tube. A volume of 300 µl isopropanol was added to the tube. The tube was vortexed for 5 s and then briefly centrifuged. Then, the tube was incubated at room temperature for 10 min to allow the DNA to bind to the particles. After that, the tube was briefly vortexed and centrifuged to resuspend the magnetic particles. The tube was placed in a magnetic separator for approximately 5 min or until the beads formed a tight pellet. The supernatant was removed and discarded. The tube was removed from the magnetic separator and the bead pellet was fully resuspended in

600 µl Wash Buffer A using brief vortex and centrifugation steps. The tube was placed in the magnetic separator for 1 min or until the beads formed a tight pellet. The supernatant was removed and discarded. These washing steps were repeated with 300 µl Wash Buffer A and then with 300 µl Wash Buffer B. After the third washing, the tube was removed from the magnetic separator and allowed to air-dry for approximately 5 min. A volume of 50 µl PrepFiler® Elution Buffer was added to the tube and vortexed at maximum speed until the bead pellet resuspened. The tube was incubated at 70 °C for 10 min. The tube was briefly vortexed and centrifuged before being placed in the magnetic separator for approximately 5 min or until the beads formed a tight pellet. The eluate was transferred to a new labelled 1.5 ml microcentrifuge tube. These elution steps were repeated once again. Both the eluates were pooled together to give a total volume of 100 µl. The tube was stored at -20 °C for further use.

2.6.5 DNA IQ™ System Extraction Kit

DNA IQ™ System Kit extraction was carried out according to the manufacturer's instructions. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. A volume of 300 µl Proteinase K Digestion Solution was added to the powdered bone sample and placed in a rotary incubator at 56 °C for 1 hour. After incubation each sample was centrifuged at 2,000 x g for 5 min. The cleared lysate was transferred to a new labelled 1.5 ml microcentrifuge tube. A volume of 600 µl DNA IQ™ Lysis Buffer was added to the tube and briefly vortexed and centrifuged. Then 15 µl of thoroughly resuspended DNA IQ™ resin was added to the tube. The tube was briefly vortexed and centrifuged. The tube was then incubated at room temperature for 10 min to allow the DNA to bind to the particles. After that, the tube was briefly vortexed and centrifuged to resuspend the magnetic particles. The tube was placed in a magnetic separator for approximately 5 min or until the beads formed a tight pellet. The supernatant was removed and discarded. The tube was removed from the magnetic separator and the bead pellet was fully resuspended in 100 µl Lysis Buffer using brief vortex and centrifugation steps. The tube was placed in the magnetic separator for 1 min

or until the beads formed a tight pellet. The supernatant was removed and discarded. The tube was removed from the magnetic separator and the bead pellet was fully resuspended in 100 µl 1 X Wash Buffer by a brief vortex and centrifuge. The tube was placed in the magnetic separator for 1 min or until the beads formed a tight pellet. The supernatant was removed and discarded. These washing steps were repeated twice. After the third washing, the tube was left in the magnetic separator and allowed to air-dry for approximately 5 min. A volume of 50 µl Elution Buffer was added to the tube and vortexed at maximum speed until the bead pellet resuspened. The tube was incubated at 65 °C for 5 min. The tube was briefly vortexed and centrifuged before being placed in the magnetic separator for approximately 5 min or until the beads formed a tight pellet. The eluate was transferred to a new labelled 1.5 ml microcentrifuge tube. These elution steps were repeated once again. Both the eluates were pooled together to give a total volume of 100 µl. The tube was stored at -20 °C for further use.

2.7 EVALUATION OF DECALCIFICATION DURING BONE EXTRACTION AND USE OF AMICON 30KDA FILTER / ETHANOL PRECIPITATION DURING PHENOL-CHLOROFOM EXTRACTION

The effect of decalcification on pulverised bone samples was studied to evaluate its necessity since only 50 mg of powdered bone samples were used for extraction. Also, the use of the Amicon 30kDa filter and ethanol precipitation methods were evaluated for the purity of DNA. Only phenol-chloroform-isoamyl alcohol extraction was used to evaluate decalcification and the Amicon 30kDa filter / ethanol precipitation studies.

2.7.1. Sample preparation

Fresh pig bone samples (femur and rib) were used. Prior to extraction any soft tissue on the bone was removed. The bone was soaked in commercial bleach (5% sodium hypochloride) for 15 min, rinsed with water and then dried. Portion of each bone was prepared weighing between 1 and 2 g; after cutting, any bone marrow was removed before pulverisation under liquid nitrogen. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube (ELKay®, UK). Total of 24 tubes (12 rib bone samples and 12 femur bone samples) were prepared. Each sample point was prepared in triplicate for decalcification and Amicon 30kDa filter / ethanol precipitation studies. The details of the samples prepared are as shown in Table 2.1.

Table 2.1: Table below shows the sample preparation for different incubation and extraction techniques using phenol-chloroform-isoamyl alcohol.

Bone type	Incubation	Extraction	Number of samples
Rib	No decalcification	Ethanol precipitation	3
		Amicon 30kDa filter	3
	Decalcification	Ethanol precipitation	3
		Amicon 30kDa filter	3
Femur	No decalcification	Ethanol precipitation	3
		Amicon 30kDa filter	3
	Decalcification	Ethanol precipitation	3
		Amicon 30kDa filter	3

2.7.2 Decalcification prior to DNA extraction

Decalcification was carried out using 0.5 M EDTA. Twelve tubes (6 rib bone samples and 6 femur bone samples) were filled with 1 ml of 0.5 M EDTA and incubated in a fridge at 4 °C overnight. The other 12 samples were not decalcified.

2.7.3 DNA extraction

After overnight incubation with 0.5M EDTA, each sample was briefly vortexed and centrifuged at maximum speed (16,000 x g) for 1 min. The lysate was removed leaving behind the bone debris. To the all the 24 samples (decalcified and non-decalcified) a volume of 400 µl of digestion buffer (10 mM Tris-HCl, 10 mM EDTA pH 8, 50 mM NaCl and 2% (w/v) SDS) and 15 µl of proteinase K (20 mg/ml) (Qiagen, UK) were added and placed in the incubator at 56 °C overnight. After incubation, the phenol-chloroform extraction

was carried out on 12 samples (6 decalcified and 6 non-decalcified) as described in Section 2.6.1.

The extraction on the other 12 samples was carried out using ethanol precipitation technique. After the sample was treated with 400 µl phenol-chloroform-isoamyl alcohol for 2 to 3 times until the aqueous layer was clear as described in phenol-chloroform-isoamyl alcohol extraction in Section 2.6.1, the aqueous layer was transferred to a new labelled 1.5 ml microcentrifuge tube. To the tube, 2 volumes of cold absolute ethanol were added and the tube was inverted several times. The tube was placed in the freezer at -20 °C for 1 h. After the incubation tube was centrifuged at maximum speed (16,000 x g) for 20 min. The ethanol was poured out carefully and the tube was left to dry for approximately 10 min. A volume of 100 µl TE buffer (10 mM Tris-HCl and 0.1 M EDTA pH 8) was added and briefly vortexed before stored in the incubator at 56 °C for 1 hour so that the DNA pellet dissolves in the TE buffer. The tube was stored at -20 °C for further use.

2.8 EVALUATION OF EXTRACTION METHODS

The efficiency of the extraction methods (phenol-chloroform-isoamyl alcohol, Chargeswitch® gDNA Plant Kit, DNeasy® Blood and Tissue Kit, Prepfiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Extraction Kit) was evaluated before extraction was carried out on main samples.

2.8.1 Sample preparation

Fresh pig bone samples (femur and rib) and degraded pig bone samples (femur and rib) of 3 months and 1 year were used. Prior to extraction any soft tissue or algae on the bone was removed. The bone was soaked in commercial bleach (5% sodium hypochloride) for 15 min, rinsed with water and then dried. Portion of each bone was prepared weighing between 1 and 2 g; after cutting, any bone marrow was removed before pulverisation under liquid nitrogen. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. Each sample point was prepared in triplicate for each extraction method. Extraction was carried out using phenol-chloroform-isoamyl alcohol, Chargeswitch® gDNA Plant Kit, DNeasy® Blood and Tissue Kit, Prepfiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Extraction Kit as described in Section 2.6.

2.9 PRE-PROCESS METHOD DEVELOPMENT FOR DEGRADED BONE SAMPLES

A pre-process method was developed since an initial evaluation of the extraction methods on degraded bone samples (3 months and 1 year) did not give satisfactory results (Chapter 5). This method was developed to increase the starting material from 50 mg to 250 mg prior to DNA extraction where five tubes of lysed 50 mg bone samples were pooled together during pre-processing as shown in Table 2.2. Also, after the digestion of the bone samples, the samples were concentrated and cleaned using the Amicon 30kDa filter (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration). Only the phenol-chloroform-isoamyl alcohol extraction method was used for the validation of this method development. This pre-process technique was applied on all degraded bone samples extraction in this thesis.

2.9.1 Sample preparation

Degraded pig bone samples (femur and rib) of 3 months and 1 year were used. Prior to extraction any soft tissue or algae on the bone was removed. The bones were soaked in commercial bleach (5% sodium hypochloride) for 15 min, rinsed with water and then dried. Portion of each bone were prepared weighing between 1 and 2 g; after cutting any bone marrow was removed before pulverisation under liquid nitrogen. For each sample, approximately 50 mg of powdered bone sample was placed in a labelled 1.5 ml microcentrifuge tube. Total of 60 tubes (15 rib bone samples and 15 femur bone samples from animals that had been exposed to the environment for 3 months and 1 year) were prepared. Each sample point was prepared in triplicate for extraction.

2.9.2 Clean-up after the digestion

A volume of 400 µl of digestion buffer (10 mM Tris-HCl, 10 mM EDTA pH 8, 50 mM NaCl and 2% (w/v) SDS) and 15 µl of proteinase K (20 mg/ml) (Qiagen, UK) was added to the powdered bone sample and placed in the incubator at 56 °C overnight. After digestion, sample was briefly vortexed and centrifuged at maximum speed (16,000 x g) for 1 min. Then cleared lysate was transferred to the Amicon 30kDa filter (Amicon Ultra-2 ml centrifugal filters for DNA purification and concentration) with the provided filtrate collection tube attached to it and labelled. At this point, the lysate of five tubes pooled together into the Amicon 30kDa filter (2 ml) to make the final volume of 2 ml. The Amicon 30kDa filter (2 ml) was capped with the labelled concentrate collection tube and was centrifuged at 7,500 x g for 20 min. Then the filtrate collection tube was removed and the Amicon 30kDa filter (2 ml) together with the concentrate collection tube was placed upside down and centrifuged at 1,000 x g for 2 min to transfer the concentrated sample from the Amicon 30kDa filter (2 ml) into the concentrate collection tube. Then the collected sample was transfer into a new labelled 1.5 ml microcentrifuge tube. After that, phenol-chloroform extraction was carried out on this concentrated sample as described in Section 2.6.1. The details of the samples extracted are as shown in Table 2.2.

Table 2.2: Table below shows the samples extracted during pre-process method development.

Bone type	Degradation duration	Number of samples prepared from pulverised bone	Number of samples pooled together during pre-process technique	Number of extracted samples
Rib	3 months	15	5	3
	1 year	15	5	3
Femur	3 months	15	5	3
	1 year	15	5	3

2.10 DNA QUANTIFICATION

2.10.1 Quantification with GoTaq® qPCR Master Mix

Quantification using GoTaq® qPCR Master Mix (Promega, UK) was carried out using non-fluorescent forward and reverse primers to give an amplicon of 70 bp. The samples were prepared according to the manufacturer's instructions but the quantification was performed at a reduced total final volume of 12.5 µl. The standard DNA dilution series was made using control DNA 9947A (Life Technologies™, UK). The reaction mix, without template DNA was prepared by combining 6.25 µl 2x GoTaq® qPCR Master Mix, 4.75 µl H_2O, and 0.25 µl of each forward and reverse 70 bp primers (10 µM each) (smallest fragment of new multiplex). The reaction mix was gently vortexed, avoiding foaming. 11.5 µl of reaction mix was loaded into appropriate wells of the MicroAmp optical 96-well reaction plate (Life Technologies™, UK). Then 1 µl of DNA standard dilutions and the DNA samples were loaded into corresponding wells. After loading, the plate was sealed with an optical adhesive cover (Life Technologies™, UK). The plate was centrifuged for 1 min with 96 well plate centrifuge to bring all reaction components together and eliminate air bubbles.

The plate was placed into an ABI 7500 Real-Time PCR machine (Life Technologies™). The thermal cycler protocol was programmed according to the manufacturer's instructions: stage 1, 95 °C for 2 min for 1 cycle; stage 2 at 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles. Dissociation curve was also added for 1 cycle; 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. After the run completed, the data were analysed and DNA concentration for each sample was estimated in ng/µl. An example of a dissociation curve obtained from this quantification method is as shown in Figure 2.1. Similar temperatures for all analysed samples indicate that the correct products were obtained from this quantification method.

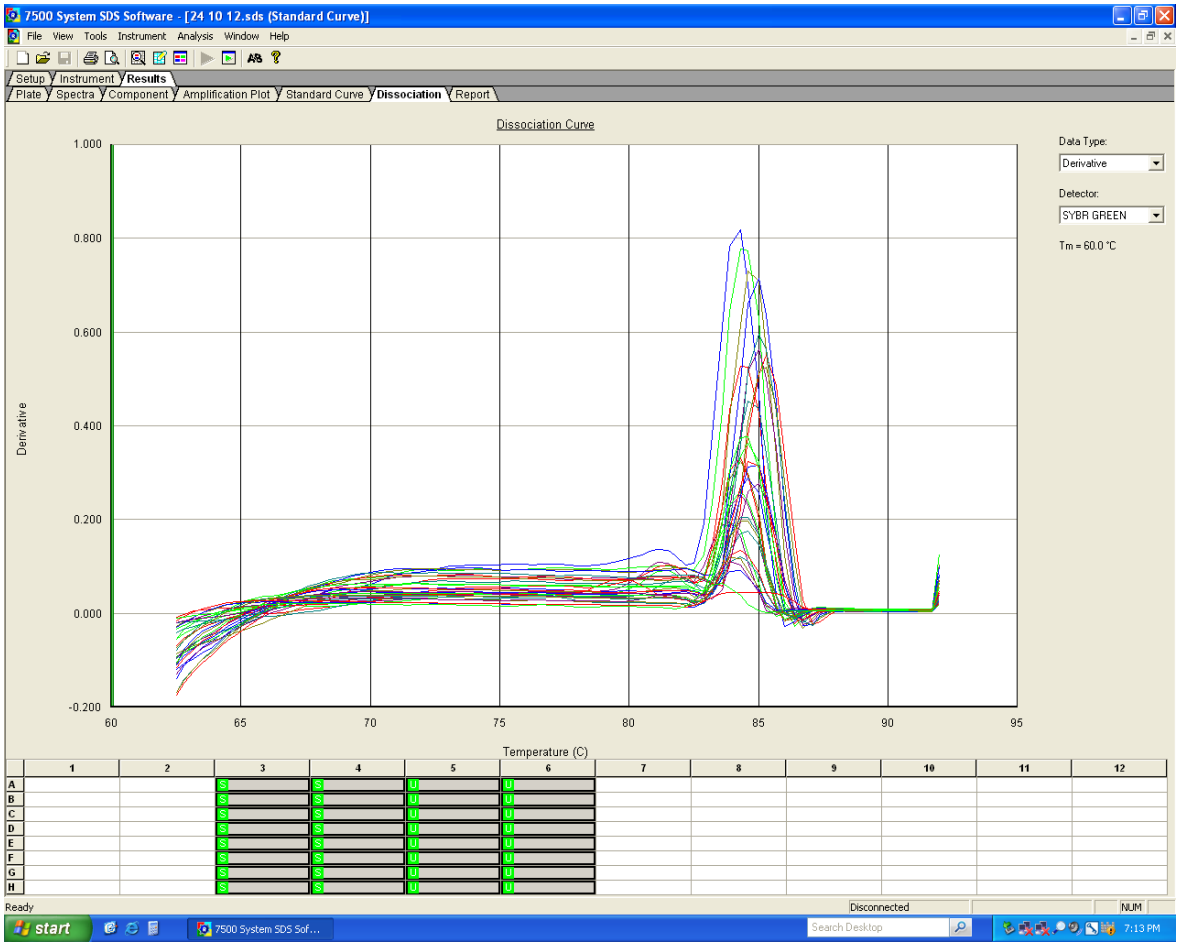


Figure 2.1: Dissociation curve obtained from the quantification using GoTaq® qPCR Master Mix.

2.10.2 Quantification of human samples with Quantifiler®

The extracted samples originated from humans were quantified using the Quantifiler® Human DNA Quantification Kit (Life Technologies™, UK) according to standard protocols recommended by the manufacturer. However, the quantification was performed at a reduced total final volume of 12.5 µl. The standard DNA dilution series was made using control DNA 9947A. The reaction mix, without template DNA was prepared by combining 6.25 µl Quantifiler® PCR Reaction Mix and 5.25 µl of Quantifiler® Human Primer Mix. The reaction mix was gently vortexed, avoiding foaming. 11.5 µl reaction mix was loaded into appropriate wells of the MicroAmp optical 96-well reaction plate. Then 1 µl of DNA standard dilutions and the DNA samples were loaded into corresponding wells. After

loading, the plate was sealed with an optical adhesive cover. The plate was centrifuged for 1 min with 96 well plate centrifuge to bring all reaction components together and to eliminate air bubbles.

The analyses of the samples were carried out using ABI 7500 Real-Time PCR machine. The thermal cycler protocol was programmed according to the manufacturer's instructions: stage 1, 95 °C for 10 min for 1 cycle; stage 2 at 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles. After each run completed the data were analysed and DNA concentration for each sample was estimated in ng/µl.

2.11 DNA AMPLIFICATION

The new multiplex was developed using four primers pairs of 4-plex and two primer pairs of IACs. Thus, this multiplex amplifies 70, 90, 194, 305, 384 and 410 bp amplicons. The primer mix was prepared according to the optimised PCR condition (Table 2.3).

The nuclear recombination activation gene 1 (RAG-1) from chromosome 11 (locus 11p13) was used to generate the 70, 194, 305 and 384 bp amplicons. This single-copy recombination activation gene 1 (RAG-1) is involved in somatic (V(D)J) rearrangement of B- and T-cell lymphocytes, which is essential for the development of a normal immune system and its functions. The RAG-1 gene is found throughout higher vertebrates and comprises a 3.1 kb exon without introns. It evolves slowly, has minimal saturation at the third position of codons, and a low frequency of indels (Nazir et al. 2013, Bernstein et al. 1996).

The sequence data for a nuclear recombination activation gene 1 (RAG-1) from human, rabbit, and pig were downloaded from GenBank and aligned using Bioedit software to identify conserved regions and the primers that would amplify 70, 194, 305, and 384 bp amplicons from the three species were identified (Nazir et al. 2013).

The 90 and 410 bp fragments (IAC_{90} and IAC_{410}) were produced from plasmid pBR322 (New England Biolabs, UK) separately, using tailed primers (please see next chapter for more details).

Table 2.3: Table below shows optimised primer concentrations of multiplex PCR reaction.

Primer	Forward and reverse primer (5'-3')	Primer concentration (µM)	Amplicon length (bp)
4-plex 70	CCTCAAAGTCATGGGCAGC	0.08	70
	GACTCTCCAGGTCAGTAGG	0.08	
4-plex 194	GCTGTTTGCTTGGCCATCCG	0.16	194
	GTGCTGGAAGACACATTCTTC	0.16	
4-plex 305	ATGAGGTCTGGCGTTCCAAC	0.20	305
	TGGTCATGAGCTTCCTGGCA	0.20	
4-plex 384	GAGCAATCTCCAGCAGTCCT	0.56	384
	GCTAAACTTCCTGTGCATGA	0.56	
IAC 90	CTGTCAAATCTAAACACCCTGATGCG	0.15	90
	GTCAGCTTGCATAATATCGAGATAACGC	0.15	
IAC 410	CTGTCAAATCTAAACACCCTGATGCG	0.45	410
	GTACAATGTTGACGTTCCTCGCTG	0.45	

2.11.1 Multiplex PCR Reaction

In a total reaction volume of 10.0 µl; 5.0 µl 2X Platinum® Multiplex PCR Master Mix (Life Technologies™, UK), 0.6 µl of primers mix, 2.4 µl of dH_2O, 1 µl of DNA template and 1 µl of IACs template were added. The amplification was carried out in GeneAmp® PCR System 9700 thermal cycler (Life Technologies™, UK). The thermal cycler conditions were prepared according to the optimised PCR condition (Table 2.4). The amplified products were stored at 4 °C for further use.

Table 2.4: Table below shows the thermal cycler conditions for multiplex PCR reaction amplification.

PCR stage		Temperature (°C)	Time (min)
Initial incubation		95	2
Denaturation	28 cycles	95	0.5
Annealing		60	1.5
Extension		72	1
Final Incubation		60	30
Hold		4	∞

2.12 CAPILLARY ELECTROPHORESIS

For fragment analysis, each sample was prepared by adding 1.0 µl of PCR product to 8.5 µl of Hi-Di™ Formamide (Life Technologies™, UK) and 0.5 µl GeneScan™ 500 LIZ™ size standard (Life Technologies™, UK). Then the samples were heated at 95 °C for 5 min and snap-cooled at 4 °C for least 3 min.

DNA fragment analysis was carried out on ABI 3500 Prism® Genetic Analyzer in a 50 cm long capillary using POP-6™ polymer (Life Technologies™, UK). Fragment analysis 50_POP6 run module was used with dye sets DS – 33 (filter set G5): 6 – FAM™ (blue), VIC® (green), NED™ (yellow), PET® (red) and LIZ® (orange). The parameters of ABI 3500 POP_6 are as shown in Table 2.5.

Table 2.5: Table below shows the parameters of ABI 3500 POP_6 module.

Parameter	Value
Run temperature	60 °C
Pre – run voltage	15 kV
Pre – run time	180 s
Injection voltage	1.6 kV
Injection time	5 s
Run voltage	15 kV
Run time	2700 s

2.13 DATA ANALYSIS

The data obtained from the capillary electrophoresis (CE) were analysed using ABI 3500 GeneMapper® ID-X Software Version 1.2 (Life Technologies™, UK). The parameters for the analysis of DNA profiles were kept the same for every run (Table 2.6).

Table 2.6: Table below shows the parameters for the analysis of PCR fragments.

Parameter	Value
Analysis Range	Full Range
Baseline Window	51 pts (points)
Minimum Peak Half Width	2 pts
Peak Detection	50 RFU
Peak Window Size	15 pts
Polynomial Degree	3 pts
Size Call Range	All Sizes
Size Calling Method	Local Southern
Slope Threshold for peak start/end	0-0

2.14 STATISTICAL ANALYSIS

Statistical analysis was carried out to investigate the differences between different methods and analyses which have been done in this research. The DNA concentrations and in some studies the peak heights (RFU) of the samples were used to carry out the statistical analysis.

Calculation of averages (avg.), the standard deviations (s.d.) and relative standard deviations (R.S.D.) were done using Excel 2010. Standard deviations were calculated to measure how data was scattered around the mean while relative standard deviations were calculated to reflect the spread of a data in percent.

R Studio software was used to perform statistical analysis such as independent t-test, analysis of variance (ANOVA) and Tukey test with α=0.05. Tukey test was used for multiple comparisons between different data sets. The term “p adj” was used for the multiple comparison statistics referring to the adjustment methods which include the Bonferroni correction where the p-values were multiplied by the number of comparisons.

CHAPTER 3

DEVELOPMENT, OPTIMISATION AND VALIDATION OF A NEW MULTIPLEX (4-PLEX & IACS) PCR ASSAY

3.1 OVERVIEW

Forensic laboratories can experience problems when trying to analyse poor quality DNA samples. One particular aspect is to differentiate between samples that are producing no or partial profiles due to DNA degradation and those that also product no or partial profiles due to PCR inhibition.

In most laboratories PCR inhibition can be detected through the use of an internal PCR control (IPC) during real-time quantification (Seo et al. 2012, Kontanis & Reed 2006). However, there are limitations with this approach as the amplicon in the real-time PCR reactions is typically short, and so does not necessarily reveal the full extent of PCR inhibition. An alternative approach, which attempted to overcome these limitations, has been to incorporate internal amplification controls (IACs) into the PCR reaction (Zahra et al. 2011).

Previous studies in this laboratory have developed a multiplex to assess DNA degradation, but this did not incorporate any features to detect PCR inhibition (Nazir 2012), making it difficult to separate the two phenomena. This part of the research aims to develop a multiplex PCR assay by combining the 4-plex system with two Internal Amplification Controls (IACs), thereby allowing the assessment of DNA degradation whilst monitoring for PCR inhibition.

3.1.1 Objectives

- To combine and optimise two existing PCR systems: a 4-plex that amplifies fragments ranging between 70 bp and 384 bp and two internally amplified controls of 90 bp and 410 bp (IAC_{90} and IAC_{410}).
- Prepare a serial dilution samples using control DNA 9947A and test the sensitivity of the multiplex.
- Test the multiplex on DNA extracted from degraded pig tissue samples to study the behaviour of the multiplex with degraded DNA.
- Prepare serial dilution samples of known PCR inhibitors to test the sensitivity of the multiplex to different inhibitors.

3.2 METHODS AND MATERIALS

3.2.1 Multiplex design

3.2.1.1 Design of 4-plex primers

Four pairs of primers were designed to amplify 70, 194, 305 and 384 bp amplicons and optimised to work with comparable efficiency using pig, rabbit and human DNA (Nazir et al. 2013).

Primer pairs with 5' fluorescein-labeled forward primers and unlabeled reverse primers were synthesized (Life Technologies™, UK) and purified using HPLC and desalting respectively: 100 µM stock solutions were prepared by adding the appropriate volume of 1X TE buffer (0.01 M Tris HCl, 0.001 M EDTA, pH 8.0; Sigma-Aldrich®, UK) and stored at -20 °C, while an aliquot of a 10 µM working solution was kept at 4 °C.

3.2.1.2 Development of Internal Amplification Controls (IACs) fragments

The 90 and 410 bp fragments (IAC_{90} and IAC_{410}) were produced from plasmid pBR322 (New England Biolabs, UK) separately, using tailed primers (Table 3.1) (Zahra et al. 2011). These fragments were generated from region 832 to 917 bp and 1682 to 2041 bp of the plasmid with a composite primer technique as shown in Figure 3.1.

Table 3.1: Table below shows the sequence of the primers used to generate the IAC_{90} and IAC_{410} fragments.

Tailed primer	Sequence 5' – 3'
IAC_{90} forward	CTGTCAAATCTAAACACCCTGATGCGGCTTGCGGTATTCGGAATCTTG
IAC_{90} reverse	GTCAGCTTGCATAATATCGAGATAACGCGAGCGAGGGCGTGCAAGATT
IAC_{410} forward	CTGTCAAATCTAAACACCCTGATGCGGATGCTGCTGGCTACCCTGT
IAC_{410} reverse	GTACAATGTTGACGTTCCTCGCTGCGTGAAGCGATTCACAGATCTCTG

Note: Colored letters show the sequence of the tailed primers

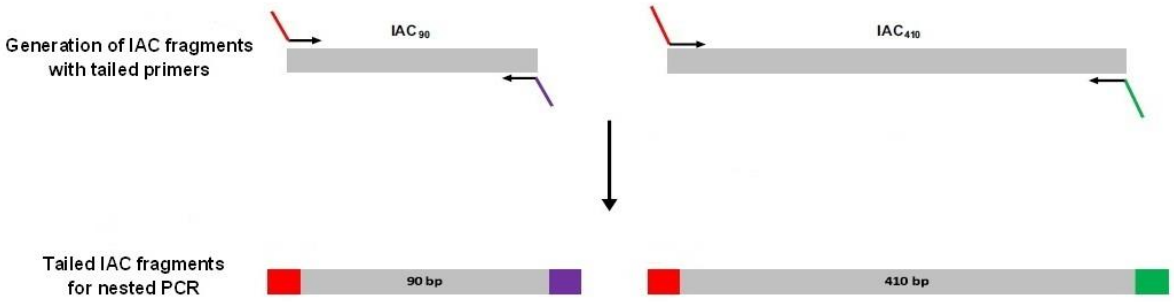


Figure 3.1: Schematic diagram shows the generation of IAC_{90} and IAC_{410} fragments from different regions of the plasmid pBR322, using tailed primers.

Amplification reactions were prepared using 10 µl of 5 ng/µl plasmid, 36 µl of 1.1X Reddy Mix PCR Master Mix (Thermo Scientific, UK) and 2 µl of 5 µM forward and reverse tailed primers to give a total reaction volume of 50 µl. Thermal cycler conditions were according to the optimised PCR conditions for IAC_{90} and IAC_{410} fragments respectively (Table 3.2). The fragments generated were purified using QIAamp Mini Elute PCR purification kit (Qiagen, UK), adopting the procedure outlined by the manufacturer (Zahra et al. 2011).

These fragments were diluted 10-fold until optimal concentration for balanced peaks on electropherograms was obtained and kept as stock.

Table 3.2: Table below shows the thermal cycler conditions for amplification of IAC_{90} and IAC_{410} fragments.

PCR stage		Temperature (°C)		Time (min)
		IAC_{90}	IAC_{410}	
Initial incubation		95	95	5
Denaturation	33 cycles	94	94	0.5
Annealing		66	58	1
Extension		72	72	1
Final Incubation		72	72	45
Hold		12	12	∞

3.2.1.3 Nested PCR using Internal Amplification Controls (IACs) primers

Two pairs of Internal Amplification Controls (IACs) primers which amplify the IAC_{90} and IAC_{410} fragments were used (Zahra et al. 2011) (Table 3.3). ROX-labeled forward primer and unlabelled reverse primers were synthesized (Life Technologies™, UK) and were purified using HPLC and desalting respectively: 100 µM stock solutions were prepared by adding the appropriate volume of 1X TE buffer (0.01 M Tris HCl, 0.001 M EDTA, pH 8.0; Sigma-Aldrich®, UK) and stored at -20 °C, while an aliquot of a 10 µM working solution was kept at 4 °C.

Table 3.3: Table below shows the sequence of the IAC_{90} and IAC_{410} ROX-labelled primers used in nested PCR.

Amplicon length (bp)	Forward and reverse primer (5' – 3')
90	CTGTCAAATCTAAACACCCTGATGCG GTCAGCTTGCATAATATCGAGATAACGC
410	CTGTCAAATCTAAACACCCTGATGCG GTACAATGTTGACGTTCCTCGCTG

3.2.1.4 Development of multiplex (4-plex & IACs)

The new multiplex was developed using four primers pairs of 4-plex and two primer pairs of IACs. Thus, this multiplex amplifies 70, 90, 194, 305, 384 and 410 bp amplicons. The primer mix was prepared according to the optimised PCR condition (refer to Table 2.3 in Chapter 2).

3.2.1.5 Multiplex PCR reaction

The thermal cycler conditions were according to the optimised PCR conditions (Table 3.4). In a total reaction volume of 10.0 µl; 5.0 µl 2X Platinum® Multiplex PCR Master Mix (Life Technologies™, UK), 0.6 µl of primer mix, 2.4 µl of dH_2O, 1 µl of DNA template and 1 µl of IAC template were added. The amplification was carried out in GeneAmp® PCR System 9700 thermal cycler (Life Technologies™, UK). The amplified products were stored at 4 °C for further use.

Table 3.4: Table below shows the thermal cycler conditions for multiplex PCR reaction amplification.

PCR stage		Temperature (°C)	Time (min)
Initial incubation		95	2
Denaturation	28 cycles	95	0.5
Annealing		60	1.5
Extension		72	1
Final Incubation		60	30
Hold		4	∞

3.2.2 Sample preparation for multiplex sensitivity studies

3.2.2.1 Control DNA

A control DNA 9947A (Life Technologies™, UK), with different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.02, and 0.01) ng was prepared by serial dilution and amplified using the optimised multiplex (4-plex & IACs) system. Each sample point was prepared in triplicate.

3.2.2.2 Degraded DNA

A degraded DNA study was carried out on extracted DNA of pig tissue samples from UCLan's experimental field site called TRACES in Burnley (Lancashire, UK). The samples were collected between 3 and 6 days interval in summer which allowed the samples to degrade in the environment. The collected samples were extracted using DNeasy® Blood and Tissue kit (Qiagen, UK) according to the manufacturer's instructions and the final

volume were standardized for all samples at 200 µl. These samples were collected and extracted by Muhammad Shahid Nazir (Nazir et al. 2013), previous PhD student, UCLan. Extracted samples were amplified directly using 1 µl with the multiplex (4-plex & IACs) system.

3.2.2.3 PCR inhibitors

The usage of the IACs in detecting the presence of PCR inhibitors was studied using human collagen type 1, humic acid, tannic acid, hematin, ethanol, phenol and 100 X TE buffer (1 M Tris HCl, 0.1 M EDTA, pH 8.0) (all from Sigma-Aldrich®, UK). The concentration range of each inhibitor tested is as shown in Table 3.5. A control DNA 9947A (1 ng/µl) was used as the template DNA. The control DNA was also amplified without any inhibitor as a blank control.

Table 3.5: Table below shows the PCR inhibitors and the concentrations used for PCR inhibitory study using multiplex system.

PCR inhibitor	Final concentration in a PCR reaction
Human Collagen Type 1	100, 50, 25, 12.5, 6.25 ng/µl
Humic Acid	40, 20, 10, 5, 2.5 and 1.25 ng/µl
Tannic Acid	40, 20, 10, 5 and 2.5 ng/µl
Hematin	40, 20, 10, 5 and 2.5 ng/µl
Ethanol	9.6, 7, 5, 2.5 and 1.25%
Phenol	9.9, 5, 2.5, 1.25 and 0.625%
TE Buffer	10, 5, 2.5, 1.25, 0.625. 0.313 and 0.156 X

3.3 RESULTS

3.3.1 Multiplex (4-plex & IACs) design

The multiplex was designed by adding four primer pairs which were designed to amplify the amplicons length of 70, 194, 305 and 384 bp for pig, rabbit and human (Nazir et al. 2013) together with two primer pairs for Internal Amplification Controls (IACs) to amplify 90 and 410 bp amplicons. The G+C contents of each primer were between 40-60% and the primer length of all the primers was less than 25 nucleotides.

At first, the amplification was carried out using AmpliTaq Gold® PCR Master Mix (Life Technologies™, UK) for the development and optimisation of the 4-plex system. The primer concentration and thermal cycler conditions for the optimised 4-plex system are as shown in the Tables 3.6 and 3.7.

Table 3.6: Table below shows the primer concentrations of 4-plex system.

Primer	Forward and reverse primer (5' – 3')	Primer concentration (µM)	Amplicon length (bp)
4-plex 70	CCTCAAAGTCATGGGCAGC	0.05	70
	GACTCTCCAGGTCAGTAGG	0.05	
4-plex 194	GCTGTTTGCTTGGCCATCCG	0.10	194
	GTGCTGGAAGACACATTCTTC	0.10	
4-plex 305	ATGAGGTCTGGCGTTCCAAC	0.15	305
	TGGTCATGAGCTTCCTGGCA	0.15	
4-plex 384	GAGCAATCTCCAGCAGTCCT	0.40	384
	GCTAAACTTCCTGTGCATGA	0.40	

Table 3.7: Table below shows the thermal cycler conditions of 4-plex system.

PCR stage		Temperature (°C)	Time (min)
Initial incubation		95	5
Denaturation	30 cycles	94	1
Annealing		60	1
Extension		72	1
Final Incubation		72	10
Hold		12	∞

But, the inclusion of IACs primers into optimised 4-plex system caused problems. The size standard was changed from GeneScan™ 500 ROX™ to GeneScan™ 500 LIZ™ because of the use of ROX-dye in the IACs primers, thus avoiding confusion in the size standard labelling and quality (Figure 3.2). The peaks became imbalanced and also split. Therefore, a different PCR master mix (Platinum® Multiplex PCR Master Mix (Life Technologies™, UK)) was tested. Optimisation of this new multiplex system was carried out by testing five different set of primer concentrations until optimal level was achieved. The annealing temperature of all these primers was 60 ± 7 °C (Table 3.8). Since the 4-plex system was optimised at 60 °C, but the annealing temperature of the IACs primers was higher, all temperatures in the range of 60 to 67 °C were also tested (Table 3.9). The primer concentration of set 5 was found to be optimal with balanced peak heights and also without split peaks (Figure 3.3) and the annealing temperature of 60 °C was found to be optimum for this multiplex (4-plex & IACs) system. The change of the PCR Master Mix between 4-plex system and the new multiplex system also caused a large increase in the peak heights of the profiles, thus the number of cycles of thermal cycler was reduced from 30 cycles to 28 cycles. The injection time on ABI 3500 Prism® Genetic Analyzer also was reduced from 10 s to 5 s.

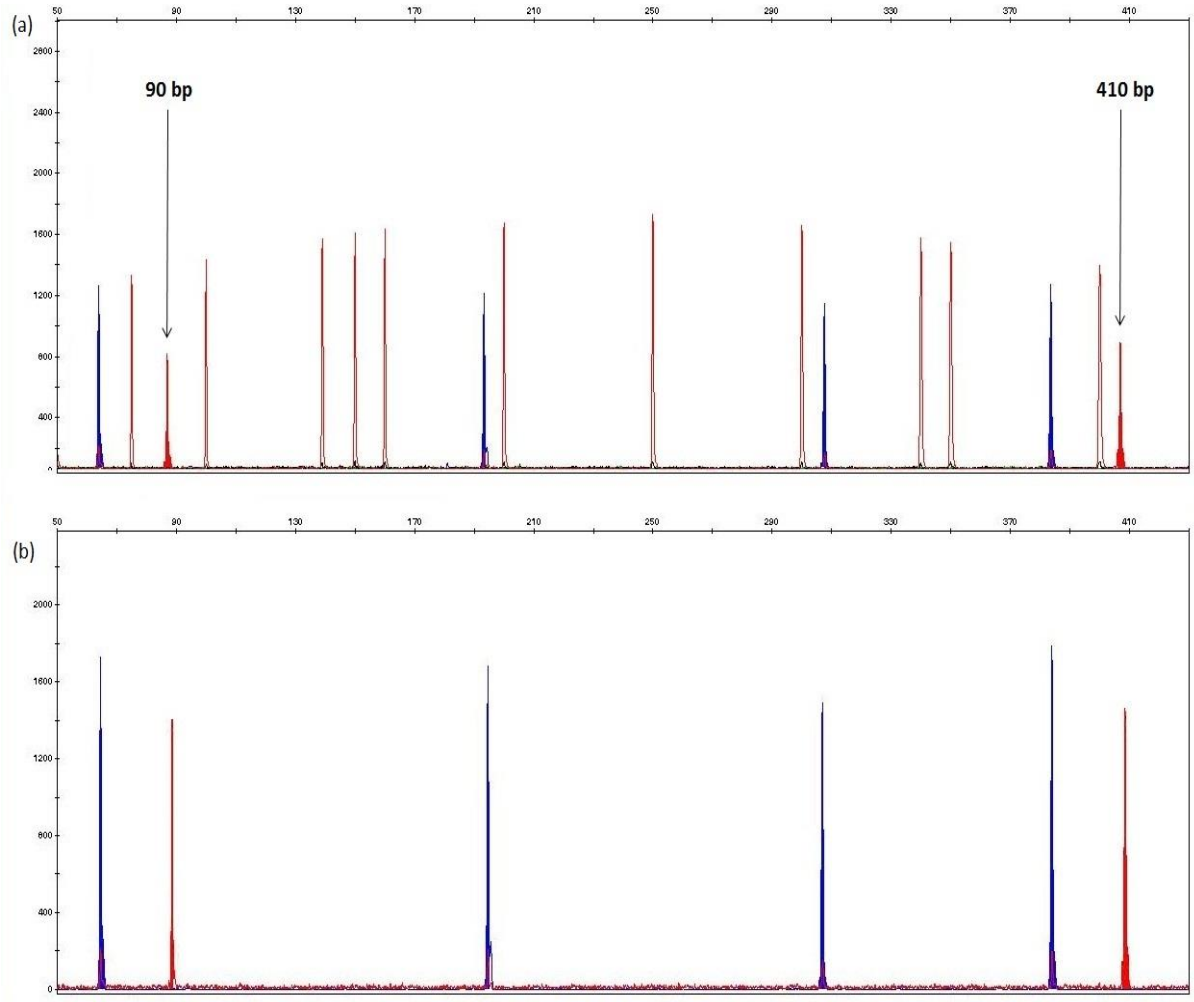


Figure 3.2: Examples of electropherograms using (a) GeneScan™ 500 ROX™ and (b) GeneScan™ 500 LIZ™ size standards. Panel can be displayed without the size standards.

Table 3.8: Table below shows the five primer sets for multiplex (4-plex & IACs) PCR reaction tested for their optimum primer concentrations.

Primer	Forward and reverse primer (5′-3′)	Annealing temperature (°C)	Primer Concentration (µM)					Amplicon length (bp)
			Set 1	Set 2	Set 3	Set 4	Set 5	
4-plex 70	CCTCAAAGTCATGGGCAGC	60	0.05	0.06	0.07	0.08	0.08	70
	GACTCTCCAGGTCAGTAGG	60	0.05	0.06	0.07	0.08	0.08	
4-plex 194	GCTGTTTGCTTGGCCATCCG	63	0.10	0.15	0.20	0.19	0.16	194
	GTGCTGGAAGACACATTCTTC	60	0.10	0.15	0.20	0.19	0.16	
4-plex 305	ATGAGGTCTGGCGTTCCAAC	60	0.15	0.20	0.20	0.15	0.20	305
	TGGTCATGAGCTTCCTGGCA	60	0.15	0.20	0.20	0.15	0.20	
4-plex 384	GAGCAATCTCCAGCAGTCCT	60	0.40	0.45	0.50	0.56	0.56	384
	GCTAAACTTCCTGTGCATGA	56	0.40	0.45	0.50	0.56	0.56	
IAC 90	CTGTCAAATCTAAACACCCTGATGCG	66	0.20	0.10	0.15	0.15	0.15	90
	GTCAGCTTGCATAATATCGAGATAACGC	67	0.20	0.10	0.15	0.15	0.15	
IAC 410	CTGTCAAATCTAAACACCCTGATGCG	66	0.20	0.30	0.40	0.45	0.45	410
	GTACAATGTTGACGTTCCTCGCTG	65	0.20	0.30	0.40	0.45	0.45	

Table 3.9: Table below shows the thermal cycler conditions tested for the optimal multiplex PCR reaction amplification.

PCR stage		Temperature (°C)	Time (min)
Initial incubation		95	2
Denaturation	28 cycles	95	1
Annealing		60 – 67[(a)]	1
Extension		72	1
Final Incubation		72	30
Hold		12	∞

[(a)] Each and every temperature from 60 to 67 °C was tested separately with each set of the primer concentrations (set 1- set 5).

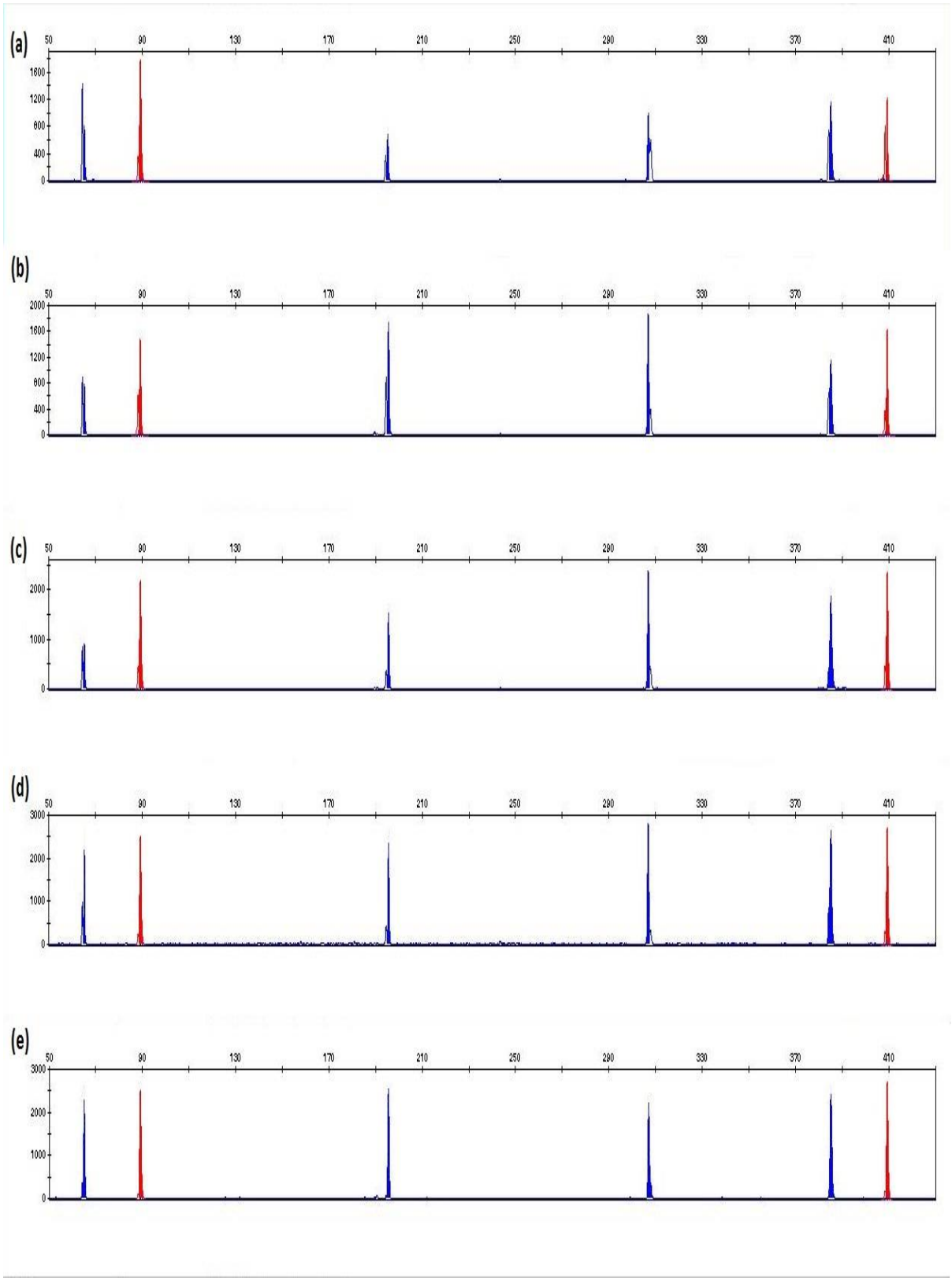


Figure 3.3: Electropherograms of (a) set 1, (b) set 2, (c) set 3, (d) set 4 and (e) set 5. Each set consisted of different primer concentrations. Imbalanced and split peaks can be observed in set 1 – set 4 but set 5 shows balanced peaks without any split peaks.

3.3.2 Amplicon specificity study

The optimised multiplex (4-plex & IACs) system was assessed for any non-specific amplification that would lead to extra peaks and could interfere with target loci. A control DNA 9947A (Life Technologies™, UK) was used as a template to evaluate the multiplex. The amplified products were analysed on ABI 3500 according to the protocol described (Methods and Materials chapter). All six amplicons were amplified and produced a specific peak for the targeted loci without any additional peaks (Figure 3.4).

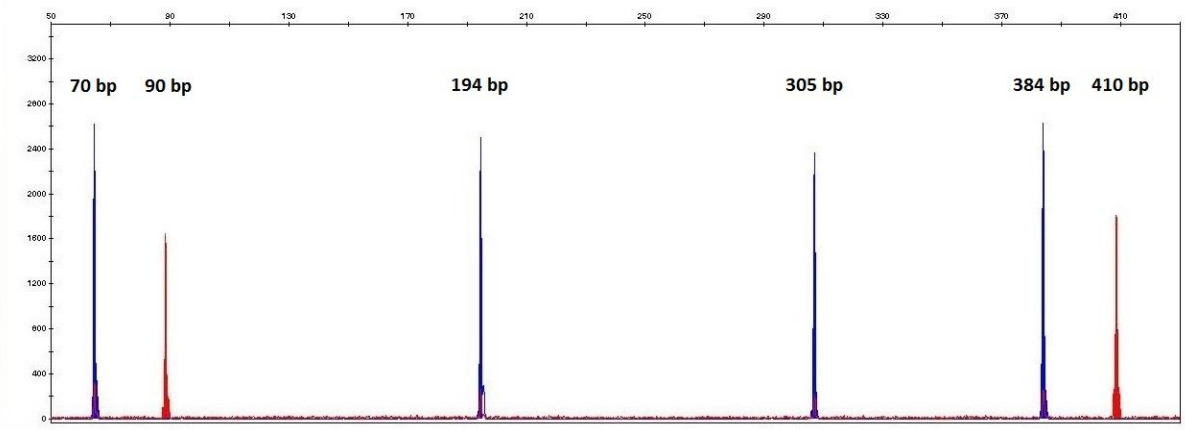


Figure 3.4: Example of electropherogram shows specific peak for each amplicon (70, 90, 194, 305, 384 and 410 bp) without any additional peaks.

3.3.3 Sensitivity of the multiplex

The results showed that full profiles were obtained from 200 ng to 0.10 ng DNA, thus the minimum amount of DNA required for this multiplex to generate full profile is 0.10 ng (Table 3.10). Below that concentration, partial profiles were generated with 0.05 ng DNA. There was only one peak detected (70 bp) in one of the triple samples (sample 2) with DNA concentration 0.02 ng. No profile was developed with 0.01 ng DNA (Figure 3.5). The minimum threshold used for peak detection was 50 RFU (Relative Fluorescence Units).

Table 3.10: Table below shows the DNA concentration and the peak heights of the profiles generated. Only 0.10 ng DNA and below were shown.

DNA concentration (ng)	DNA sample	4-plex peak height (RFU)				IACs peak height (RFU)	
		70	194	305	384	90	410
0.10	Sample 1	130	142	86	92	1560	1648
	Sample 2	83	63	53	66	1433	1522
	Sample 3	151	101	106	191	1716	1909
0.05	Sample 1	61	n.d.	n.d.	n.d.	1411	1449
	Sample 2	57	52	55	n.d.	1409	1452
	Sample 3	52	n.d.	n.d.	n.d.	1494	1602
0.02	Sample 1	n.d.	n.d.	n.d.	n.d.	1360	1427
	Sample 2	50	n.d.	n.d.	n.d.	1460	1550
	Sample 3	n.d.	n.d.	n.d.	n.d.	1508	1637
0.01	Sample 1	n.d.	n.d.	n.d.	n.d.	1590	1724
	Sample 2	n.d.	n.d.	n.d.	n.d.	1408	1489
	Sample 3	n.d.	n.d.	n.d.	n.d.	1466	1539

Note: n.d.: not detected

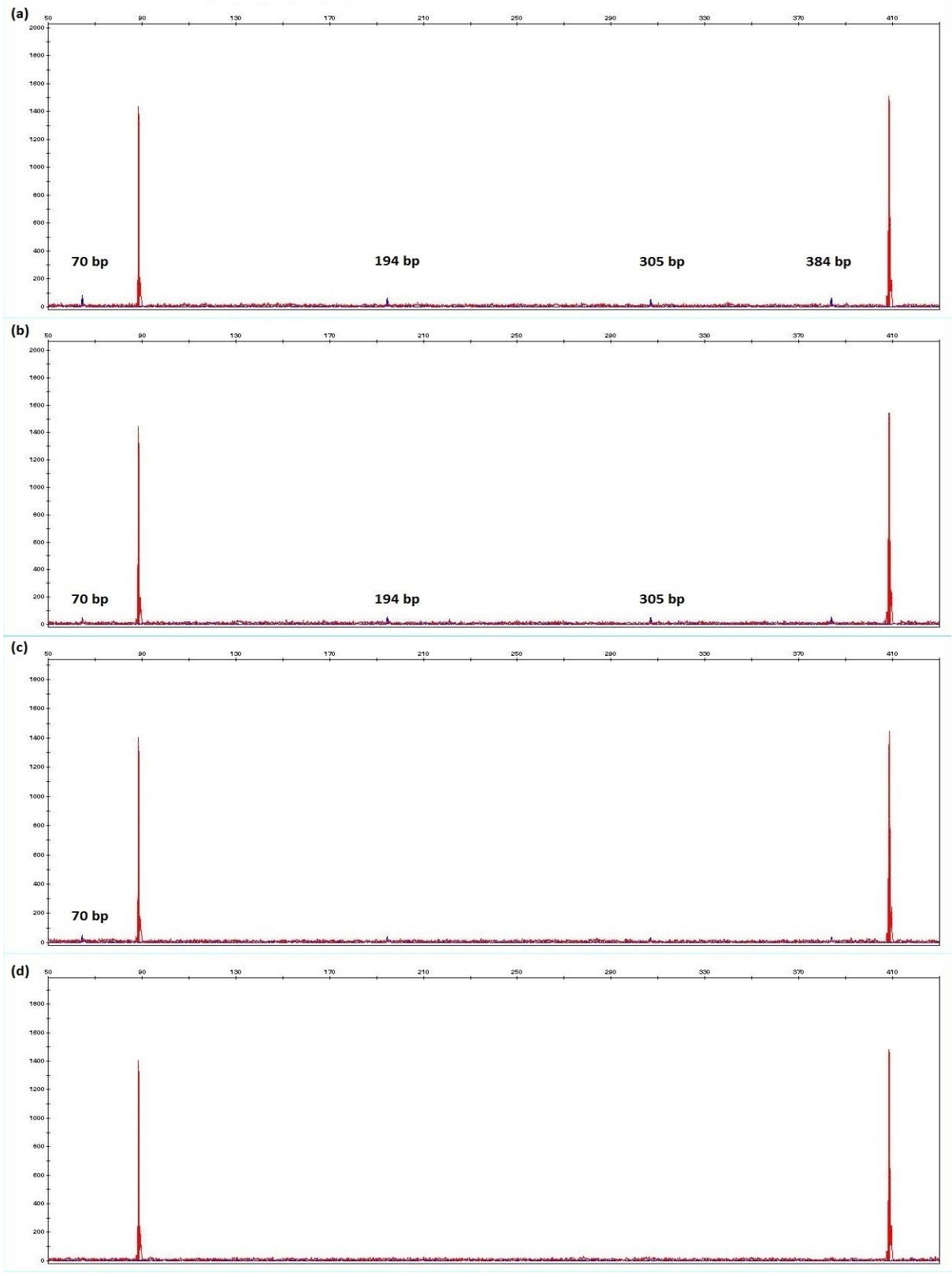


Figure 3.5: Electropherograms generated from samples from set 2 with (a) 0.10 ng, (b) 0.05 ng, (c) 0.02 ng and (d) 0.01 ng DNA. Full profile was produced with 0.10 ng DNA but only partial profiles were produced below that and no DNA profile was produced with 0.01 ng DNA.

3.3.4 Degraded DNA study using the multiplex system

The DNA extract volume used for degradation study was kept constant at 200 µl for each sample for comparative study (Nazir et al. 2013). 1 µl of each sample was used for amplification. As the time of collection delayed, the larger fragments were degraded. Full profiles could be obtained up to and including day 24 (295 ADD). The IACs peaks are well balanced in all the electropherograms indicating the absence of PCR inhibitors (Figure 3.6).

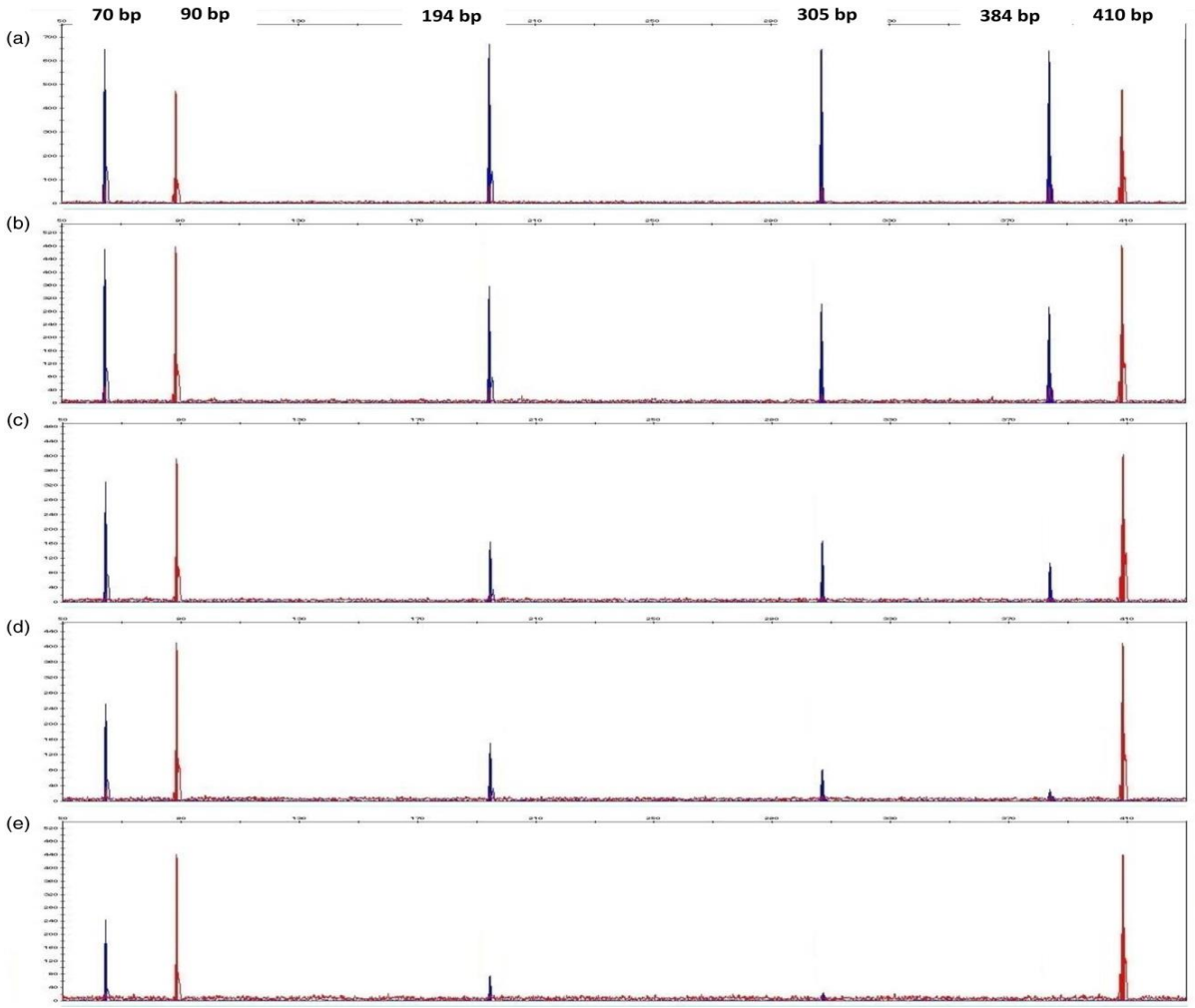


Figure 3.6: Examples of electropherograms generated from 1 µl of DNA extract (from a total of 200 µl) extracted from pig soft muscle tissues at (b) 159 ADD, (c) 203 ADD, (d) 295 ADD and (e) 338 ADD. Control DNA 9947A (a) is also shown.

3.3.5 PCR inhibitors study using the multiplex system

Different types of inhibitors were prepared to test the efficiency of the IACs in the multiplex system to detect the inhibitors (please refer to Table 3.5). The study was carried out using 1 µl of prepared concentrations from human collagen type 1, humic acid, tannic acid, hematin, ethanol, phenol and 100X TE buffer (10 mM Tris-HCl pH 8, 1mM EDTA) with 1 ng/µl control DNA 9947A in total of 10 µl PCR reaction, as shown in Tables 3.11 and 3.12. Each samples point was prepared in triplicate but only one sample was chosen to represent them in the ratio calculation since all the triplicate samples produced similar results. The control DNA also was amplified without any inhibitor as a blank control. The results are as shown in Tables 3.11 and 3.12 and Figures 3.7 to 3.14.

Table 3.11: Table below shows the properties of DNA profiles developed with human collagen type 1, humic acid, tannic acid and hematin with different concentrations.

Inhibitor	Final concentration	IACs PH (RFU)		IAC_{410}/IAC_{90}	4-plex peak height (RFU)				4-plex peak imbalance
		IAC_{90}	IAC_{410}		70	194	305	384	
Human Collagen Type 1	100 ng/µl	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	50 ng/µl	1510	n.d.	N/A	1543	499	157	n.d.	PP
	25 ng/µl	1969	1608	0.82	1930	1575	1358	1249	0.65
	12.5 ng/µl	1655	1610	0.97	2085	2030	1729	1649	0.79
	6.25 ng/µl	1586	1609	1.01	2231	2219	2180	2160	0.97
Humic Acid	40 ng/µl	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	20 ng/µl	634	n.d.	N/A	127	n.d.	n.d.	n.d.	PP
	10 ng/µl	942	n.d.	N/A	1569	710	60	n.d.	PP
	5 ng/µl	1635	257	0.16	2448	2069	1588	873	0.36
	2.5 ng/µl	1750	989	0.57	2107	1787	1382	1188	0.56
	1.25 ng/µl	1571	1919	1.22	2118	1944	2150	1998	0.92
Tannic Acid	40 ng/µl	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	20 ng/µl	1232	n.d.	N/A	367	167	n.d.	n.d.	PP
	10 ng/µl	1204	440	0.36	2578	1887	1461	1399	0.54
	5 ng/µl	1607	1283	0.80	2473	2001	1688	1539	0.62
	2.5 ng/µl	1231	1387	1.13	2319	2223	2362	2270	0.96
Hematin	40 ng/µl	1174	n.d.	N/A	711	n.d.	n.d.	n.d.	PP
	20 ng/µl	1392	n.d.	N/A	2198	986	193	n.d.	PP
	10 ng/µl	1585	798	0.50	2366	2008	1433	1311	0.55
	5 ng/µl	1683	1694	1.00	2353	2275	2235	2109	0.90
	2.5 ng/µl	1652	1888	1.14	2196	2041	2001	1922	0.88

Note: n.d.: not detected, N/A: Not Applicable, NP: No Profile, PP: Partial Profile.

Table 3.12: Table below shows the properties of DNA profiles developed with ethanol, phenol and TE buffer with different concentrations. Control DNA is also shown.

Inhibitor	Final concentration	IACs PH (RFU)		IAC_{410}/IAC_{90}	4-plex peak height (RFU)				4-plex peak imbalance
		IAC_{90}	IAC_{410}		70	194	305	384	
Ethanol	9.6%	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	7%	1507	n.d.	N/A	1391	1056	245	n.d.	PP
	5%	1390	527	0.38	2241	1862	1533	889	0.40
	2.5%	1567	978	0.62	2288	1749	1368	1075	0.47
	1.25%	1546	1239	0.80	2222	2171	2008	1914	0.86
Phenol	9.9%	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	5%	288	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	2.5%	1431	308	0.22	1715	1483	1027	613	0.36
	1.25%	1810	1200	0.66	2477	1624	1569	1378	0.56
	0.625%	1765	1777	1.01	2332	2294	2311	2254	0.97
TE Buffer	10X	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	5X	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	2.5X	n.d.	n.d.	N/A	n.d.	n.d.	n.d.	n.d.	NP
	1.25X	1021	n.d.	N/A	2497	1697	62	n.d.	PP
	0.625X	1328	840	0.63	2387	1856	1543	1103	0.46
	0.313X	1546	1484	0.96	2274	1988	1550	1250	0.55
	0.156X	1529	1669	1.09	1982	1931	1715	1699	0.86
Control DNA	1 ng/µl	1549	1652	1.07	2200	2057	2000	2193	0.91

Note: n.d.: not detected, N/A: Not Applicable, NP: No Profile, PP: Partial Profile.

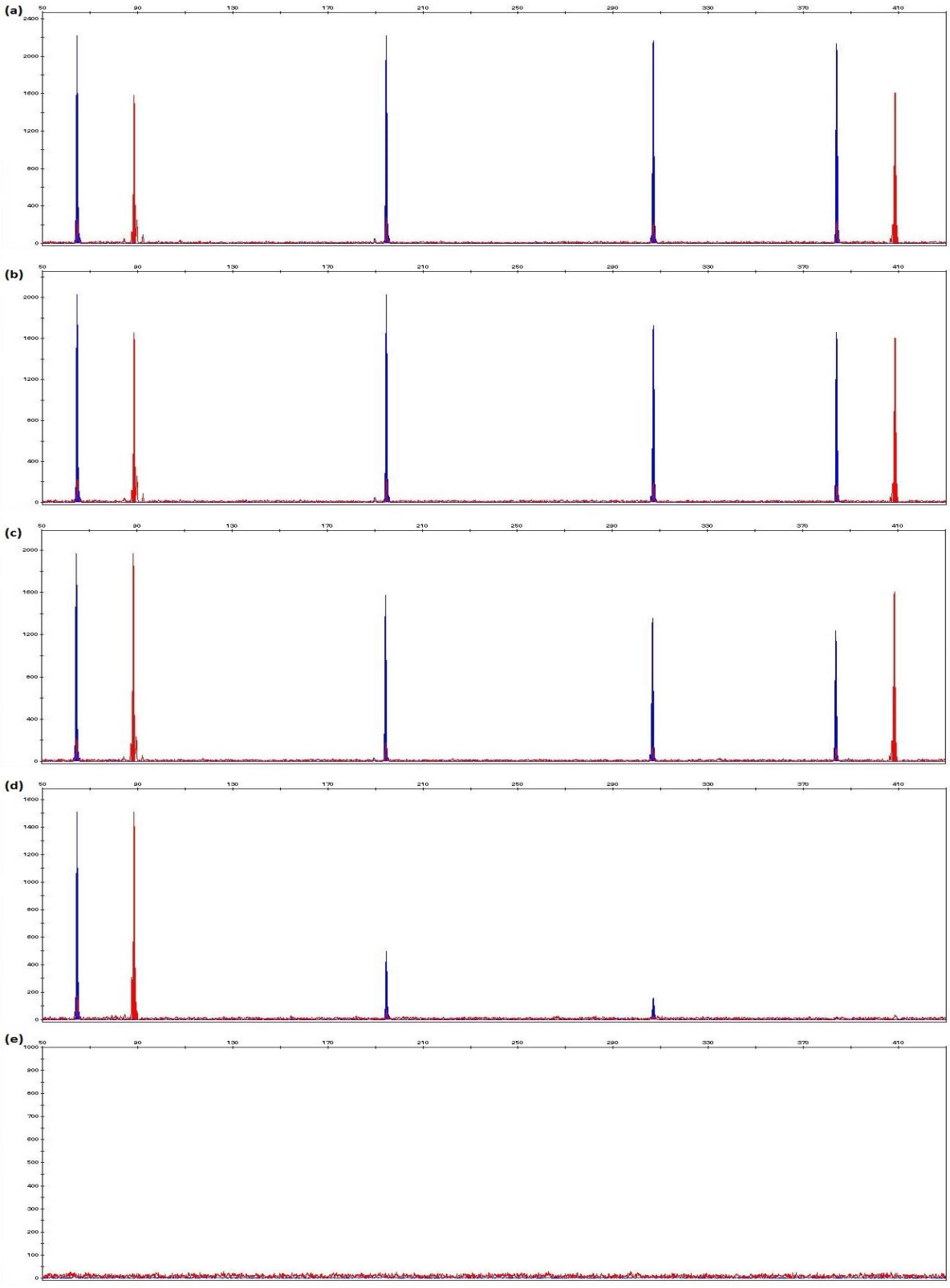


Figure 3.7: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 6.25 ng/µl, (b) 12.5 ng/µl, (c) 25 ng/µl, (d) 50 ng/µl and (e) 100 ng/µl Human Collagen Type 1 in 10µl PCR reaction.

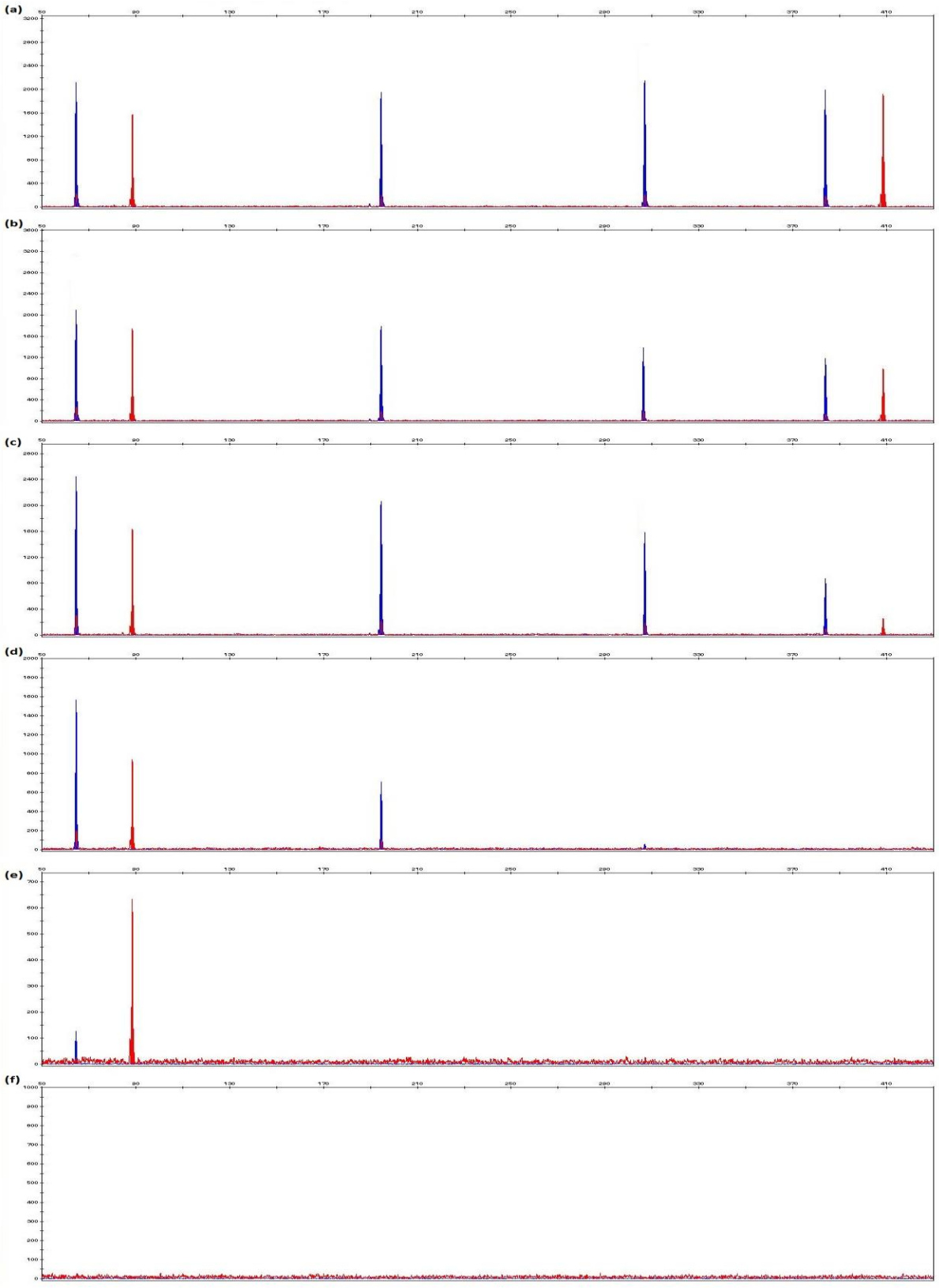


Figure 3.8: Electropherograms generated from 1 ng of control DNA 9947A with final concentration of (a) 1.25 ng/µl, (b) 2.5 ng/µl, (c) 5 ng/µl, (d) 10 ng/µl, (e) 20 ng/µl and 40 ng/µl humic acid in 10µl PCR reaction.

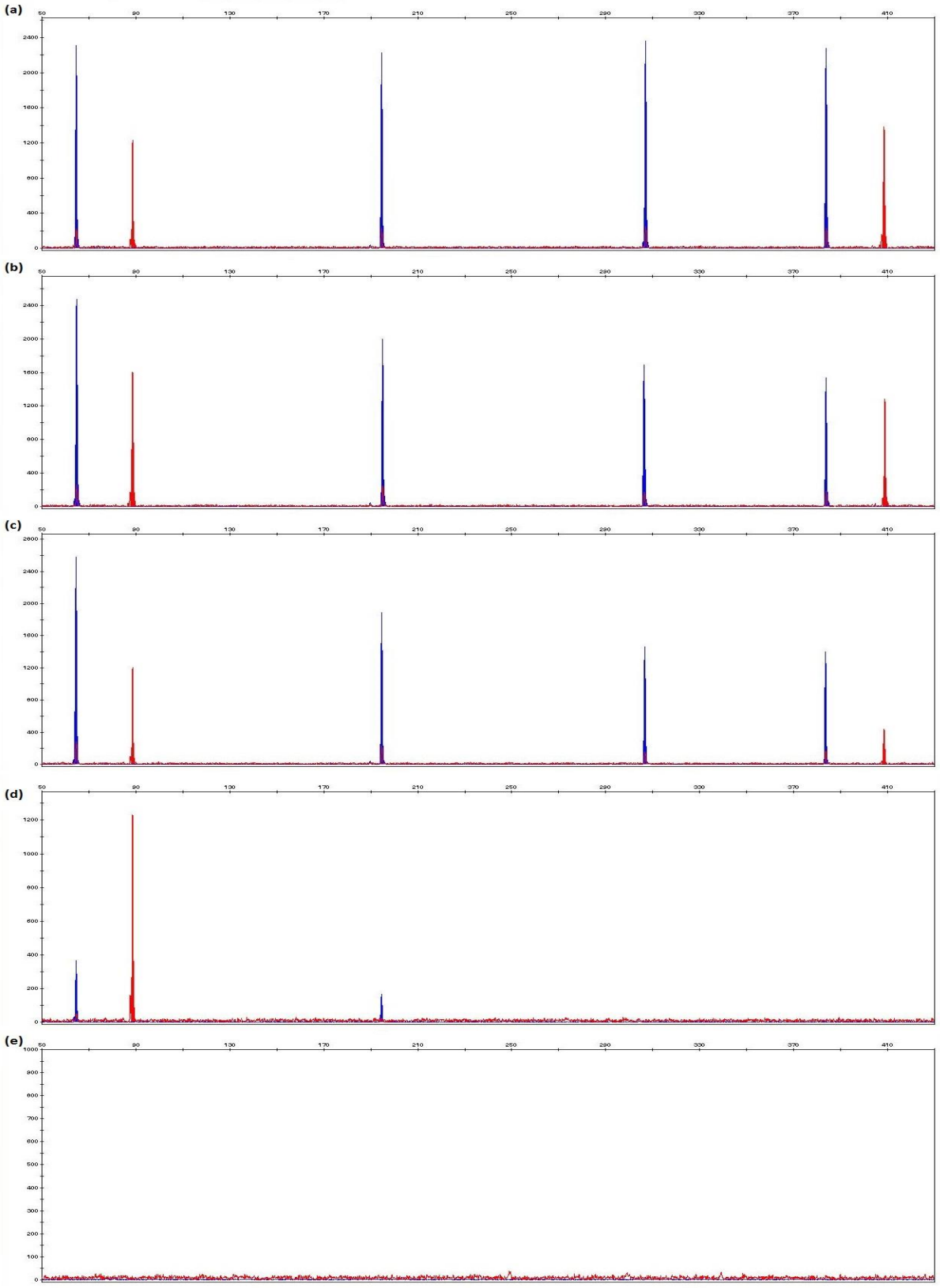


Figure 3.9: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 2.5 ng/µl, (b) 5 ng/µl, (c) 10 ng/µl, (d) 20 ng/µl and (e) 40 ng/µl tannic acid in 10µl PCR reaction.

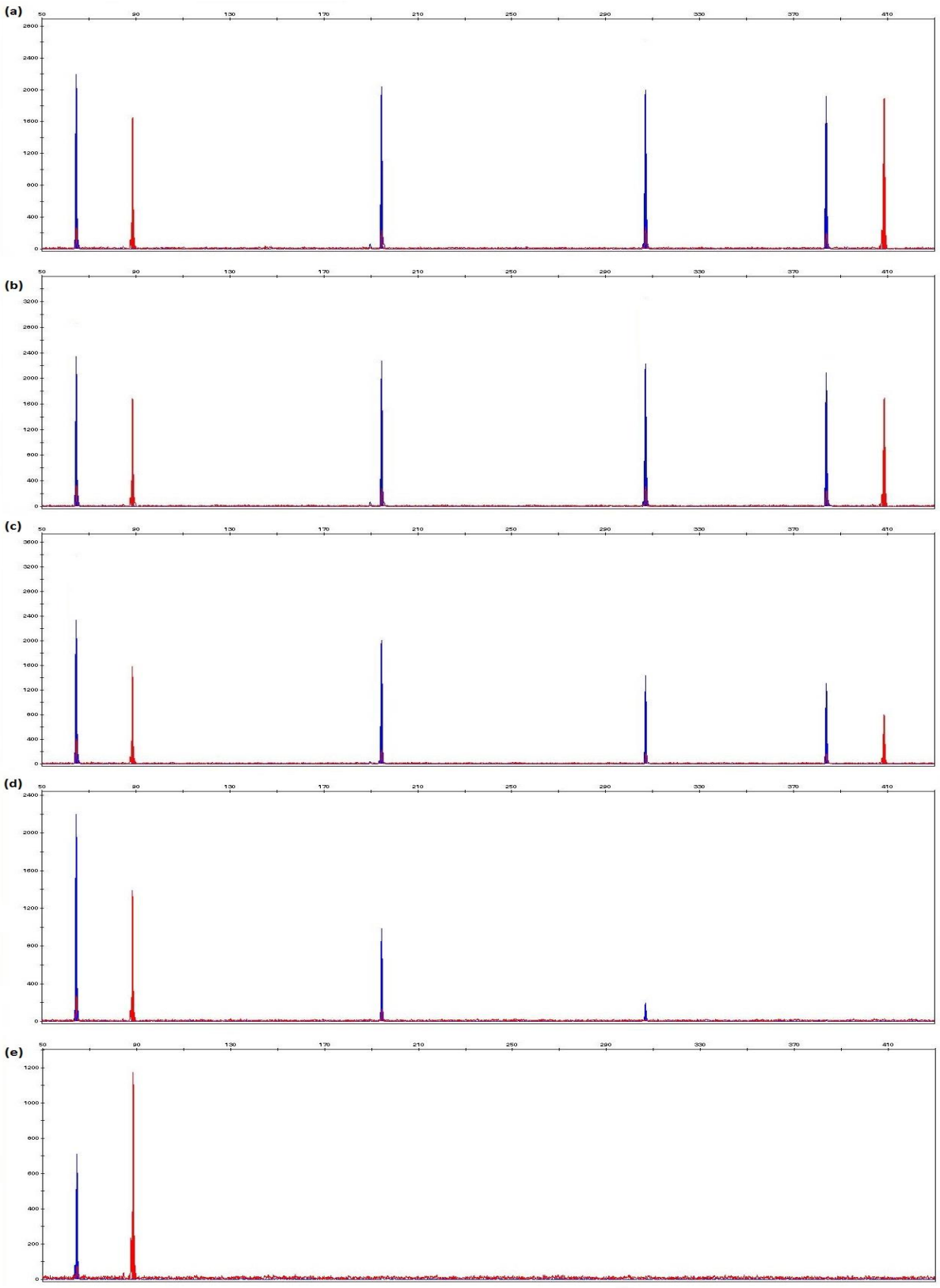


Figure 3.10: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 2.5 ng/µl, (b) 5 ng/µl, (c) 10 ng/µl, (d) 20 ng/µl and (e) 40 ng/µl hematin in 10µl PCR reaction.

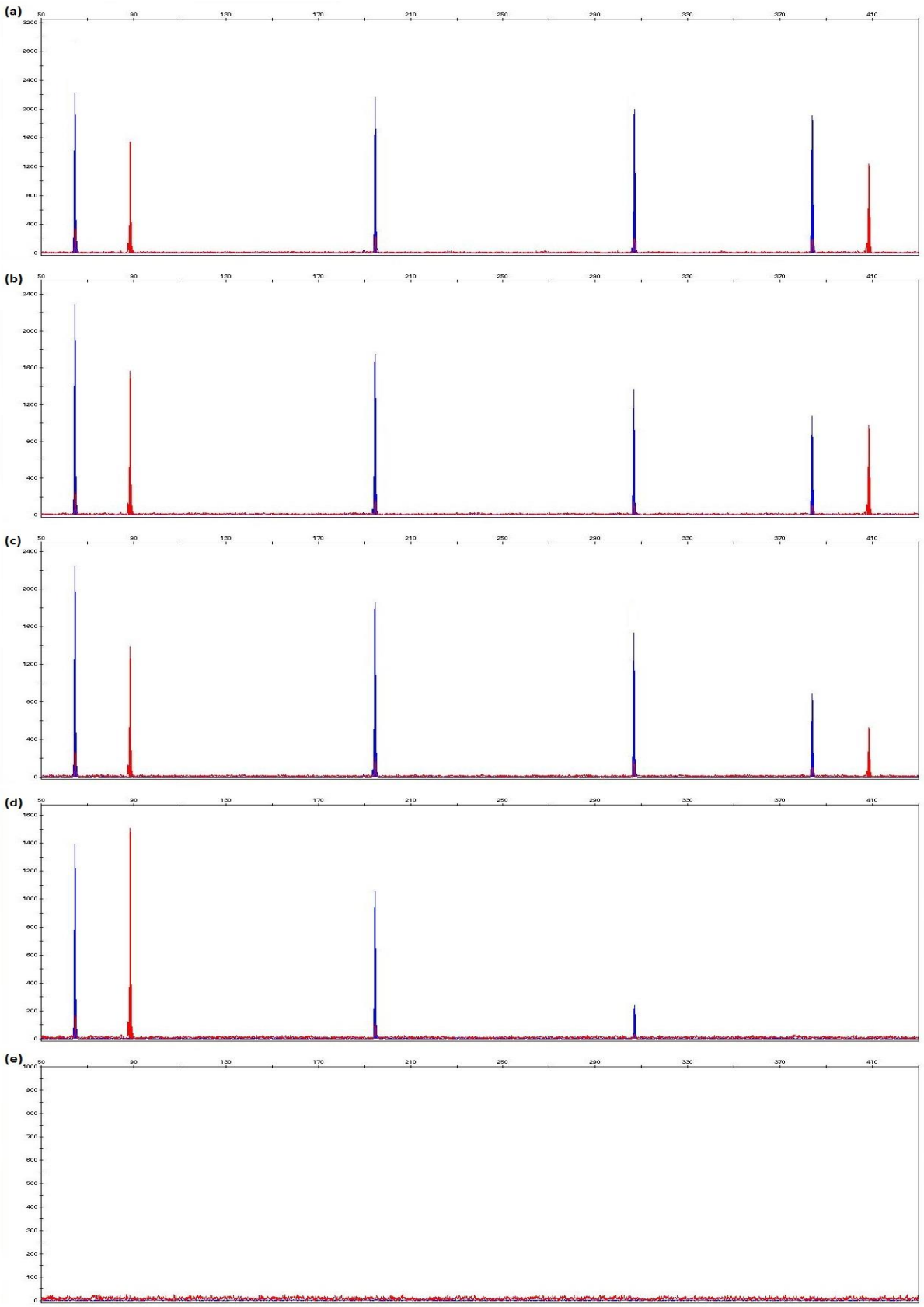


Figure 3.11: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 1.25%, (b) 2.5%, (c) 5%, (d) 7% and (e) 9.5% ethanol in 10µl PCR reaction.

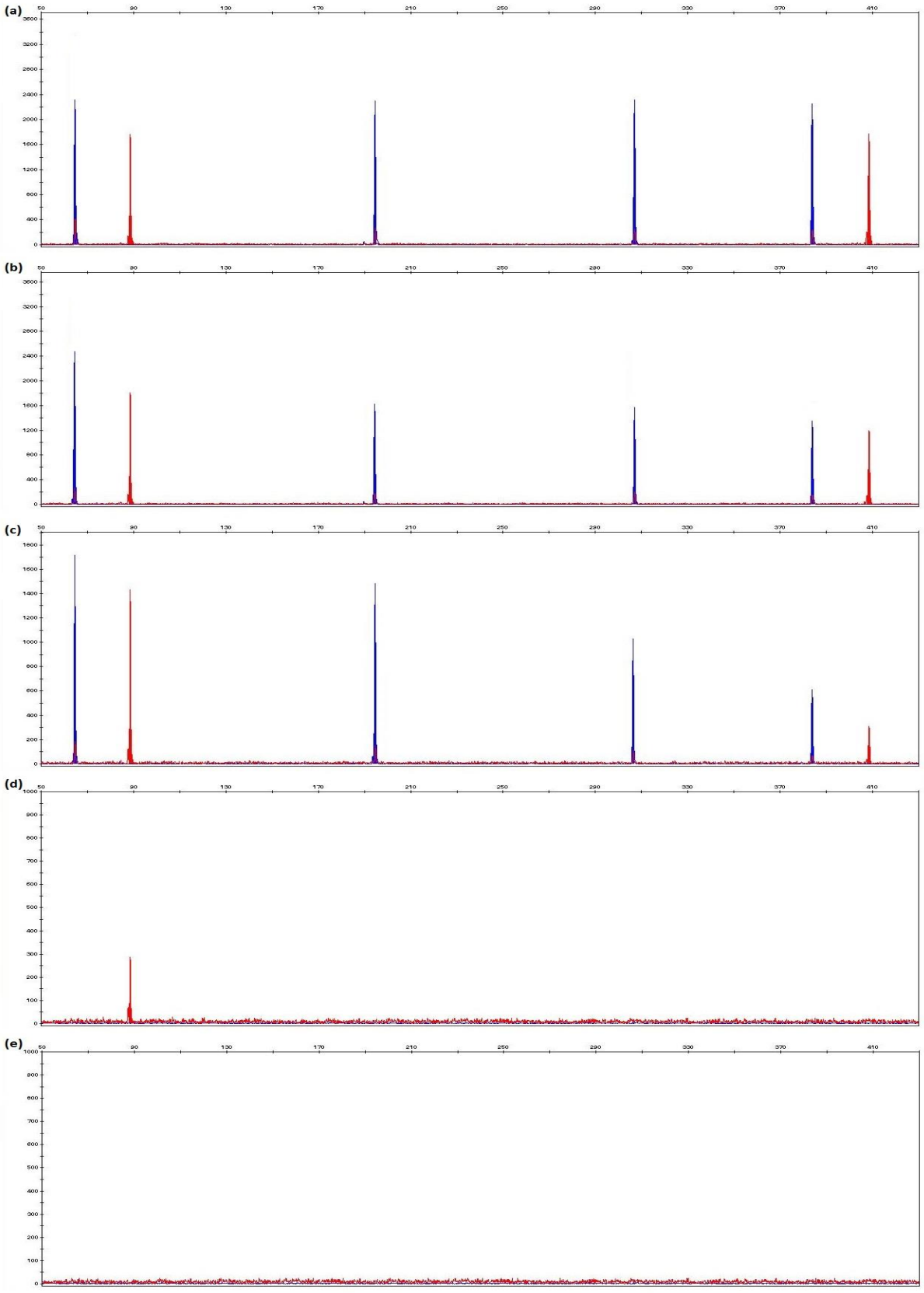


Figure 3.12: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 0.625%, (b) 1.25%, (c) 2.5%, (d) 5% and (e) 9.9% phenol in 10µl PCR reaction.

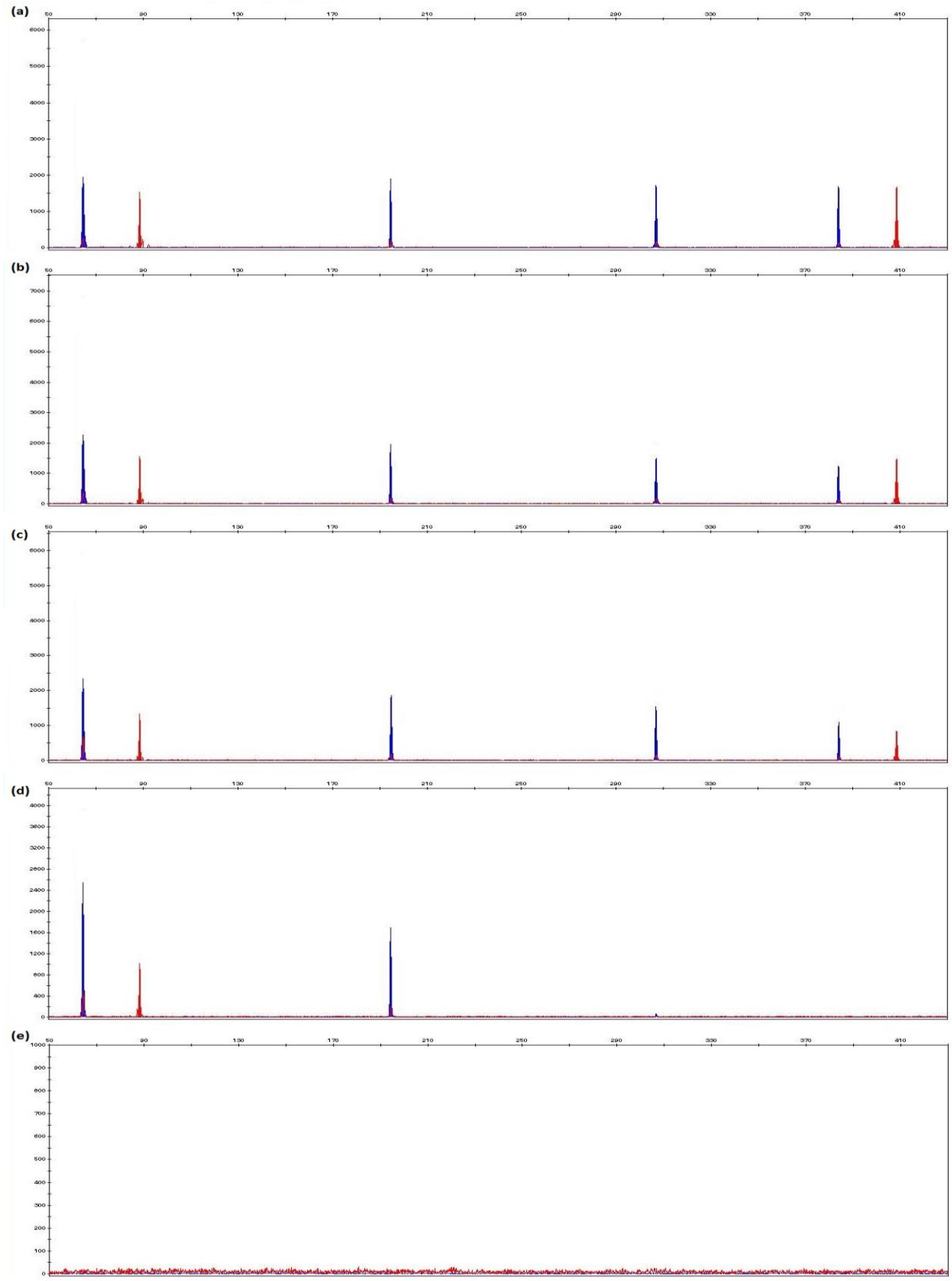


Figure 3.13: Electropherograms generated from 1 ng of control DNA 9947A with final concentrations of (a) 0.156 X, (b) 0.313 X, (c) 0.625 X, (d) 1.25 X and (e) 2.5 X TE buffer in 10µl PCR reaction.

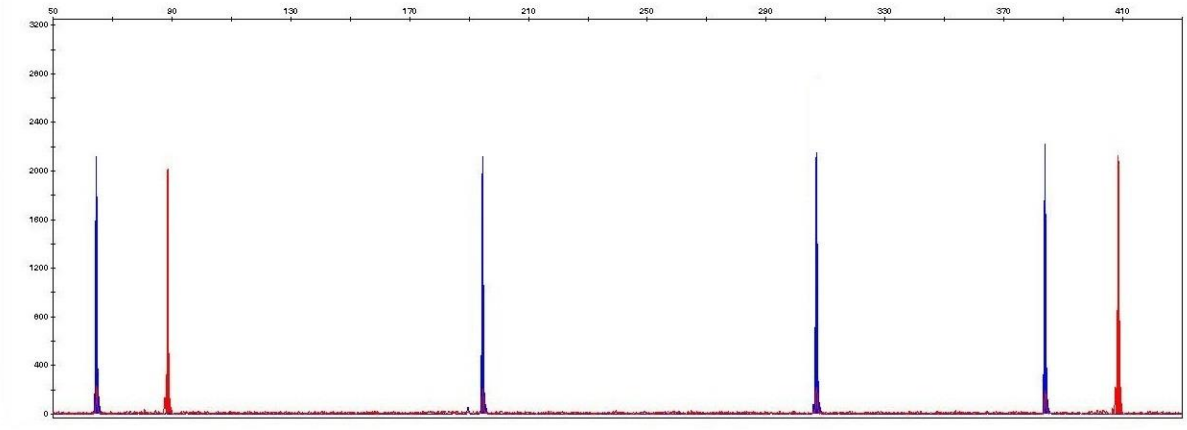


Figure 3.14: Electropherogram generated from 1 ng of control DNA 9947A without any inhibitor in 10µl PCR reaction.

3.4 DISCUSSION

3.4.1 Multiplex (4-plex & IACs) design and validation

At the beginning stage, the 4-plex multiplex with the IACs was very useful to study the degradation pattern of the samples (Nazir et al. 2012). But, problems arise when inhibited samples are being analysed using this 4-plex multiplex. This is because highly degraded DNA samples produce either partial DNA profiles or negative results when amplified using multiplex PCR. At the same time, inhibition of amplifications can also result in reduced product yield or complete failure and in some cases the inhibited samples are mistakenly assumed to be degraded DNA (Kontanis & Reed 2006).

To overcome this problem, this 4-plex multiplex was improved with two Internal Amplification Controls (IACs) to develop a multiplex (4-plex & IACs) system which can differentiate between degraded and inhibited DNA samples. Internal Amplification Controls (IACs) assist in avoiding false interpretation of the DNA profiles which could be caused by PCR inhibitors (Sachadyn & Kur 1998).

There were many difficulties in developing this multiplex. Since IACs primers are longer than 4-plex primers, they have higher annealing temperatures. Thus, a range of temperatures (60 – 67 °C) were studied and 60 °C was found to be the optimum annealing temperature for this multiplex. The PCR master mix also was changed since the inclusion of IACs primers caused unbalanced peak heights and split peaks thus the AmpliTaq Gold® PCR Master Mix (Life Technologies™, UK) which was used for the 4-plex was replaced with Platinum® Multiplex PCR Master Mix since Platinum® Multiplex PCR Master Mix which works well with multiple loci system (Liu et al. 2011). With the new PCR master mix, the concentrations of primers also were optimised to produce balanced peak heights.

After the development and optimisation of this multiplex, it was tested on different concentrations of template DNA to study its sensitivity. It was found that this multiplex worked efficiently on DNA template as low as 0.10 ng. Partial profiles were developed until 0.05 ng DNA but no profile was obtained below that concentration. Identifiler® Plus kit (Life Technologies™) gives full profile with minimum of 0.125 ng DNA (Wang et al. 2012) and PowerPlex® 16 HS System (Promega) also gives full profile with minimum of 0.125 ng DNA (Ensenberger et al. 2010). Thus, this new multiplex system shows that it has a sensitivity level comparable to the leading commercial kits in the market (Gill et al. 2005). Also, this multiplex does not produce any non-specific peaks, and only amplified the targeted loci.

3.4.2 DNA degradation study

Forensic samples which are exposed to environmental insults will normally degrade and the DNA will be fragmented. Longer fragments have more chances to be fragmented than the shorter fragments (Butler et al. 2003, Takahashi et al. 1997). This can be observed in the DNA profiles but can be misinterpreted as PCR inhibition since PCR amplification failure or a reduced sensitivity of detection usually for the larger fragments also happens in PCR inhibited samples.

Therefore, extracted DNA from degraded pig soft tissue samples were studied in this part of research using the multiplex (4-plex & IACs) system to show that these are degraded samples and not PCR inhibited samples. These samples were collected from a pig carcass which was left in the environment during summer and the samples were collected every 3 to 6 days (Nazir et al. 2013).

The profiles developed using the multiplex system show that full profiles could be obtained from samples collected up to and including day 24 (295 ADD). The degradation pattern becomes very obvious after that and could be clearly observed on the DNA

profiles of samples collected after 338 ADD. The IAC peaks are undisturbed and well balanced in all the electropherograms from this study which indicates the absence of PCR inhibitors and confirms that the samples analysed are degraded samples.

A precious study has been done using multiplexes to identify DNA degradation in the samples. A triplex assay which was developed using a short fragment (nuCSF 67 bp), a long fragment (nuTH01, 170 – 190 bp) and an internal PCR control was used to study the sample quality together with the assessment on PCR inhibition. This triplex assay which was tested on several forensic samples showed that it was a good tool to evaluate the quality of the samples (Swango et al. 2006). Compare to that triplex assay, the multiplex assay developed in this chapter has more markers (4-plex) and covers a broader range (70 – 384 bp), thus a clearer assessment and understanding can be obtained from the samples.

3.4.3 PCR inhibitor study

Several PCR inhibitors were studied to test the efficiency of IACs in detecting inhibitors. As indicated by the results obtained, higher concentrations of the inhibitors causes a decay in the DNA profiles obtained. This can be seen in the peak height ratio of the IACs and also the 4-plex products.

As commonly used in the forensic community, the ratio of 0.6 for heterozygote balance was fixed to identify good DNA profiles; if the ratio of IAC peak heights is above 0.6, that profile will be accepted (Kelly et al. 2012, Bright et al. 2010, Petricevic et al. 2010). The heterozygote imbalance was calculated by dividing the peak height of IAC_{410} with the peak height of IAC_{90}. Based on this ratio, the maximum PCR inhibitor concentration that could be present in the samples before this multiplex system failed to produce acceptable DNA profiles was 25 ng/µl human collagen type 1; 1.25 ng/µl humic acid; 5 ng/µl tannic acid; 5 ng/µl hematin; 2.5% ethanol; 1.25% phenol and 0.625X TE buffer in a PCR reaction.

A previous study using the same IAC_{90} and IAC_{410} with AmpFlSTR® SGM Plus® kit showed that a full profile was obtained below 8 ng/µl humic acid in 12.5 µl PCR reactions (Zahra et al. 2011) while, in the new multiplex system, the maximum concentration of humic acid that could present was 12.5 ng/µl in 10 µl PCR reaction, higher than the previous study. The IAC_{90} and IAC_{410} in the new multiplex system are likely more compatible with this inhibitor because of the number of loci involved in AmpFlSTR® SGM Plus® kit which is 11 loci (including amelogenin) compared to 4 loci in the new multiplex system.

An earlier study of inhibitory effects on real-time PCR showed that reliable results could be obtained when tannic acid was less than 0.4 ng in a 25 µl PCR reaction (Kontanis & Reed 2006). With the new multiplex system, the maximum amount of tannic acid before PCR inhibition detected was 50 ng in a 10 µl PCR reaction. Other than that, the targeted fragment size in that study is 132 bp, smaller than the $IAC_{410.}$ This shows that the new multiplex system is more reliable and with the real-time PCR shows high level of PCR inhibitors, satisfactory profiles still can be obtained with the multiplex.

Another study showed that usage of phenol-saturated phosphate-buffered saline with concentrations as high as 15% did not affect the Tth DNA polymerase mediated reverse transcriptase activity (Katcher & Schwartz 1994). While with the usage of Platinum® Multiplex PCR Master Mix, the concentration of phenol as high as 12.5% did not inhibit the amplification of this new multiplex system.

Humic acid which is commonly present in the soil has been found to inhibit the amplification and caused total failure of DNA quantification in a real-time PCR at concentration more than 4.8 ng/µl when Quantifiler® Human DNA Quantification Kit was used (Seo et al. 2012). While using this new multiplex, the level of humic acid concentration which caused the IAC peaks imbalance ratio to less than 0.6 was at 1.25 ng/µl. However, these results should not be directly compared to each other since real-time result was based on total inhibition while the multiplex result was based on the IAC peaks imbalance. IAC peaks in this multiplex were totally undetected when the humic

acid concentration was at 40 ng/µl (Table 3.11). If this result is compared with the study by Seo et al. (2012), then the new multiplex system appears more resistant to humic acid inhibition.

Many PCR inhibition studies carried out using real-time PCR were based on qualitative indication rather than quantitative measurement for PCR inhibitors (Seo et al. 2010, Keyser et al. 2009, Vanek et al. 2009, Gojanovic & Sutlovic 2007), thus quantitative comparison could not be carried with other PCR inhibitors which were tested with this multiplex system.

Control DNA without the PCR inhibitor produced a clean DNA profile with expected ratios of the IACs and 4-plex peak heights.

CHAPTER 4

DNA QUANTIFICATION USING THE NEW MULTIPLEX (4-PLEX & IACS)

4.1 OVERVIEW

Quantification of DNA is an important process to produce proper amplification and DNA profiles. Real-time PCR application for DNA quantification has overcome the old techniques since it is faster and less expensive than the traditional methods such as UV spectrometry, gel-based and blotting techniques (Nicklas & Buel 2003a, Nicklas & Buel 2003b). However, during DNA quantification using real-time PCR, the actual quality of the samples cannot be determined and this can cause difficulty to obtain good DNA profiles.

In the previous chapter it was demonstrated that the new multiplex was effective at assessing DNA degradation and PCR inhibition. Further experiments were designed to evaluate whether the multiplex could be useful for quantifying the amount of DNA present using the peak heights of the electropherograms; this method was compared to an in-house and a commercial real-time quantification method.

4.1.1 Objective

- To compare the accuracy and precision of the multiplex for DNA quantification using an in-house and a commercial real-time system.

4.2 RESULTS

4.2.1 Quantification of serial dilution samples using real-time PCR

A serial dilution was carried out using stock 200 ng/µl control DNA (9947A, Life Technologies™) until the final concentration was 0.01 ng/µl. Each point of the concentration was prepared in triplicate and the final volume of each sample was 10 µl. This serial dilution samples were quantified using GoTaq® qPCR Master Mix and Quantifiler® Human DNA Quantification kit. The quantification results are as shown in the Tables 4.1 and 4.2.

Table 4.1: Table below shows the concentrations of the DNA samples which were prepared by serial dilution using control DNA 9947A and quantified using GoTaq® qPCR Master Mix.

DNA concentration (ng/µl)	Quantification using GoTaq® qPCR Master Mix (ng/µl)					
	Set A	Set B	Set C	Avg.	S.d.	R.S.D. (%)
200	192.9	209.0	192.9	198.3	9.30	4.69
100	103.4	104.6	96.6	101.5	4.31	4.25
50	47.8	52.3	48.7	49.6	2.38	4.80
25	26.1	24.2	24.9	25.1	0.96	3.83
12.5	12.83	12.05	12.26	12.38	0.40	3.26
6.25	6.128	6.038	6.345	6.170	0.16	2.56
3.13	3.074	2.989	3.209	3.091	0.11	3.59
1.56	1.618	1.527	1.549	1.565	0.05	3.03
0.78	0.995	0.999	0.902	0.965	0.05	5.69
0.39	0.426	0.484	0.402	0.437	0.04	9.64
0.20	0.245	0.259	0.207	0.237	0.03	11.35
0.10	0.156	0.071	0.095	0.107	0.04	40.83
0.05	0.093	0.068	0.051	0.071	0.02	29.90
0.02	0.018	0.042	0.015	0.025	0.01	59.19
0.01	0.022	0.030	0.011	0.021	0.01	45.43

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation

Table 4.2: Table below shows the concentrations of the DNA samples which were prepared by serial dilution using control DNA 9947A and quantified using Quantifiler® Human DNA Quantification kit.

DNA concentration (ng/µl)	Quantification using Quantifiler® Human DNA Quantification kit (ng/µl)					
	Set A	Set B	Set C	Avg.	S.d.	R.S.D. (%)
200	196.7	192.9	199.0	196.2	3.08	1.57
100	99.5	103.4	95.7	99.5	3.85	3.87
50	52.3	48.2	49.2	49.9	2.14	4.28
25	24.4	26.1	25.7	25.4	0.89	3.50
12.5	12.05	12.30	11.96	12.10	0.18	1.46
6.25	6.038	6.220	6.088	6.115	0.09	1.54
3.13	3.172	3.074	3.014	3.087	0.08	2.58
1.56	1.555	1.522	1.532	1.536	0.02	1.10
0.78	0.747	0.802	0.793	0.781	0.03	3.78
0.39	0.381	0.383	0.377	0.380	$3.1e^{-03}$	0.80
0.20	0.201	0.198	0.202	0.200	$2.1e^{-03}$	1.04
0.10	0.096	0.094	0.102	0.097	$4.2e^{-03}$	4.28
0.05	0.050	0.050	0.051	0.050	$5.8e^{-04}$	1.15
0.02	0.024	0.026	0.024	0.025	$1.2e^{-03}$	4.68
0.01	0.013	0.013	0.012	0.013	$5.8e^{-04}$	4.56

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation

The results obtained show that there is not much difference between actual concentrations of the control DNA with the estimated concentration from real-time PCR. The samples quantified using GoTaq® qPCR Master Mix showed low R.S.D percentage until the samples concentrations went below 0.20 ng/µl where the R.S.D start to increase up to 59.19%. This error was not observed in the Quantifiler® Human DNA Quantification kit quantification where the R.S.D stayed below 5% until the lowest concentration of 0.01 ng/µl. This shows that Quantifiler® Human DNA Quantification kit is more robust for the quantification of samples with low amounts of DNA.

4.2.2 Amplification and analysis of the serial dilution samples using multiplex (4-plex & IACs)

After the serial dilution samples were quantified using real-time PCR, the remaining samples were used to amplify the multiplex (4-plex & IACs) using 1 µl of each sample. Then the electrophoresis was carried out on the amplified samples and the results obtained from the electropherograms are as shown in the Table 4.3.

Table 4.3 Table below shows the average peak heights of the electropherograms produced using the serial diluted control DNA samples.

DNA concentration (ng/µl)	Average peak height (RFU)					
	Set A	Set B	Set C	Avg.	S.d.	R.S.D. (%)
200	32482.50	32580.00	32254.75	32439.08	166.92	0.51
100	30675.00	31551.50	31295.75	31174.08	450.74	1.45
50	30078.00	25832.75	19626.25	25179.00	5256.45	20.88
25	16107.00	14499.00	19513.50	16706.50	2560.44	15.33
12.5	12506.25	9659.00	12309.75	11491.67	1590.17	13.84
6.25	5467.75	9137.25	7141.75	7248.92	1837.10	25.34
3.13	2921.25	3536.50	4088.00	3515.25	583.67	16.60
1.56	2062.75	2528.25	1671.75	2087.58	428.79	20.54
0.78	895.25	820.50	685.00	800.25	106.58	13.32
0.39	500.25	321.50	291.50	371.08	112.86	30.41
0.20	215.25	227.25	282.25	241.58	35.73	14.79
0.10	112.50	66.25	137.25	105.33	36.04	34.21
0.05	15.25	41.00	13.00	23.08	15.56	67.40
0.02	0.00	12.50	0.00	4.17	7.22	N/A
0.01	0.00	0.00	0.00	0.00	0.00	N/A

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation, N/A: Not Applicable.

The results showed that as the concentrations went down, precision decreased. This can been seen at the DNA concentration of 0.05 ng/µl where the R.S.D went up to 67.40% and the concentrations below that were not good enough for the statistical calculation. This is mainly because of the partial profiles which were generated at those concentrations. Full profiles were obtained from 0.10 ng/µl and above. The examples of the electropherograms produced are as shown in the Figures 4.1, 4.2 and 4.3.

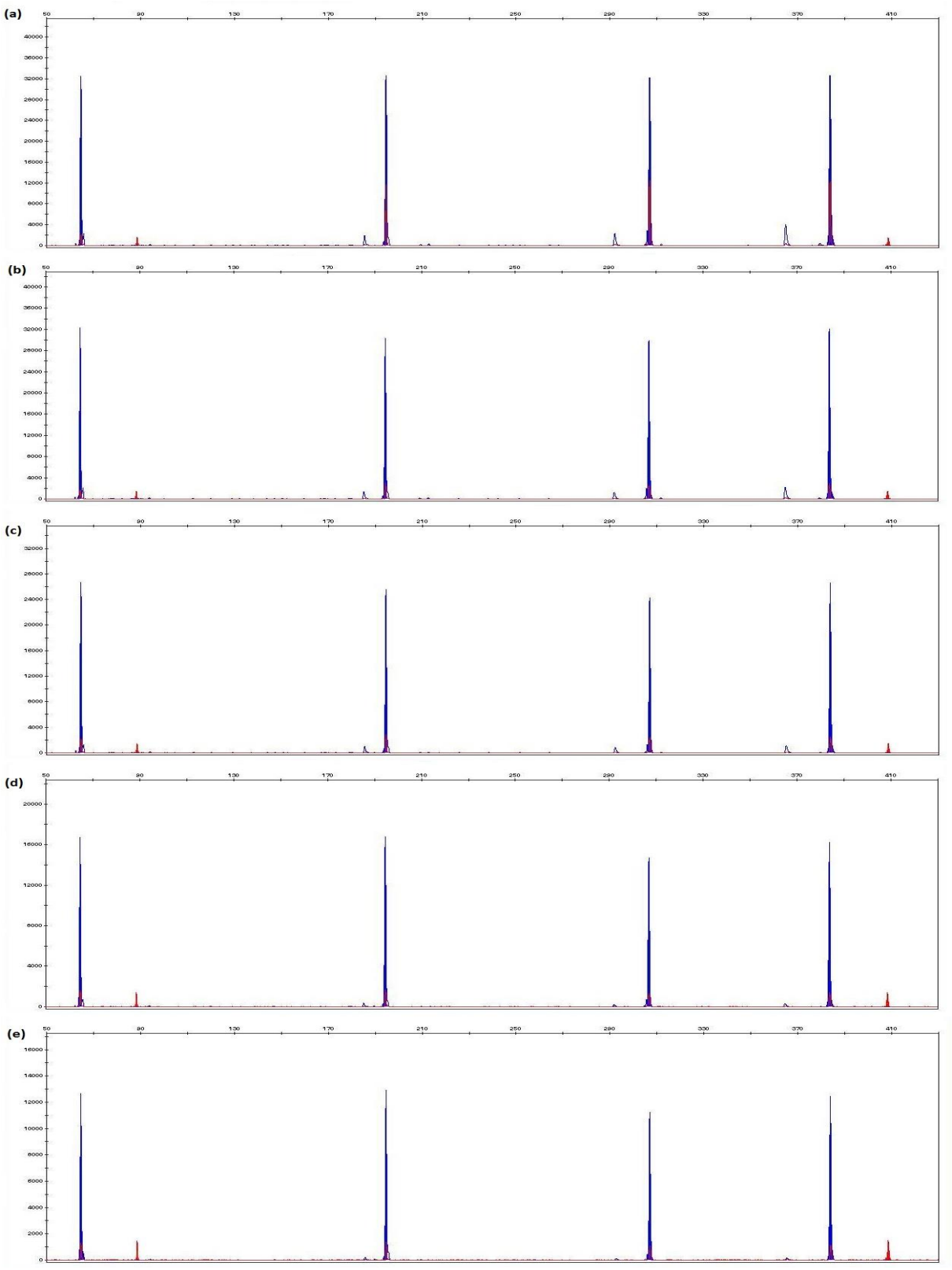


Figure 4.1: Electropherograms generated from serial diluted control DNA 9927A with (a) 200 ng/µl, (b) 100 ng/µl, (c) 50 ng/µl, (d) 25 ng/µl and (e) 12.5 ng/µl final concentrations. 1 µl of each sample was used for amplification.

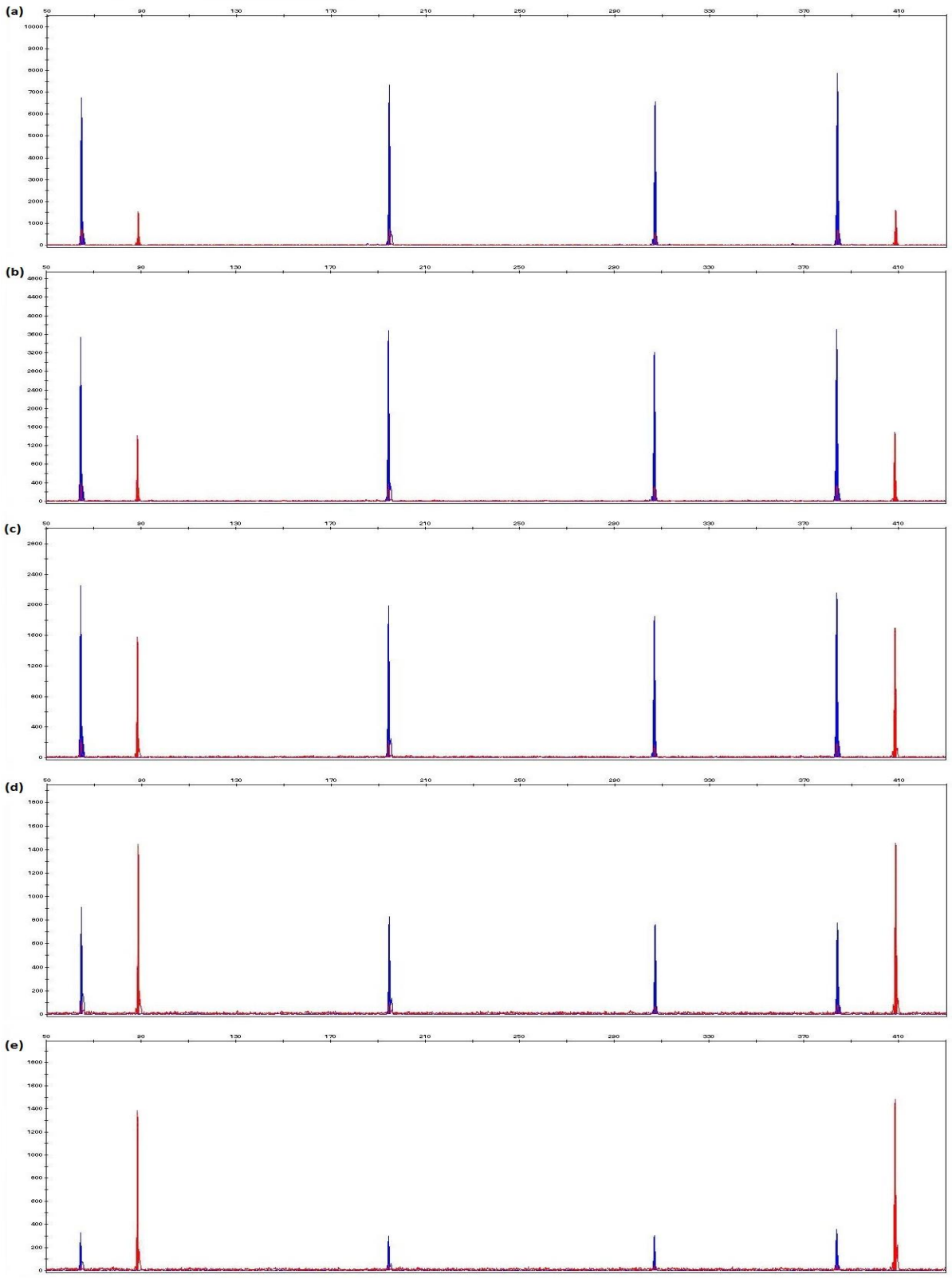


Figure 4.2: Electropherograms generated from serial diluted control DNA 9927A with (a) 6.25 ng/µl, (b) 3.13 ng/µl, (c) 1.56 ng/µl, (d) 0.78 ng/µl and (e) 0.39 ng/µl final concentrations. 1 µl of each sample was used for amplification.

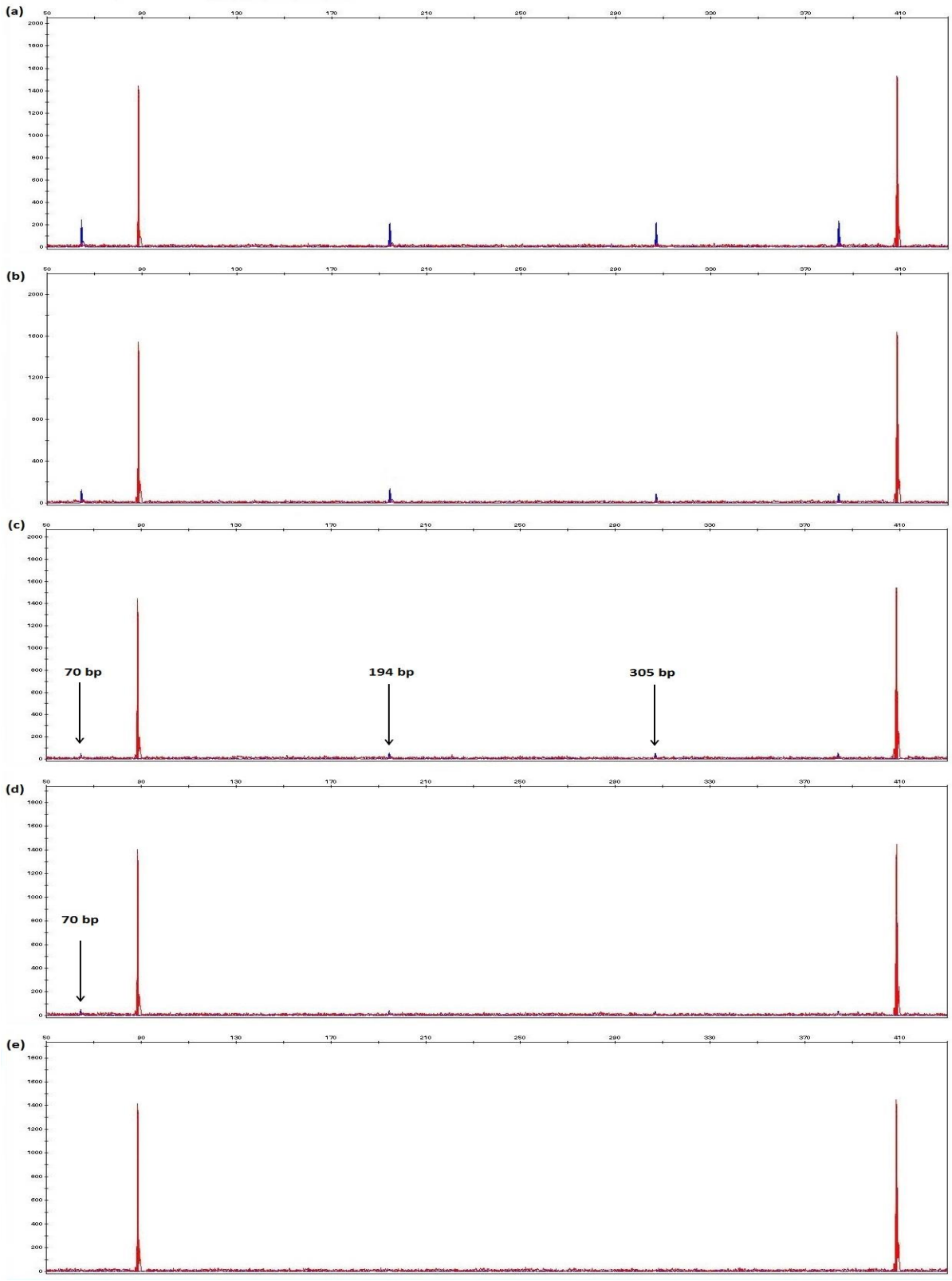


Figure 4.3: Electropherograms generated from serial diluted control DNA 9927A with (a) 0.20 ng/µl, (b) 0.10 ng/µl, (c) 0.05 ng/µl, (d) 0.02 ng/µl and (e) 0.01 ng/µl final concentrations. 1 µl of each sample was used for amplification.

4.2.3 Correlation graph plotting using the average peak heights and DNA concentrations

Before the correlation graph was plotted, the data obtained was statically evaluated for its suitability to form a linear regression. Unfortunately, this data set showed exponential distribution (Figure 4.4) and also not normally distributed (Figure 4.5).

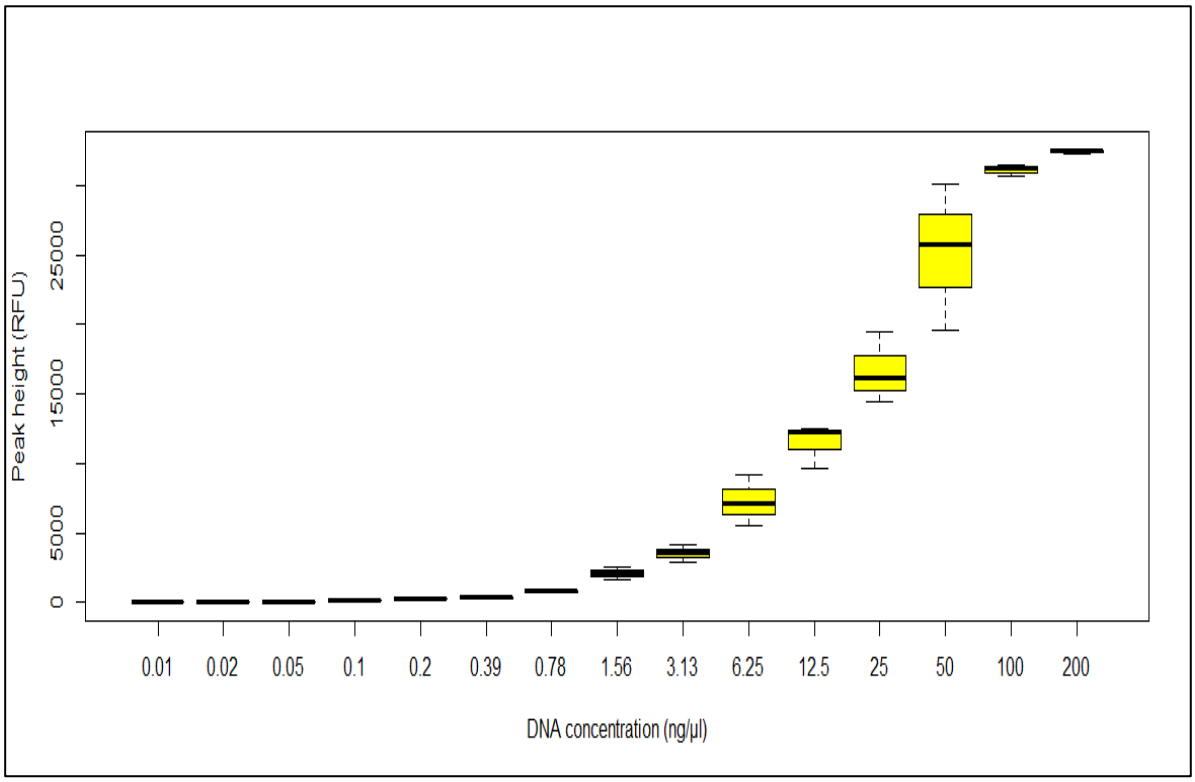


Figure 4.4: Boxplots showing the interaction between the serial diluted control DNA samples and the peak heights of the electropherograms generated by those samples.

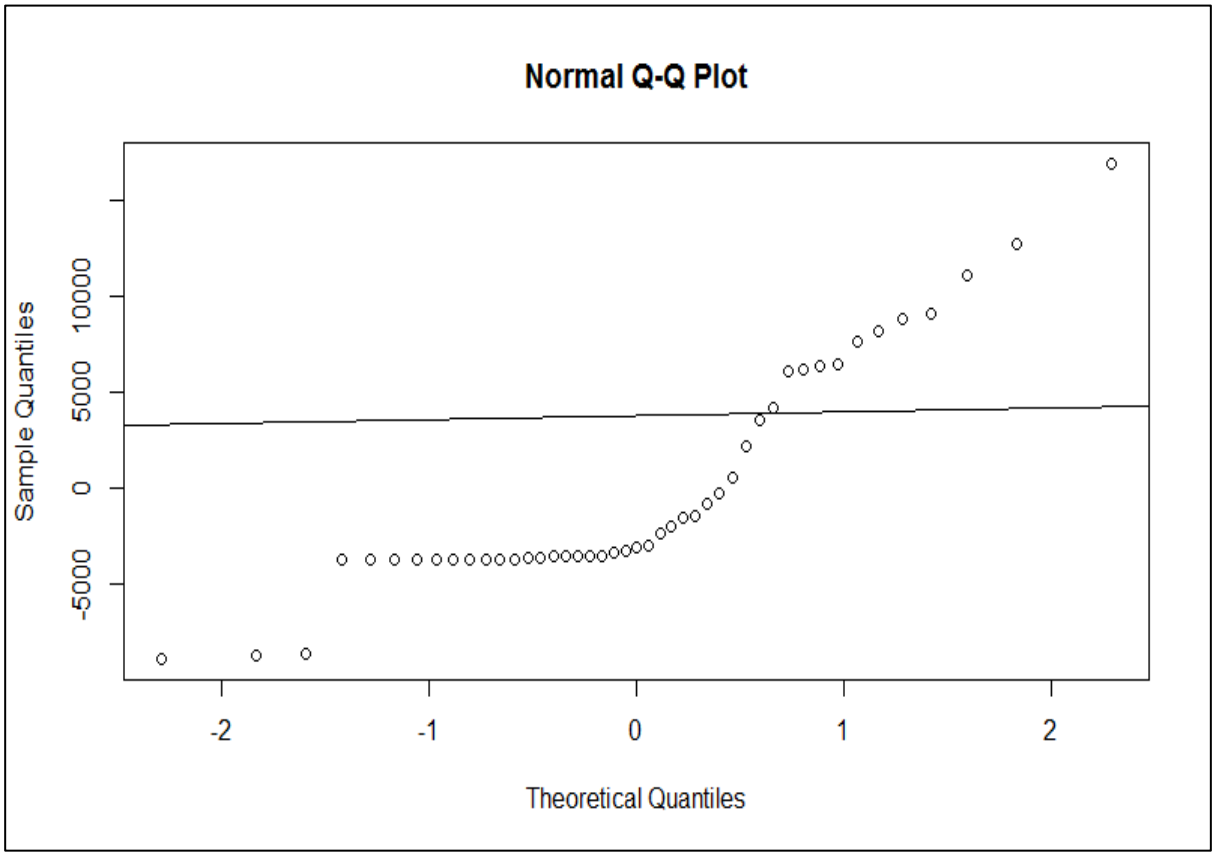


Figure 4.5: Normal q-q plot showing that the data are not normally distributed, thus not suitable to generate a linear line using the sample points.

The whole data set was not suitable for a linear regression formation since the data has exponential distribution. Also, the data are not normally distributed thus no correction could be carried out to make a linear regression. Thus the significant differences between each serial dilution points were calculated to identify the range of dilution points suitable to form the linear regression. The results of these statistical calculations are as shown in the Table 4.4.

Table 4.4: Table below shows the statistical data of the comparison between each serial dilution points in the sequence.

Comparison between dilution points	p adj
0.01 : 0.02	1.000
0.02 : 0.05	1.000
0.05 : 0.10	1.000
0.10 : 0.20	1.000
0.20 : 0.39	1.000
0.39 : 0.78	1.000
0.78 : 1.56	0.999
1.56 : 3.13	0.999
3.13 : 6.25	0.314
6.25 :12.5	0.159
12.5 : 25	**0.032**
25 : 50	**$5.46e^{-05}$**
50 : 100	**0.008**
100 : 200	0.999

From the results obtained, it was concluded that the range from 12.5 ng/µl to 100 ng/µl is suitable for the correlation graph plotting. This is because there are significant differences between these concentration points and the p-values were below 0.05. Thus these points were chosen to plot the correlation graph. Also, this statistical calculation showed that this graph will be useful for samples with DNA concentrations between 12.5 ng/µl to 100 ng/µl since there is significant difference between each samples between that concentrations ($p<0.05$). The plotted correlation graph is as shown in the Figure 4.6.

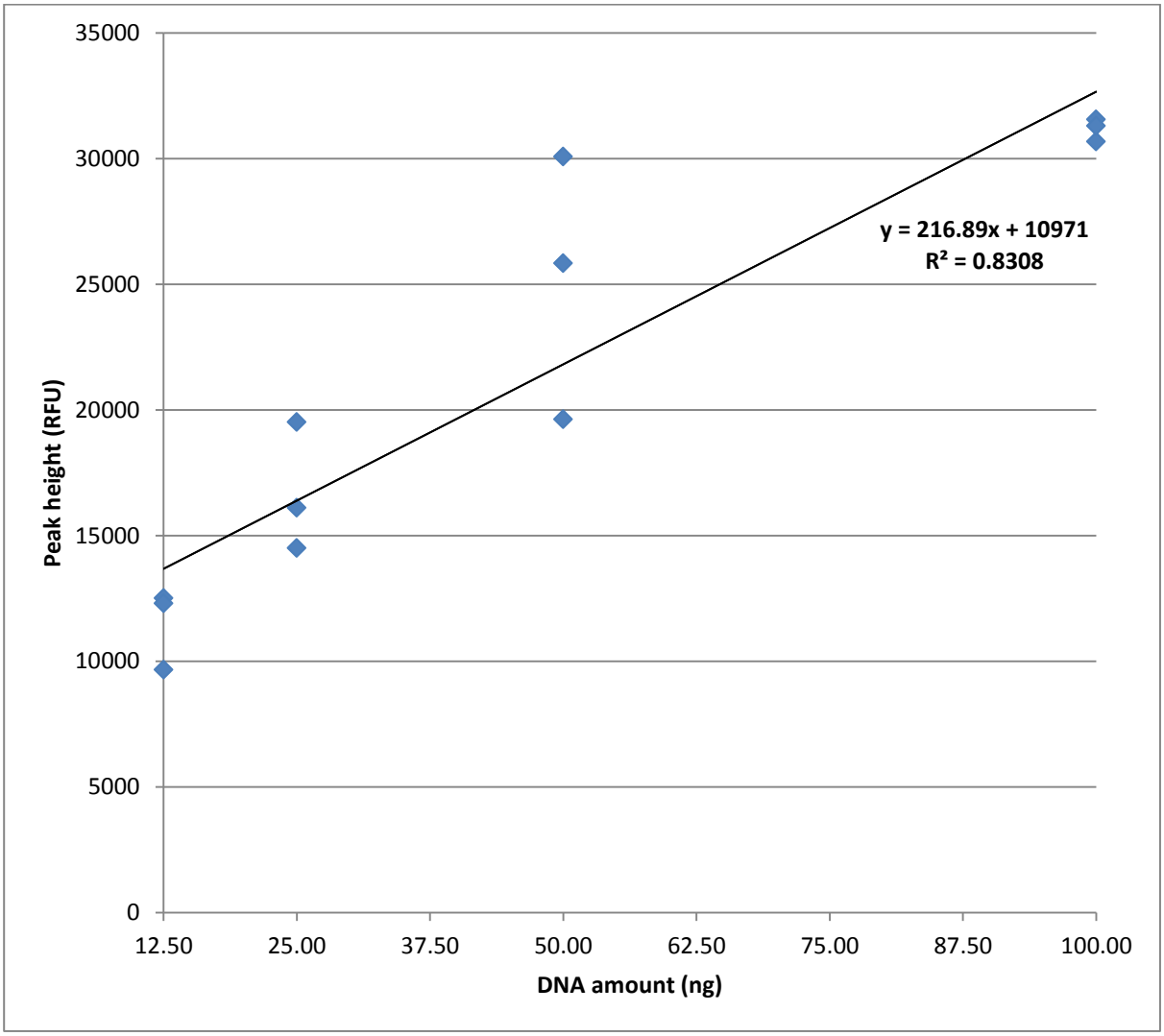


Figure 4.6: Figure above shows the graph represents correlation between average peak heights of 4-plex with different DNA amounts.

The linear regression formula of y=216.89x+10971 was obtained from the correlation graph. Also the coefficient of determination (R^2) was 0.8308.

4.2.4 Evaluation of the correlation graph

Once the correlation graph was obtained, its efficiency to estimate the DNA concentration using the peak height was tested. This test was carried out using the same samples which were used to create this correlation graph (Table 4.3, Section 4.2.2).

4.2.4.1 Concentration estimation using control DNA 9947A

For the estimation of DNA concentration using the correlation graph, the serial dilution samples with DNA concentrations between 12.5 ng/µl and 100 ng/µl were chosen since this correlation graph is useful for samples with DNA concentration up to 12.5 ng/µl. The average peak heights were calculated using the linear regression formula (y=216.89x+10971) to estimate the DNA concentration. The results are as shown in the Table 4.5.

Table 4.5: Table below shows the comparison of estimated DNA concentrations with their theoretical DNA concentration.

Average peak height (RFU)	Theoretical DNA concentration (ng/µl)	Estimation from correlation graph		
		DNA concentration (ng/µl)	S.d.	R.S.D (%)
31174.08	100	93.15	4.84	4.84
25179.00	50	65.51	10.97	21.93
16706.50	25	26.44	1.02	4.09
11491.67	12.5	2.40	7.14	57.13

Note: S.d.: Standard deviation, R.S.D.: Relative Standard Deviation.

The estimated DNA results showed that this correlation graph is not very useful for DNA concentration estimation. The samples with concentration of 25 ng/µl and 100 ng/µl have R.S.D. of 4.09% and 4.84% respectively showing that the estimated DNA concentrations are more precise. However, samples with concentration of 12.5 ng/µl and 50 ng/µl have R.S.D. of 57.13% and 21.93% showing that less precise DNA estimation were calculated. The fluctuation of estimation among these DNA concentrations indicating that this correlation graph is not a useful tool for DNA concentration estimation.

4.2.4.2 Concentration estimation using reference samples

For further evaluation of the correlation graph, several buccal swab samples which were extracted using PureGene extraction method where chosen as reference samples. These samples were extracted by Kosrat Najm (Najm 2013), previous Masters student, UCLan. These samples were quantified using Quantifiler® Human DNA Quantification kit and were amplified using the multiplex (4-plex & IACs). The statistical data of this study are as shown in the Table 4.6.

Table 4.6: Table below shows the comparison of estimated DNA concentrations with their theoretical DNA concentration.

Average peak height (RFU)	DNA concentration (ng/µl)	Estimation from correlation graph		
		DNA concentration (ng/µl)	S.d.	R.S.D (%)
16147.50	17.35	23.87	4.61	26.56
15359.00	15.81	20.23	3.12	19.75
18109.75	23.58	32.91	6.60	27.98
17797.50	22.21	31.47	6.55	29.48
15919.50	16.51	22.82	4.46	26.98
15136.75	17.65	19.21	1.10	6.23
13895.75	14.14	13.48	0.47	3.29
16323.50	17.09	24.68	5.37	31.43
14179.25	17.09	14.79	1.63	9.51
14277.25	14.97	15.24	0.19	1.29

Note: S.d.: Standard deviation, R.S.D.: Relative Standard Deviation.

The results showed that, 3 out of 10 samples have R.S.D. below 10% thus good DNA concentration estimations for these samples were obtained. However, the success rate of this correlation graph for this batch of samples was only 30%. Even though unsatisfactory result obtained, the efficiency of this correlation graph is based on the accuracy of each batch of analysis samples. If there is an error in the amplification process or electrophoresis analysis, differences in peak heights can occur, thus estimation of the DNA concentration using this correlation graph becomes inaccurate. However, the evaluations of this correlation graph showed that it is not very useful for DNA concentration estimation.

4.2.5 Correlation graph of Internal Amplification Controls (IACs)

A correlation graph was plotted between the average peak heights of IACs and the DNA concentrations to identify if there is any affect caused by the high amount of tested DNA on amplification efficiency of IACs. The average peak heights of IACs which were used to plot this correlation graph were obtained from the same electropherograms which were used to plot the correlation graph of 4-plex. The details obtained from the electropherograms are as shown in the Table 4.7.

Table 4.7 Table below shows the average peak heights of IACs which were obtained from the electropherograms produced using the serial diluted control DNA samples.

DNA concentration (ng/µl)	Average peak height (RFU)					
	Set A	Set B	Set C	Avg.	S.d.	R.S.D. (%)
200	1471.5	1739.5	1663.0	1624.67	138.05	8.50
100	1634.0	1787.0	1526.0	1649.00	131.14	7.95
50	1428.0	1744.5	1816.0	1662.83	206.49	12.42
25	1549.5	1716.5	1493.0	1586.33	116.21	7.33
12.5	1493.5	1497.0	1540.5	1510.33	26.18	1.73
6.25	1348.5	1684.0	1621.5	1551.33	178.42	11.50
3.13	1392.0	1469.0	1533.0	1464.67	70.60	4.82
1.56	1639.0	1722.0	1439.5	1600.17	145.20	9.07
0.78	1429.5	1454.5	1441.5	1441.83	12.50	0.87
0.39	1449.5	1441.5	1663.0	1518.00	125.64	8.28
0.20	1577.5	1490.0	1426.5	1498.00	75.82	5.06
0.10	1604.0	1477.5	1812.5	1631.33	169.16	10.37
0.05	1657.0	1505.0	1502.5	1554.83	88.49	5.69
0.02	1393.5	1403.5	1572.5	1456.50	100.58	6.91
0.01	1430.0	1448.5	1548.0	1475.50	63.46	4.30

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation.

The results showed that the similarity between each IACs of the same concentration is very high as the highest R.S.D. is only 12.42%. To confirm that IAC neither changed much when the tested DNA concentration increased, the correlation graphs using average peak heights of the IACs and the DNA concentrations was plotted. The plotted correlation graph is as shown in the Figure 4.7.

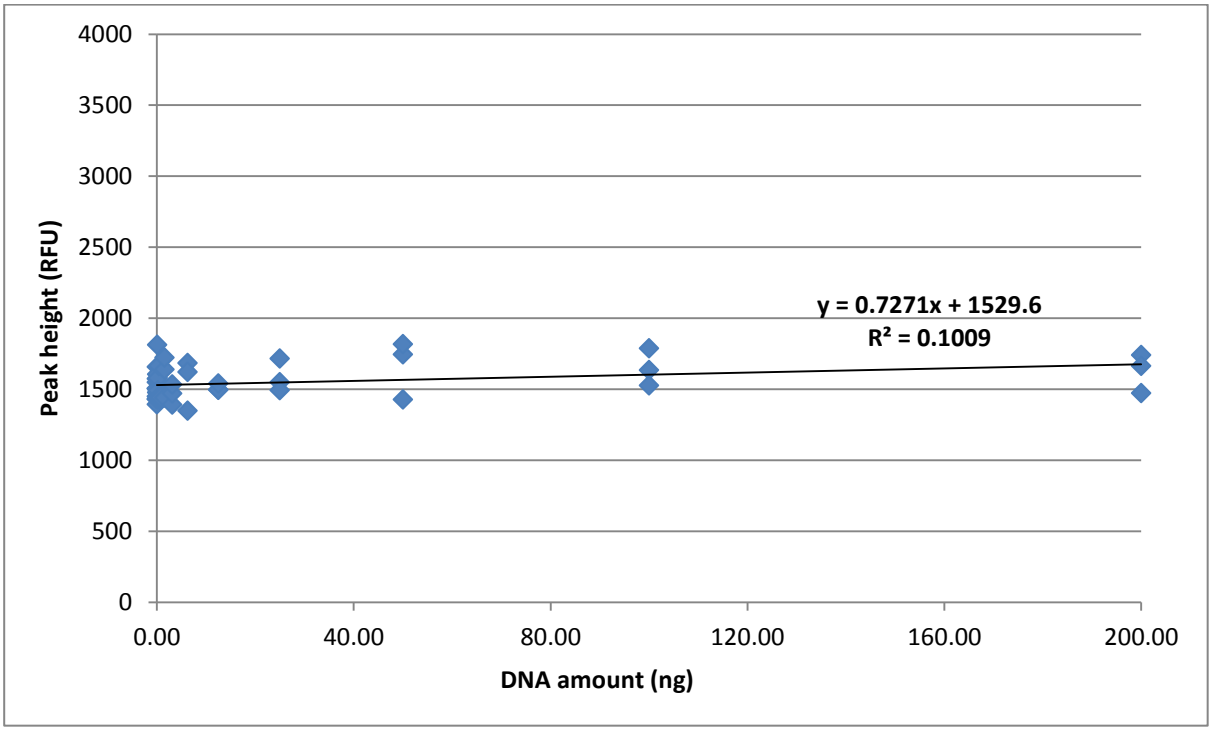


Figure 4.7: Figure above shows the graph represents correlation between average peak heights of IACs with DNA concentrations.

The coefficient of determination (R^2) obtained from this correlation graph was as low as 0.101 showing that not much difference between the average peak heights of IACs which were developed in different DNA concentrations. This result showed that high amounts of tested DNA will not affect the amplification of the IACs. Thus, this multiplex system is suitable for quantification purpose as the IACs only detect the PCR inhibitions and would not be affected by the DNA concentrations.

4.3 DISCUSSION

DNA quantification is an essential process in the DNA profiling. With the estimation of the DNA concentrations obtained, normalization can be carried out on the DNA samples before the proper DNA amount is used for the PCR amplification based on the recommendation of the amplification kits. Errors in DNA quantification and normalization processes can cause problems with the DNA profiles (Pascali & Merigioli 2014, Gill et al. 2005).

Several methods have been used to quantify the DNA amount: UV spectrometry, gel-based techniques, blotting techniques and DNA amplification using real-time PCR (Alonso et al. 2004, Nicklas, Buel 2003a, Nicklas, Buel 2003b). Earlier techniques measured total DNA in the sample but the latest techniques specifically measure human DNA. Real-time PCR kits such as Quantifiler® Human DNA Quantification were specifically developed for this purpose. This quantification kit is robust and has sensitivity to detect the DNA concentration as low as 16 pg/µl (Green et al. 2005). A similar result was also obtained from the analysis presented in this chapter where the Quantifiler® Human DNA Quantification kit gave a more accurate result for the lowest DNA concentration tested (0.01 pg/µl) with R.S.D. below 5%, while the GoTaq® qPCR Master Mix gave R.S.D. as high as 45.43% for the same DNA concentration. GoTaq® qPCR Master Mix uses Sybr® Green dyes which are non-specific fluorescent dyes that bind with any double stranded DNA while the Quantifiler® Human DNA Quantification contains sequence specific DNA probes which are labelled with a reporter dye and emit fluorescence only once the probe has bound with its complementary DNA target (Life Technology 2014). This further explains the accuracy and specificity of Quantifiler® Human DNA Quantification kit for human DNA quantification.

Even though real-time PCR techniques provide good DNA quantification results, only the quantity of the DNA can be obtained. But with the DNA quantification using capillary electrophoresis, both the quality and quantity of the DNA can be analysed. Even though there is no specific study on this, in general, allelic peak heights can be used as an

estimator of DNA concentration as there is an approximately linear relationship between peak heights and DNA quantity. Tvedenbrink et al. (2009) estimated the allele drop-out probability which can be caused by the amount of DNA used, thus the regression model which was developed to estimate the allele drop-out probability can also be the indicator of the amount of DNA. Similar type of studies using the allelic drop-out probability to estimate the amount of DNA also has been carried out in recent years (Haned et al. 2011, Tvedebrink et al. 2010, Tvedebrink et al. 2009).

A correlation graph was developed in this study using the average peak heights against the DNA concentrations for quantification. The electrophrograms to obtain this correlation graph were developed using the control DNA 9947A with serial dilution from 200 - 0.01 ng/µl. Previous study has shown that as the DNA concentration increases the peak heights start to become static or cause split peaks (Kukita et al. 2002), thus the DNA concentration as high as 200 ng/µl was selected to identify any such problems. No split peak was observed from the electropherograms produced but the peak height started to become static above 100 ng/µl of DNA concentration (please refer Figure 4.4).

The developed correlation graph has a linear regression formula of y=216.89x+10971 and coefficient of determination (R^2) of 0.8308. This correlation graph was developed using DNA concentrations in the range of 12.5 – 100 ng/µl. Compared to Quantifiler® Human DNA Quantification kit (0.01 – 200 ng/µl) and GoTaq® qPCR Master Mix (0.20 – 200 ng/µl) the correlation graph has a narrow range. A success rate of only 30% was obtained using this correlation graph to estimate the concentration of an unknown DNA samples when the actual DNA concentration was between 12.5 – 100 ng and also the evaluations of this correlation graph showed that it is not very useful for DNA concentration estimation. Furthermore, this correlation graph cannot be applied on degraded DNA samples since the estimation is based on the average peak heights of the 4-plex and larger peaks will not be amplified with degraded samples. A previous study also showed that the peak height values sometimes show poor reproducibility even in standard DNA analysis (Manabe et al. 2013), thus DNA concentrations may vary from the real quantity if estimated using a correlation graph.

However, with the Internal Amplification Controls (IACs), this multiplex still a useful tool to assess the quality of the sample. The study on IACs peak heights also indicated that there is no interaction between the 4-plex amplification and IACs amplification. This can be seen as the correlation graph of average IACs peak heights against DNA concentration has an almost flat line with coefficient of determination (R^2) of 0.101 indicating the amplification occurred at each point of different DNA concentrations without any interference. Thus this multiplex is useful to detect any PCR inhibitors in the samples while quantitating them, as the IACs peaks are known to be not affected by the tested DNA concentrations.

CHAPTER 5

DNA EXTRACTION FROM BONE SAMPLES

5.1 OVERVIEW

The ultimate aim of the DNA extraction process is to obtain maximum amounts of DNA from the samples submitted. At the same time, it is also important to get a pure DNA extraction by eliminating inhibitors which can reduce the efficiency of the amplification process, thus selection of extraction techniques is very important for samples containing PCR inhibitors.

The main aim of the research presented in this chapter was to assess the capability of five extraction methods on bone samples. These five extraction methods (ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and phenol-chloroform-isoamyl alcohol) were assessed for their capability to yield DNA from bone samples and at the same time the quality of the extracted DNA also was taken into consideration to identify the best extraction. Extractions were carried out using rib and femur samples that were either fresh or had been exposed to the environment for 3 months and 1 year.

Prior to the main testing, the decalcification process and the usage of Amicon 30kDa filter (Amicon ultra-0.5 centrifugal filter unit with ultracel-30 membrane, Merck Millipore) were evaluated using the phenol-chloroform-isoamyl alcohol extraction method to identify the best technique for bone extraction. The decalcification process was tested for its effect on DNA in the bone extraction, while the usage of Amicon 30kDa filter was assessed

against the ethanol precipitation technique to identify the best technique to carry out on the aqueous phase of the phenol-chloroform-isoamyl alcohol extraction.

The optimised methods were also used to extract DNA from bone samples that had been subjected to different preservation regimes: cell lysis solution (with 1% sodium azide), dehydration/freeze drying, ethanol (96%), freezing and room temperature storage (see Chapter 2 and 6 for further details).

PART 1: EVALUATION OF EXTRACTION METHODS

5.2 OBJECTIVES

- Evaluate the effect of the decalcification process during DNA extraction from bone.
- Evaluate the use of Amicon 30kDa filters compared to ethanol precipitation for the purification of DNA using the phenol-chlorofom-isoamyl alcohol extraction method.
- Evaluate all five extraction methods: ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and phenol-chloroform-isoamyl alcohol, to test their performance on both fresh and degraded bone samples.

5.3 RESULTS

5.3.1 Evaluation of decalcification during bone extraction and use of Amicon 30kDa filter / ethanol precipitation during phenol-chloroform extraction

A total of 24 samples were extracted to assess the effect of decalcification and use of both Amicon 30kDa filters and ethanol precipitation when using the phenol-chloroform-isoamyl alcohol extraction. The details of the samples are as described in the Table 5.1. Each tested combination was prepared in triplicate (Sample 1 – Sample 3) and the final volume was standardized at 100 µl for comparison. The detail of methods and materials used in this evaluation study are as described in Chapter 2 (Section 2.7).

5.3.1.1 Comparison of DNA concentrations

After the extraction, quantification was carried out on all extracted samples using GoTaq® qPCR Master Mix quantification method. The DNA concentration results obtained are shown in Table 5.1. The decalcification process is hereafter referred to as incubation technique to avoid confusion of word 'decalcification' between decalcification process and samples with decalcification and non-decalcification.

Table 5.1: Table below shows the DNA concentrations extracted from different incubation and DNA concentration techniques using phenol-chloroform-isoamyl alcohol extraction method.

Bone type	Incubation technique	DNA concentration technique	DNA concentration (ng/µl)				
			Sample 1	Sample 2	Sample 3	Avg.	S.d.
Rib	No decalcification	Ethanol precipitation	128.27	137.02	136.26	133.85	4.85
		Amicon 30kDa filter	104.41	123.38	124.18	117.32	11.19
	Decalcification	Ethanol precipitation	86.01	79.00	66.88	77.30	9.68
		Amicon 30kDa filter	28.52	23.83	33.00	28.45	4.59
Femur	No decalcification	Ethanol precipitation	30.69	61.12	60.90	50.90	17.50
		Amicon 30kDa filter	34.28	29.27	34.57	32.71	2.98
	Decalcification	Ethanol precipitation	31.92	31.91	30.58	31.47	0.78
		Amicon 30kDa filter	9.36	10.93	7.84	9.38	1.54

Note: Avg.: Average, S.d.: Standard deviation.

The average values indicating that the highest DNA yields (133.85 ng/µl) were obtained from rib bone samples which were extracted without decalcification and with ethanol precipitation. Lowest mean DNA yields (9.38 ng/µl) were obtained from the femur bone samples which were decalcified and also extracted using Amicon 30kDa filter.

5.3.1.2 Comparison of DNA concentrations using ANOVA

Analysis of variance (ANOVA) was carried out using the R Studio software, on the DNA concentration data (Table 5.2 and Figure 5.1) to see if there was a significant difference between the combination of the incubation and DNA concentration techniques and also the incubation and DNA concentration techniques alone. The bone type was ignored in this study since the intention of this study was to evaluate the incubation and DNA concentration techniques.

Table 5.2: Table below shows the mean DNA concentrations extracted from combination of different incubation and DNA concentration techniques using phenol-chloroform-isoamyl alcohol.

Combination of techniques	Mean DNA concentration (ng/µl)	Standard deviation
Non-decalcification – Ethanol precipitation	92.38	46.86
Non-decalcification – Amicon 30kDa filter	75.01	46.92
Decalcification – Ethanol precipitation	54.38	25.84
Decalcification – Amicon 30kDa filter	18.92	10.89

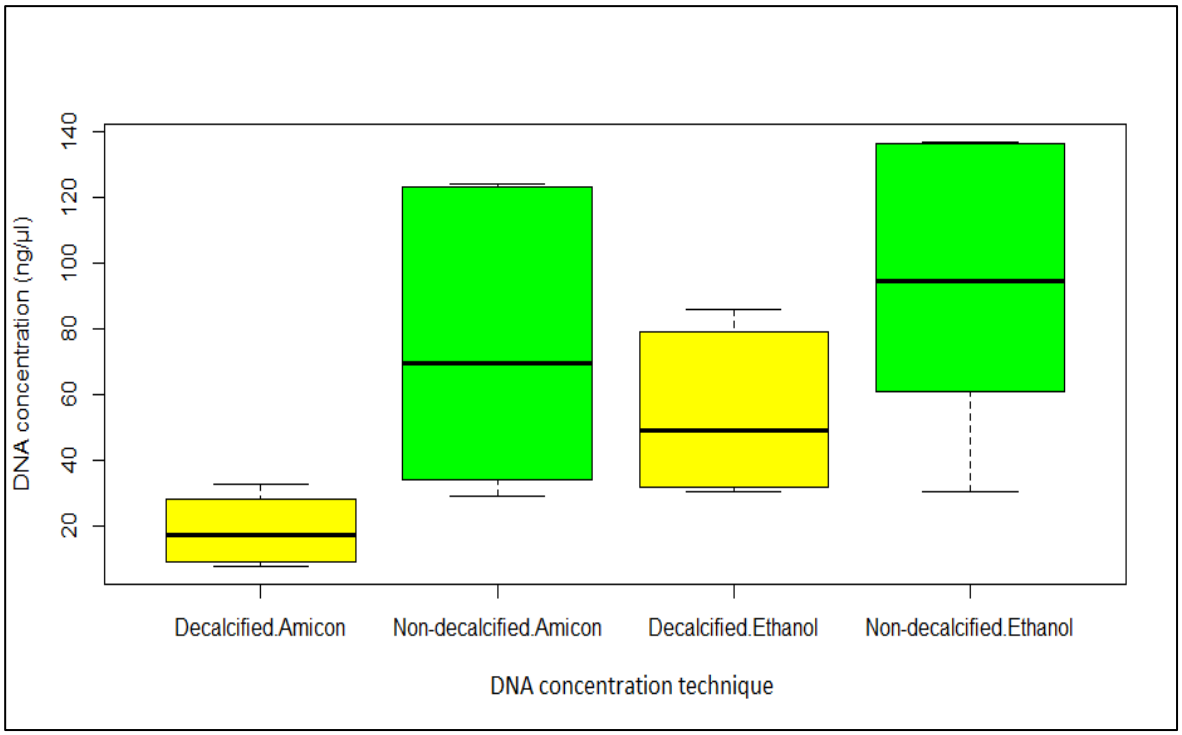


Figure 5.1: Boxplots generated from the concentrations of DNA extracted using combinations of different incubation and DNA concentration techniques.

The combination of incubation and DNA concentration techniques shows that highest mean DNA yields (92.38 ng/µl) were obtained when the bone samples were extracted without decalcification and with the ethanol precipitation technique during phenol-chroloform-isoamyl alcohol extraction. The combination of decalcification and Amicon 30kDa filter technique produced lowest mean DNA yield (18.92 ng/µl).

Also based upon the ANOVA results, the extracted DNA concentration of bone samples was significantly affected by use of the incubation technique ($F_{1,20}$ = 10.25, p = 0.004) but not by DNA concentration technique alone ($F_{1,20}$ = 3.23, p = 0.087). This means without incubation technique, similar result could be obtained from both DNA concentration techniques but with either one of the DNA concentration technique, both incubation techniques would give significantly different results.

Also, the multiple comparison data shows that there is a significant difference between the combination of the non-decalcification and ethanol precipitation with the combination of decalcification and Amicon 30kDa filter methods (p=0.010). This can be seen in Figure 5.1. However, there is no significant difference between the other combinations.

5.3.1.3 DNA amplification and analysis of extracted bone samples

All the extracted samples in this evaluation study were amplified using the multiplex (4-plex & IACs). 1 µl of each extract was used for amplification.

Although all the previous results showed that the ethanol precipitation produces a higher DNA yield, better quality electropherograms were produced using DNA extracts from the phenol-chloroform-isoamyl alcohol extraction using Amicon 30kDa filters (Figure 5.2). Inhibition was detected in the samples extracted using ethanol precipitation, which caused the drop in the peak heights (RFU) of the larger amplicons and also caused imbalance in the internal amplification controls while, samples extracted using Amicon 30kDa filters produced more balanced peaks. The decalcified bone samples generated lower peak heights compared to the non-decalcified bone samples, which was concordant with the DNA quantification results.

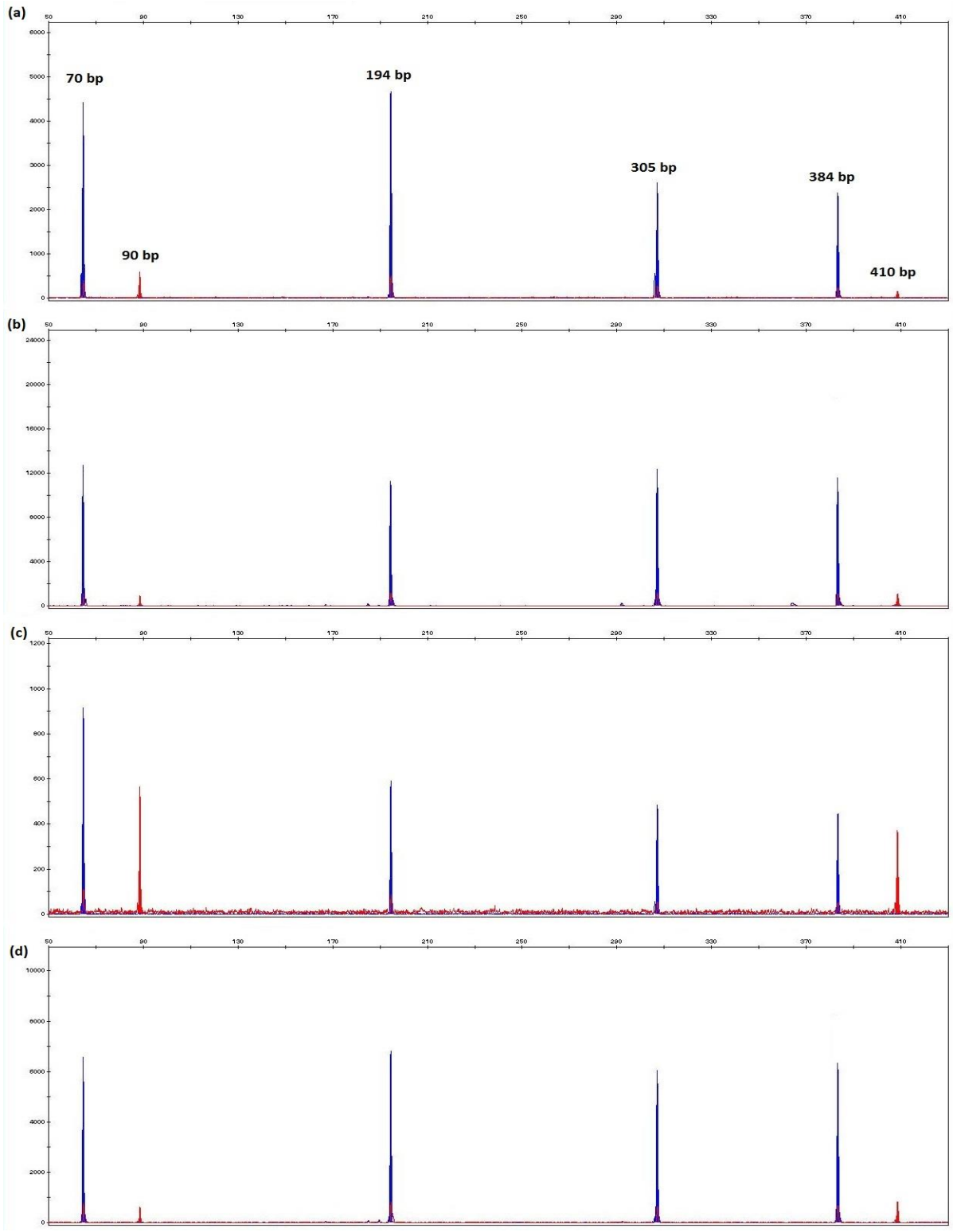


Figure 5.2: Examples of electropherograms generated from extracted DNA of fresh femur bone samples using (a) non-decalcification – ethanol precipitation, (b) non-decalcification – Amicon 30kDa filter, (c) decalcification – ethanol precipitation and (d) decalcification – Amicon 30kDa filter extraction techniques. 1 µl of each extract was used.

5.3.2 Evaluation of extraction methods

Five extraction methods were evaluated: ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit and PrepFiler® BTA Forensic DNA Extraction Kit and Phenol-chloroform-isoamyl alcohol, all using non-decalcified bone samples and Amicon filtration method for DNA concentration. The extractions were carried out on fresh rib and femur samples and rib and femur samples recovered from animals that had been exposed to the environment for 3 months and 1 year. Each extracted sample was prepared in triplicate (Sample 1 – Sample 3). The final volume was standardized at 100 µl for each extraction for comparison. The detail of methods and materials used in this evaluation study are as described in Chapter 2 (Section 2.8).

5.3.2.1 Fresh bone samples extraction

DNA was successfully extracted from fresh bone samples using all tested extraction methods. Quantification was carried out on all extracted samples using GoTaq® qPCR Master Mix quantification method. The DNA concentrations obtained are as shown in the Tables 5.3 and 5.4.

Table 5.3: Table below shows the DNA concentrations extracted from fresh rib bone samples using five extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	55.31	64.31	52.87	57.60	6.20
DNA IQ™ System Kit	6.25	6.41	7.83	6.83	0.87
DNeasy® Blood & Tissue Kit	39.52	30.57	37.36	35.82	4.67
Phenol-chloroform-isoamyl alcohol	95.13	91.58	111.93	99.55	10.87
PrepFiler® BTA Forensic DNA Extraction Kit	33.31	33.84	27.69	31.61	3.41

Note: Avg.: Average, S.d.: Standard deviation.

Table 5.4: Table below shows the DNA concentrations extracted from fresh femur bone samples using five extraction methods.

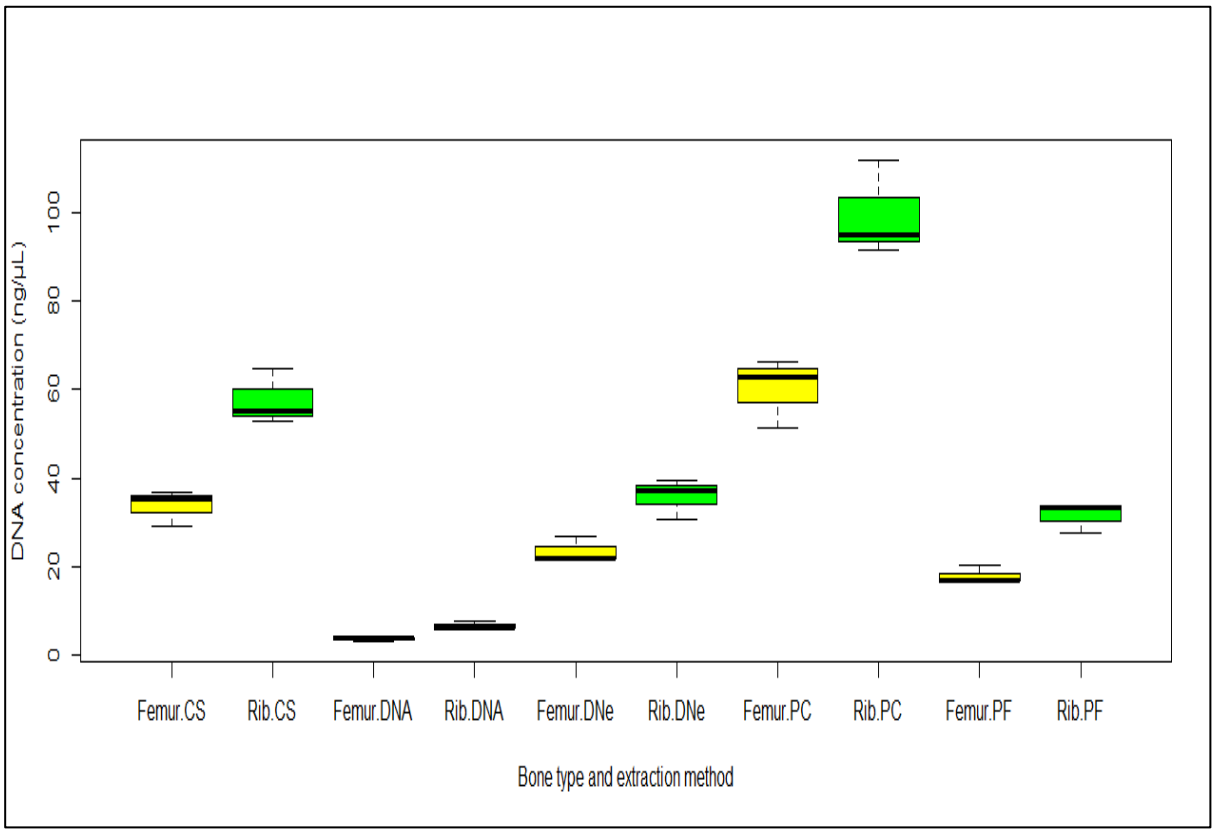
Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	36.87	29.02	35.47	33.78	4.19
DNA IQ™ System Kit	4.40	3.78	3.09	3.76	0.66
DNeasy® Blood & Tissue Kit	21.43	26.93	22.04	23.47	3.02
Phenol-chloroform-isoamyl alcohol	66.26	62.98	51.53	60.26	7.73
PrepFiler® BTA Forensic DNA Extraction Kit	16.92	16.66	20.37	17.98	2.07

Note: Avg.: Average, S.d.: Standard deviation.

ANOVA results showed that there is a significant difference between the extraction methods in rib ($F_{4,10} = 95.29$, $p = 6.41e^{-08}$) and femur ($F_{4,10} = 73.34$, $p = 2.27e^{-07}$), but that there is no significant difference between the types of bones since same amount of samples from both bone types were used for extraction ($F_{1,28} = 3.48$, $p = 0.078$). Significant differences were seen when same extraction methods were used with different types of bones with phenol-chloroform-isoamyl alcohol and the ChargeSwitch® gDNA Plant Kit as shown in Table 5.5 and Figure 5.3.

Table 5.5: Table below shows the statistical data of the comparison between different bone types which were extracted using same extraction method.

Combination of techniques comparison	p adj
Rib – ChargeSwitch : Femur – ChargeSwitch	**0.001**
Rib – DNA IQ : Femur – DNA IQ	0.999
Rib – DNeasy : Femur – DNeasy	0.185
Rib – Phenol : Femur – Phenol	**$6.00e^{-07}$**
Rib – Prepfiler : Femur – Prepfiler	0.109



Note: CS.: ChargeSwitch, DNA: DNA IQ, DNe: DNeasy, PC: Phenol, PF: Prepfiler

Figure 5.3: Boxplots generated from the concentrations of DNA extracted using different extraction methods on femur and rib bones.

5.3.2.2 DNA amplification and analysis on extracted fresh bone samples

All the extracted samples in this evaluation study were amplified using the multiplex (4-plex & IACs). 1 µl of each extract was used for amplification.

The electropherogram results showed that although the phenol-chloroform method produced the highest DNA yields, all samples could be successfully amplified using the 4-plex & IACs multiplex (Figure 5.4). No inhibition was detected in any of the samples when amplifying both Internal Amplification Controls (IACs).

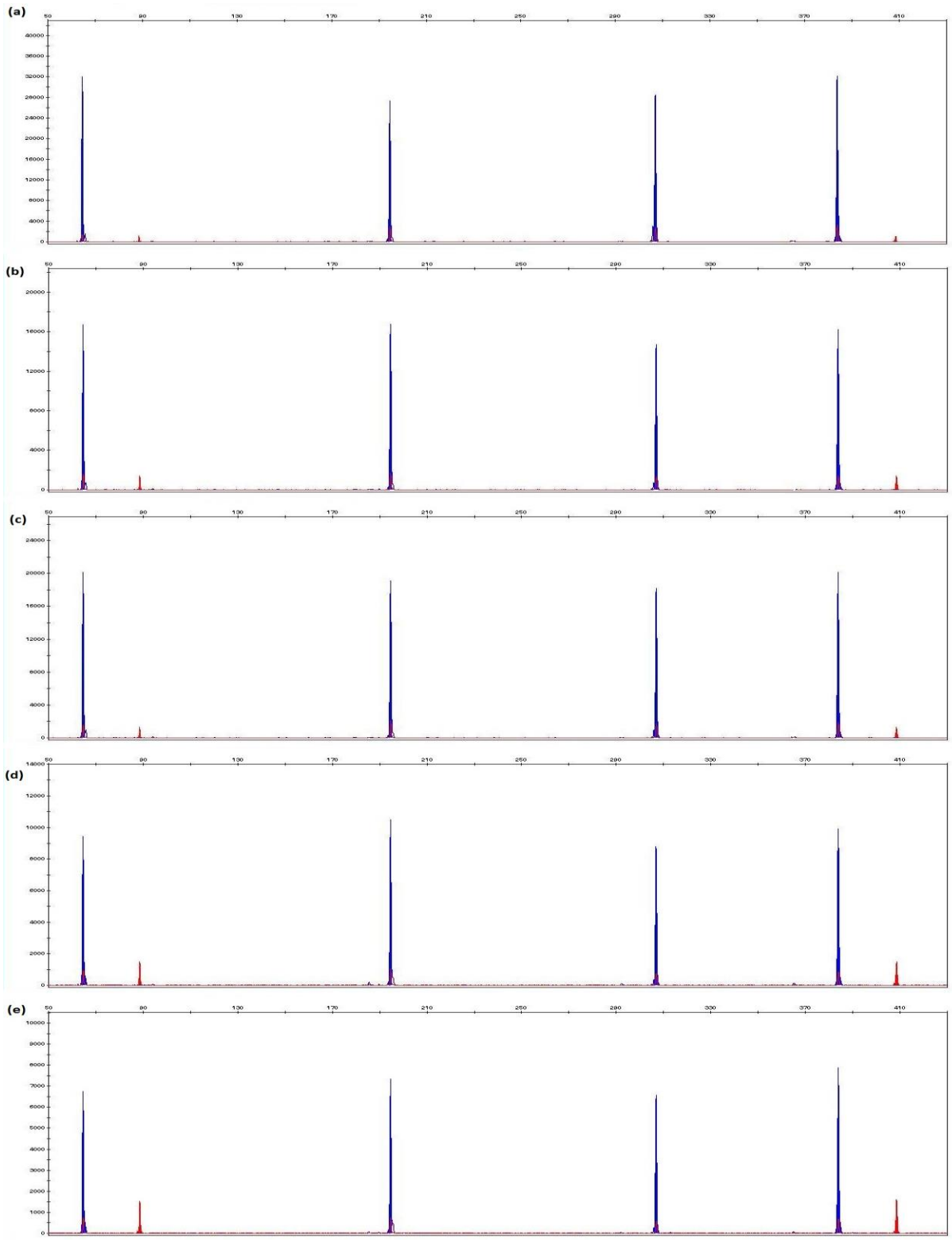


Figure 5.4: Examples of electropherograms generated from extracted DNA of fresh rib bone samples using (a) phenol-chloroform-isoamyl alcohol, (b) DNeasy® Blood & Tissue Kit, (c) ChargeSwitch® gDNA Plant Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. 1 µl of each extract was used.

5.3.2.3 Degraded bone samples

Rib and femur samples recovered from animals that had been exposed to the environment for 3 months and 1 year were used for evaluation study of the extraction methods on degraded bone samples. Extracted samples were quantified using GoTaq® qPCR Master Mix quantification method.

Following extraction of 50 mg of bone no DNA was detected through either real-time PCR or amplification of the 4-plex. The electropherogram results (Figure 5.5) identified the presence of presence of inhibitors in the different extraction methods in several of the methods and only ChargeSwitch® gDNA Plant Kit extracts gave balanced IAC peaks. The same pattern was observed in both rib and femur that had been exposed to the environment for 3 months and 1 year.

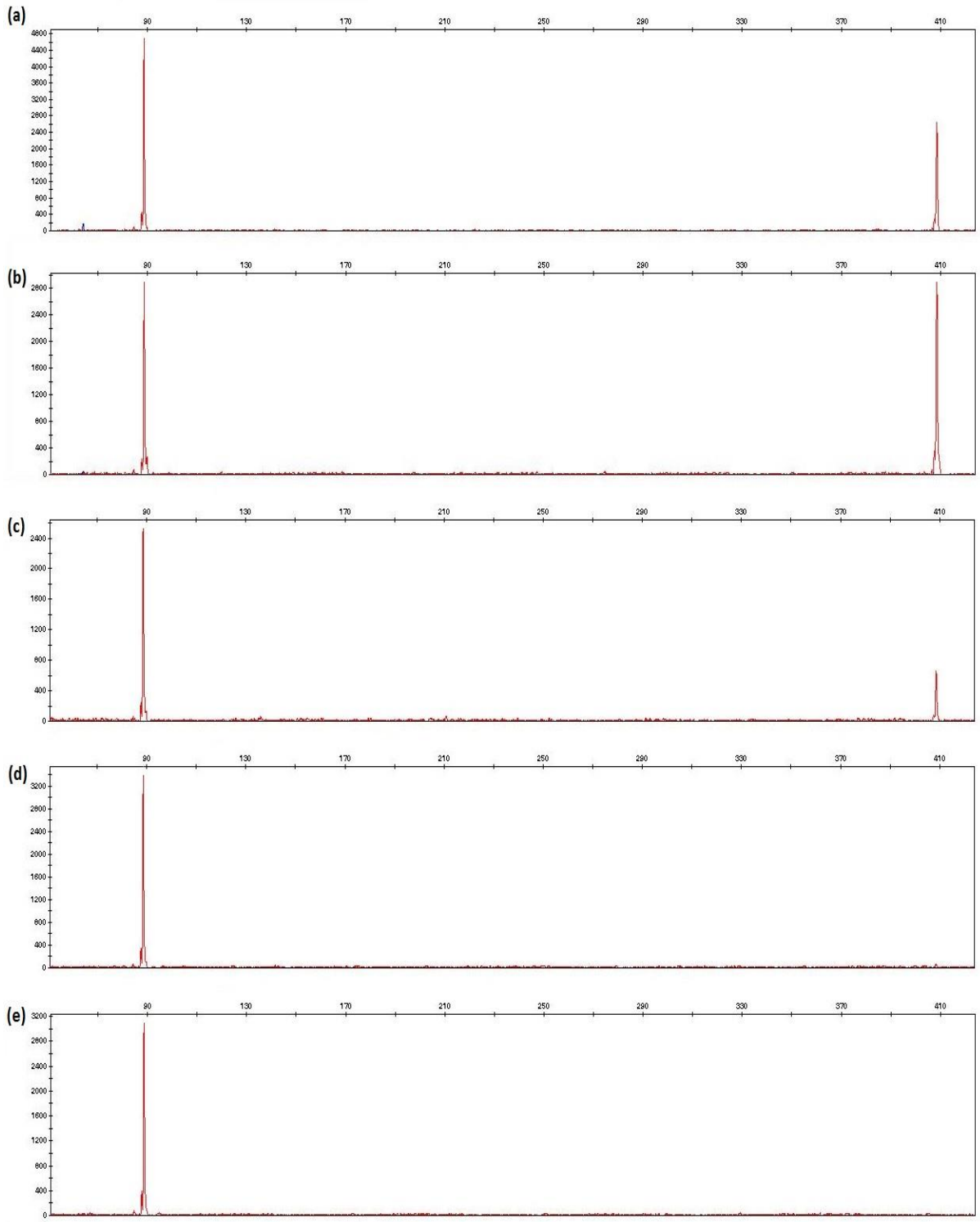


Figure 5.5: Examples of electropherograms generated from rib bone samples recovered from animals that had been exposed to the environment for 3 months that were extracted using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. 1 µl of each extract was amplified.

5.3.3 Pre-process method development for degraded bone samples

Since no DNA yield was observed with the degraded bone samples a pre-process method was developed to concentrate and clean-up the digested bone samples. The same rib and femur samples were used for this method development.

This method increased the starting material (pulverised bone samples) from 50 mg to 250 mg. For each sample 5 separate 50 mg samples were digested using the method-specific protocol. The samples were then pooled and concentrated and cleaned using the Amicon 30kDa filter (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration). Each extracted sample was prepared in triplicate (Sample 1 – Sample 3). The final volume was standardized at 100 µl for each extraction for comparison study. The detail of methods and materials used in this method developmental study are as described in Chapter 2 (Section 2.9).

5.3.3.1 DNA quantification on degraded bone samples

Extracted samples were quantified using GoTaq® qPCR Master Mix quantification method. The DNA concentrations obtained are as shown in the Tables 5.6, 5.7, 5.8 and 5.9.

Table 5.6: Table below shows the DNA concentrations from rib bone samples recovered from animals that had been exposed to the environment for 3 months using five extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	45.85	50.08	62.9	52.94	8.88
DNA IQ™ System Kit	5.46	6.28	7.77	6.50	1.17
DNeasy® Blood & Tissue Kit	37.75	28.39	39.65	35.26	6.03
Phenol-chloroform-isoamyl alcohol	86.56	82.81	114.10	94.49	17.09
PrepFiler® BTA Forensic DNA Extraction Kit	25.26	25.14	33.09	27.83	4.56

Note: Avg.: Average, S.d.: Standard deviation.

Table 5.7: Table below shows the DNA concentrations from femur bone samples recovered from animals that had been exposed to the environment for 3 months using five extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	30.57	22.49	33.91	28.99	5.87
DNA IQ™ System Kit	2.94	3.70	3.64	3.43	0.42
DNeasy® Blood & Tissue Kit	20.47	25.01	23.39	22.96	2.30
Phenol-chloroform-isoamyl alcohol	60.29	56.95	57.13	58.12	1.88
PrepFiler® BTA Forensic DNA Extraction Kit	12.83	12.38	18.02	14.41	3.13

Note: Avg.: Average, S.d.: Standard deviation.

Table 5.8: Table below shows the DNA concentrations from rib bone samples recovered from animals that had been exposed to the environment for 1 year using five extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	11.46	12.52	18.22	14.07	3.64
DNA IQ™ System Kit	1.37	1.57	1.94	1.63	0.29
DNeasy® Blood & Tissue Kit	9.44	7.10	9.91	8.82	1.51
Phenol-chloroform-isoamyl alcohol	21.64	20.70	31.02	24.45	5.71
PrepFiler® BTA Forensic DNA Extraction Kit	6.32	6.28	9.52	7.37	1.86

Note: Avg.: Average, S.d.: Standard deviation.

Table 5.9: Table below shows the DNA concentrations from femur bone samples recovered from animals that had been exposed to the environment for 1 year using five extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Sample 1	Sample 2	Sample 3	Avg.	S.d.
ChargeSwitch® gDNA Plant Kit	7.64	5.62	9.23	7.50	1.81
DNA IQ™ System Kit	0.61	0.93	1.06	0.87	0.23
DNeasy® Blood & Tissue Kit	5.12	6.25	5.85	5.74	0.57
Phenol-chloroform-isoamyl alcohol	16.07	13.24	14.28	14.53	1.43
PrepFiler® BTA Forensic DNA Extraction Kit	3.21	3.09	5.01	3.77	1.08

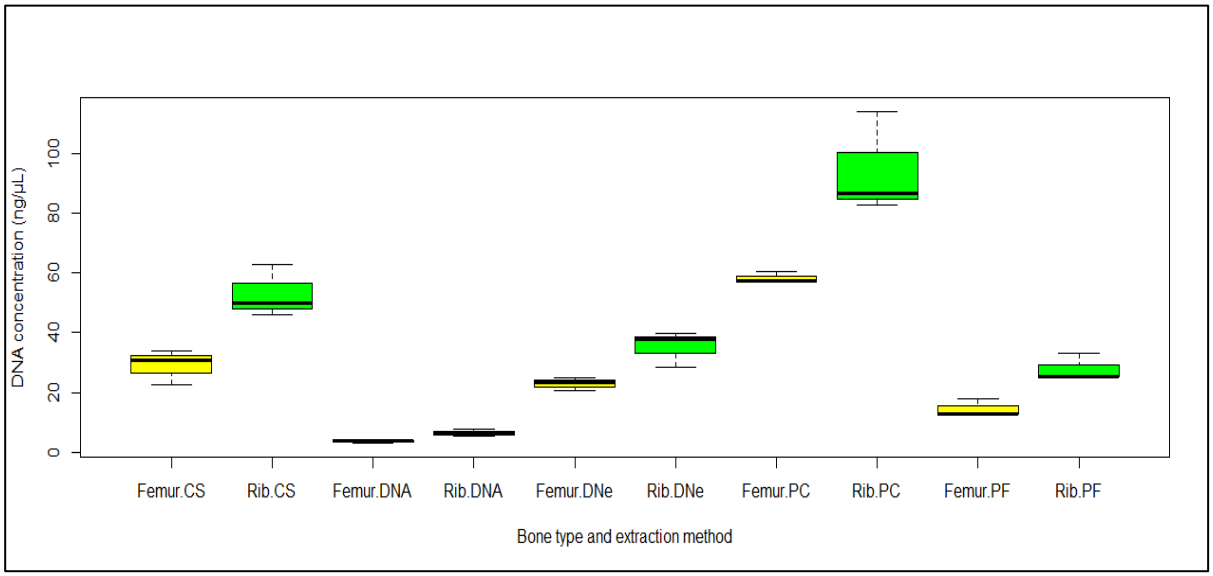
Note: Avg.: Average, S.d.: Standard deviation.

ANOVA results showed that there is a significant difference between the extraction methods in 3 months rib ($F_{4,10}$ =38.19, p = $4.98e^{-06}$), 3 months femur ($F_{4,10}$ = 119.12, p = $2.17e^{-08}$), 1 year rib ($F_{4,10}$ = 21.50, p = $6.71e^{-05}$) and 1 year femur ($F_{4,10}$ = 57.52, p = $7.26e^{-07}$)

The ANOVA result also showed that there is no significant difference between the types of bones used for the extraction from 3 months ($F_{1,28}$ = 3.52, p = 0.073) and 1 year ($F_{1,28}$ = 3.65, p = 0.067). However, multiple pairwise analyses using the Tukey test showed that there is a significant difference when the same extraction methods were applied to different types of bones in phenol-chloroform-isoamyl alcohol (bones degraded for both 3 months and 1 year) and ChargeSwitch® gDNA Plant Kit (3 months) extraction methods as shown in Table 5.10 and Figures 5.6 and 5.7.

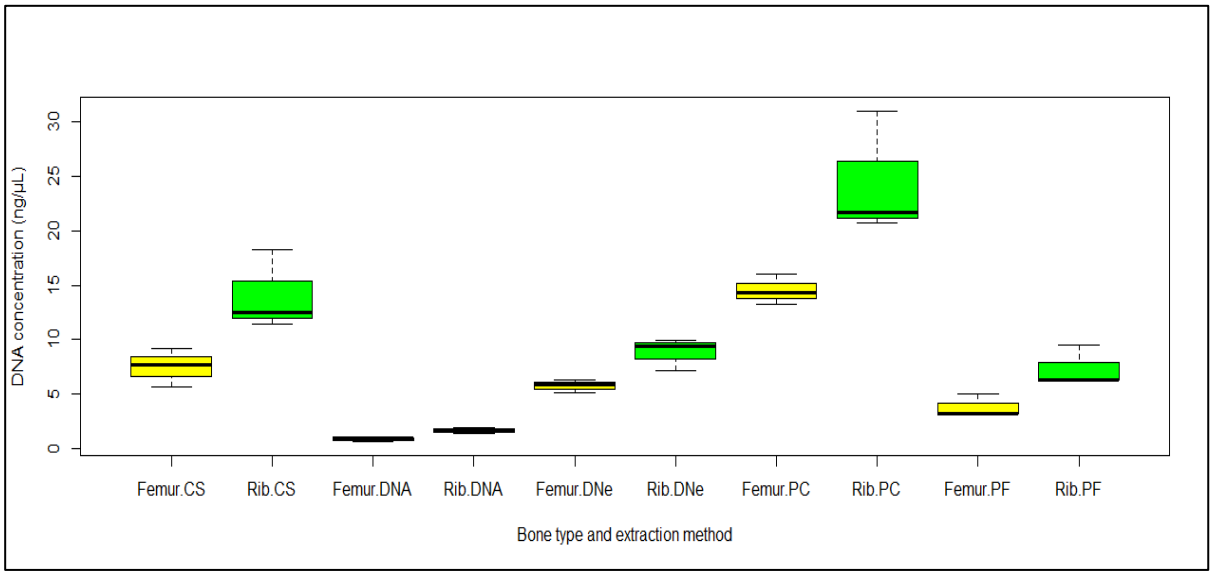
Table 5.10: Table below shows the statistical data of the comparison between different bone types which were extracted using same extraction method.

Combination of techniques comparison	p adj	
	3 months	**1 year**
Rib – ChargeSwitch : Femur – ChargeSwitch	**0.012**	0.076
Rib – DNA IQ : Femur – DNA IQ	0.999	0.999
Rib – DNeasy : Femur – DNeasy	0.505	0.852
Rib – Phenol : Femur – Phenol	**$1.05e^{-04}$**	**0.002**
Rib – Prepfiler : Femur – Prepfiler	0.393	0.713



Note: CS.: ChargeSwitch, DNA: DNA IQ, DNe: DNeasy, PC: Phenol, PF: Prepfiler

Figure 5.6: Boxplots generated from the concentrations of DNA extracted from rib and femur bones recovered from animals that had been exposed to the environment for 3 months using different extraction methods.



Note: CS.: ChargeSwitch, DNA: DNA IQ, DNe: DNeasy, PC: Phenol, PF: Prepfiler

Figure 5.7: Boxplots generated from the concentrations of DNA extracted from rib and femur bones recovered from animals that had been exposed to the environment for 1 year using different extraction methods.

5.3.3.2 DNA amplification and analysis of extracted degraded bone samples

All the extracted samples in this method developmental study were amplified using the multiplex (4-plex & IACs). 1 μl of each extract was used for amplification.

The electropherograms produced show that the DNA was successfully extracted from the degraded bone samples, after using this pre-process method. The electropherograms also show that no PCR inhibitors were observed in the extracted DNA samples since both the IAC peaks are balanced. Also, the DNA degradation can be observed in both rib and femur samples recovered from animals that had been exposed to the environment for 3 months and 1 year (Figures 5.8 and 5.9).

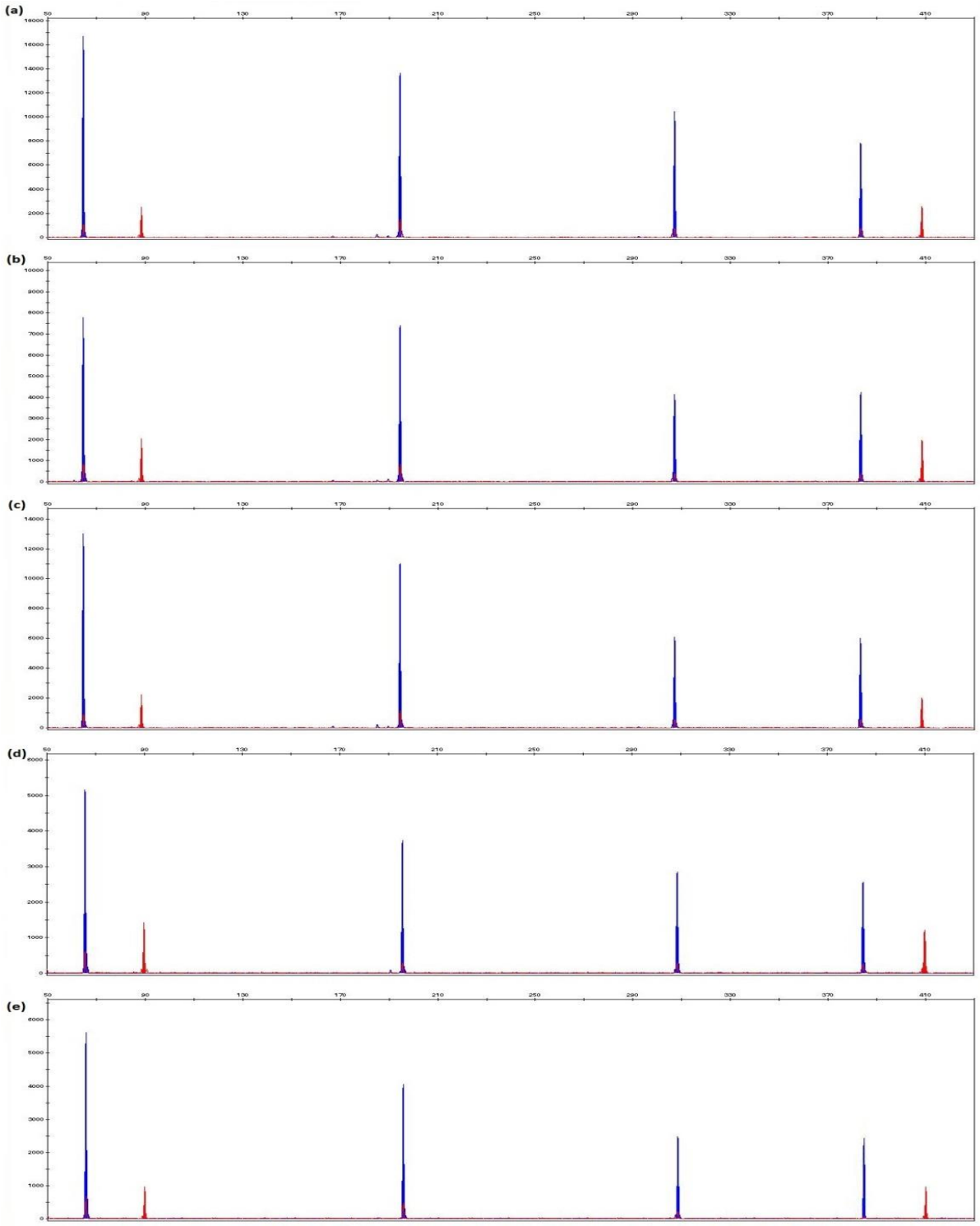


Figure 5.8: Examples of electropherograms generated from extracted DNA of rib bone samples recovered from animals that had been exposed to the environment for 3 months using pre-process technique and (a) phenol-chloroform-isoamyl alcohol, (b) DNeasy® Blood & Tissue Kit, (c) ChargeSwitch® gDNA Plant Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. 1 µl of each extract was used.

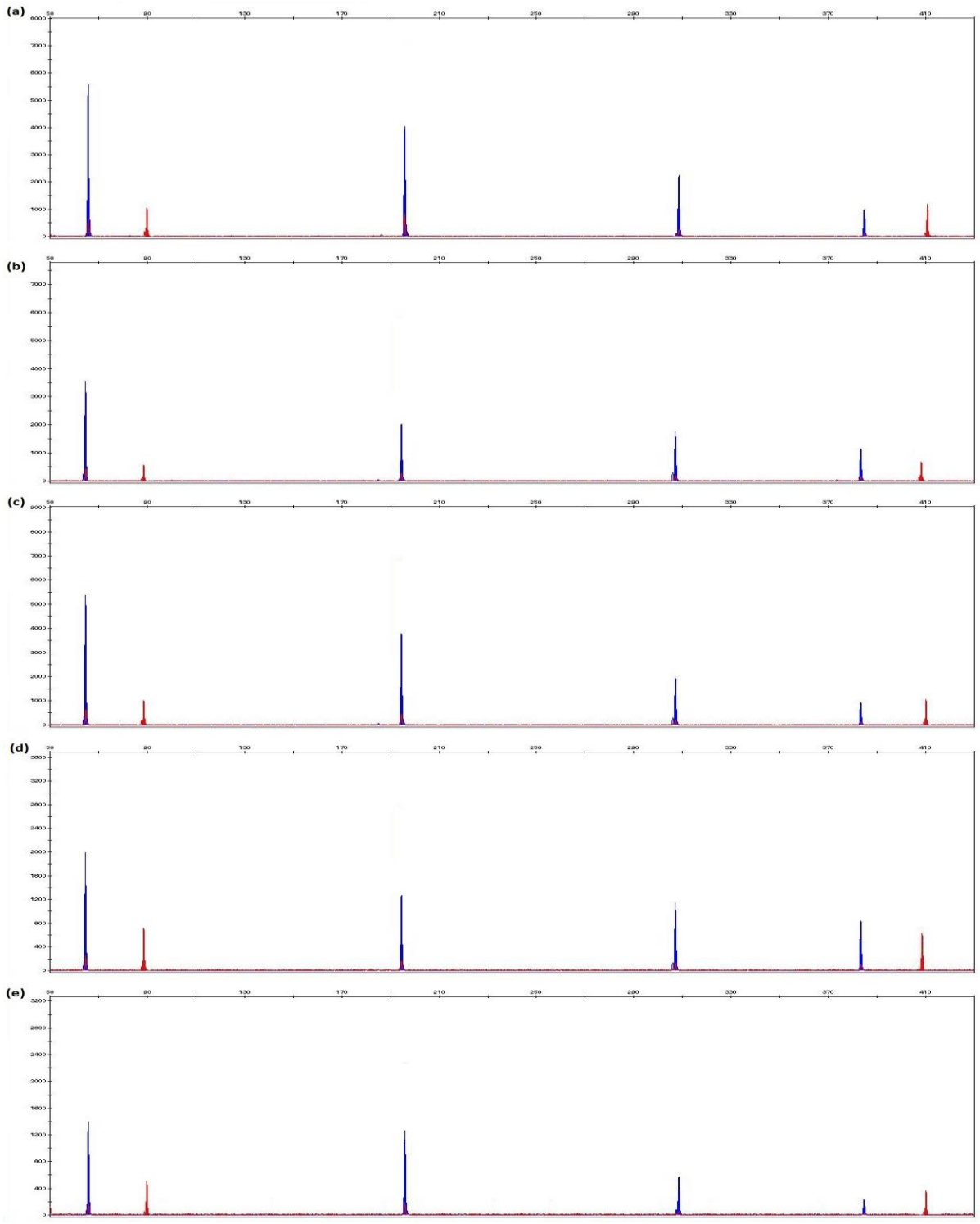


Figure 5.9: Examples of electropherograms generated from extracted DNA of rib bone samples recovered from animals that had been exposed to the environment for 1 year using pre-process technique and (a) phenol-chloroform-isoamyl alcohol, (b) DNeasy® Blood & Tissue Kit, (c) ChargeSwitch® gDNA Plant Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. 1 µl of each extract was used.

PART 2: DNA EXTRACTION FROM PRESERVED BONE SAMPLES

5.4 OBJECTIVE

- Extract all 180 preserved bone samples (fresh and degraded) using the five optimised extraction methods and further evaluate their performances on bone samples.

5.5 RESULTS

After all the evaluation studies and the optimisations on the extraction methods, extraction of preserved bone samples was carried out. A total of 180 bone pieces (rib and femur) (90 fresh and 90 degraded (for 3 months)) which were preserved using cell lysis solution (with 1% sodium azide), dehydration / freeze drying, ethanol (96%), freezing and room temperature storage were extracted. In total 900 DNA extractions were carried out. The details of methods and materials used for bone preservations are as described in Chapter 2 (Section 2.4)

These preserved bone samples were extracted using optimised extraction methods: phenol-chloroform-isoamy alcohol, DNeasy® Blood & Tissue Kit, ChargeSwitch® gDNA Plant Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit after 6 weeks, 6 months and 1 year of preservation. The final volume was standardized at 100 µl for comparison study. The details of methods and materials used for each extraction method are as described in Chapter 2 (Section 2.6). The pre-process technique was used for degraded bone samples as described in Chapter 2 (Section 2.9).

Even though preserved bones were used for extraction, the statistical calculations were only carried out on the extraction methods to evaluate their capability to extract DNA from the bone samples. Thus, the preservation methods were not taken into consideration here (but please see Chapter 6 for these results).

5.5.1 DNA concentration from different bone types

After the extraction, quantification was carried out on all extracted samples using GoTaq® qPCR Master Mix quantification method. The mean DNA concentration results obtained are shown in Table 5.11. The mean DNA concentrations showed that rib bone samples produced more DNA yield compare to the femur bone samples and that the DNA recovery gradually decreased with the time of preservation with both fresh and degraded bone samples.

Table 5.11: Table below shows the mean DNA concentrations of preserved bone samples (femur and rib) extraction after 6 weeks, 6 months and 1 year. Each mean value represents 75 samples which were preserved in 5 different preservation methods, extracted using 5 different extraction methods and extracted in triplicate.

Preservation period	Mean DNA concentration (ng/µl)			
	Fresh bone sample		Degraded bone sample	
	Femur	Rib	Femur	Rib
6 weeks	19.33 (30.72)	45.00 (56.77)	16.15 (26.55)	38.82 (49.63)
6 months	14.09 (21.01)	34.91 (44.51)	11.51 (19.56)	31.37 (42.28)
1 year	6.22 (14.98)	21.02 (38.02)	7.80 (16.42)	23.36 (38.29)

Note: Standard deviation showed in the bracket.

5.5.2 Evaluation of bone type for DNA extraction using ANOVA

The two types of bones (femur and rib) which were used in the preserved bone samples extraction study were statistically analysed to see either there is a difference in the DNA yield from different bone types. The ANOVA results showed that there is a significant difference between the bone types at 6 weeks ($F_{1,148}$ = 11.87, p = 7.44e^{-04}), 6 months ($F_{1,148}$ = 13.42, p = 3.46e^{-04}) and 1 year ($F_{1,148}$ = 9.84, p = 2.06e^{-03}) preserved fresh bone samples. Significant difference was also present between the bone types at 6 weeks ($F_{1,148}$ = 12.16, p = 6.44e^{-04}), 6 months ($F_{1,148}$ = 13.63, p = 3.13e^{-04}) and 1 year ($F_{1,148}$ = 10.46, p = 1.51e^{-03}) preserved degraded bone samples.

Following ANOVA the Tukey test showed no significant difference between bone types when same extraction method applied to them except phenol-chloroform-isoamyl alcohol extraction method, which had a p-value below 0.05 at all preservation points in both fresh and degraded bone samples (Table 5.12).

Table 5.12: Table below shows the statistical data of the comparison between different bone types which were extracted using same extraction method.

Bone type with same extraction comparison	p adj					
	Fresh bone sample			Degraded bone sample		
	6 weeks	6 months	1 year	6 weeks	6 months	1 year
Rib – ChargeSwitch : Femur – ChargeSwitch	1.000	0.999	0.997	0.999	0.999	0.998
Rib – DNA IQ : Femur – DNA IQ	0.442	0.981	0.999	0.891	0.981	0.999
Rib – DNeasy : Femur – DNeasy	0.948	0.809	0.438	0.762	0.634	0.541
Rib – Phenol : Femur – Phenol	**0.008**	**1.60e^{-06}**	**5.11e^{-04}**	**3.25e^{-04}**	**1.96e^{-05}**	**1.10e^{-04}**
Rib – Prepfiler : Femur – Prepfiler	0.744	0.644	0.999	0.817	0.916	0.999

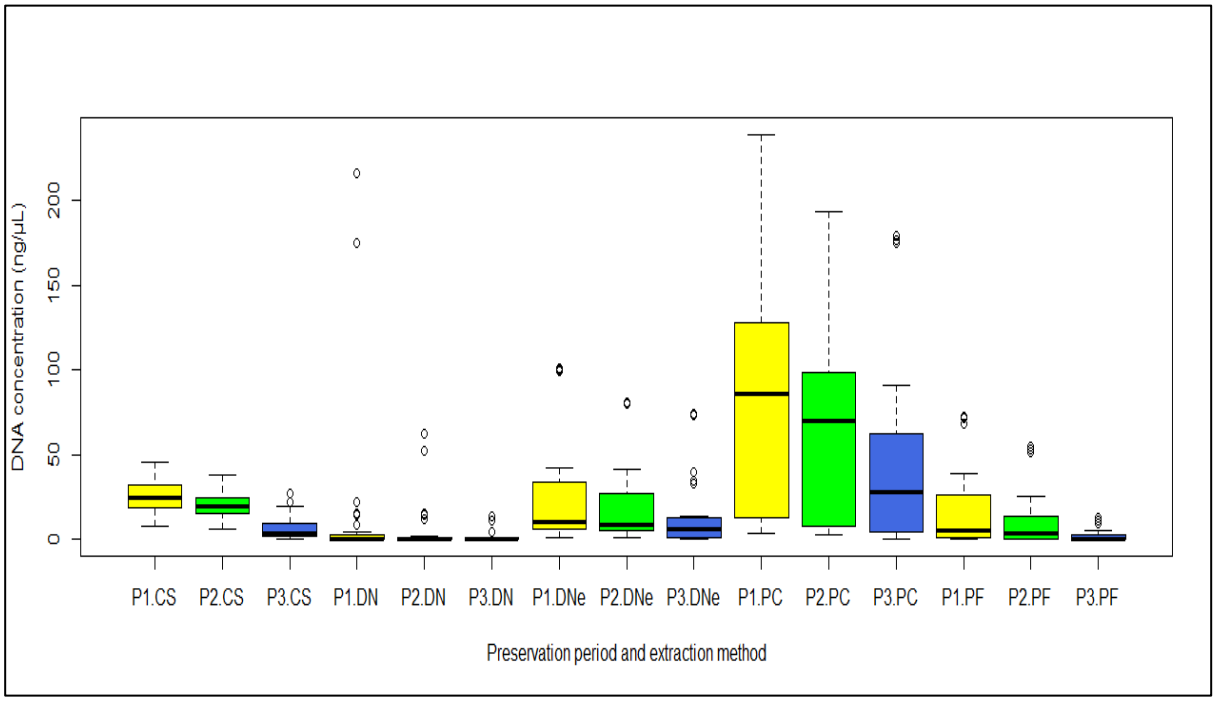
5.5.3 Extraction methods comparison for preserved fresh bone samples

The mean DNA concentration for each extraction method was calculated without considering the bone type, based on the lack of significant differences between the DNA quantities from different bone types, for the majority of methods. The results are as shown in Table 5.13 and Figure 5.10.

Table 5.13: Table below shows the mean DNA concentrations extracted from preserved fresh bone samples after 6 weeks, 6 months and 1 year using different extraction methods. Each mean value represents 30 samples; two bone types which were preserved in 5 different preservation methods and extracted in triplicate.

Extraction method	Mean DNA concentration (ng/µl)					
	6 weeks	6 months	1 year	Avg.	S.d.	R.S.D. (%)
ChargeSwitch® gDNA Plant Kit	25.44	20.67	6.60	17.57	9.80	55.75
DNA IQ™ System Kit	15.49	5.40	1.83	7.57	7.08	93.55
DNeasy® Blood & Tissue Kit	22.50	19.08	14.87	18.82	3.82	20.31
Phenol-chloroform-isoamyl alcohol	81.89	66.51	42.59	63.66	19.80	31.11
PrepFiler® BTA Forensic DNA Extraction Kit	15.52	10.85	2.21	9.53	6.75	70.88

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation.



Note: P1: 6 weeks, P2: 6 months, P3: 1year, CS.: ChargeSwitch, DN: DNA IQ, DNe: DNeasy, PC: Phenol, PF: Prepfiler.

Figure 5.10: Boxplots generated from the concentrations of DNA extracted from preserved fresh bone samples after 6 weeks, 6 months and 1 year using different extraction methods. Each boxplot represents 30 samples which were preserved in 5 different preservation methods.

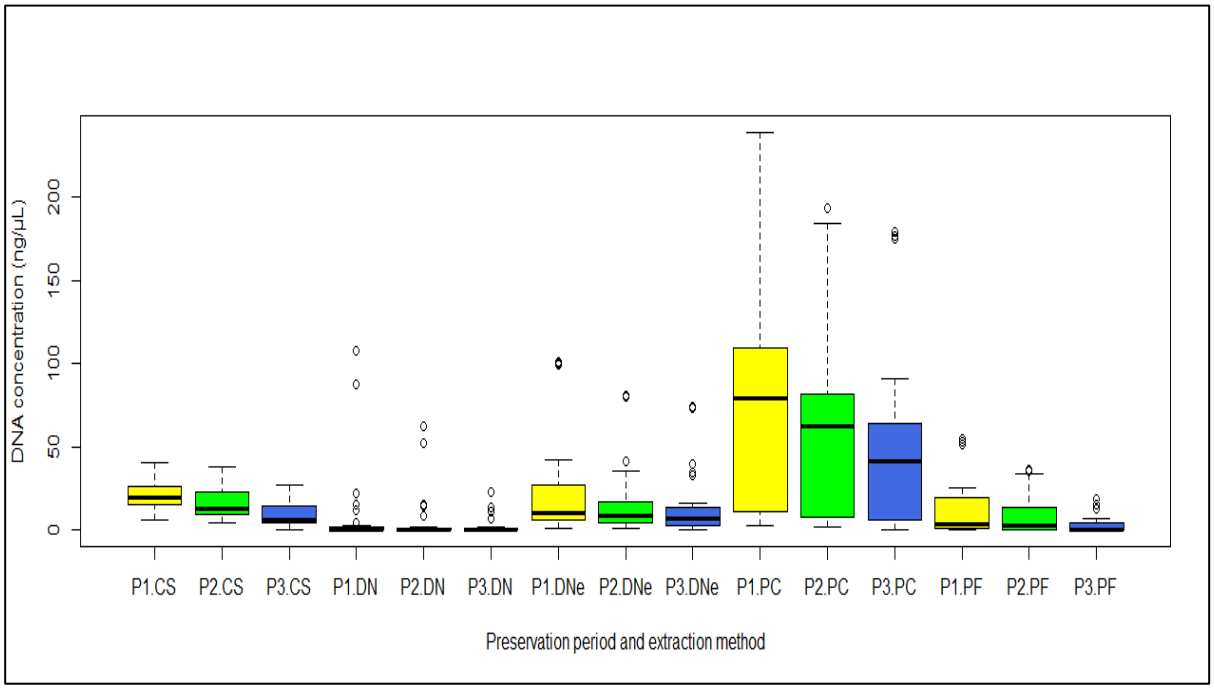
5.5.4 Extraction methods comparison for preserved degraded bone samples

Same as the preserved fresh bone extraction methods comparison, the mean DNA concentration for each extraction method used to extract the DNA from preserved degraded bone samples was calculated without considering the bone type. The results are as shown in Table 5.14 and Figure 5.11.

Table 5.14: Table below shows the mean DNA concentrations extracted from preserved degraded bone samples after 6 weeks, 6 months and 1 year using different extraction methods. Each mean value represent 30 samples; two bone types which were preserved in 5 different preservation methods and extracted in triplicate.

Extraction methods	Mean DNA Concentrations (ng/µl)					
	6 weeks	6 months	1 year	Avg.	S.d.	R.S.D. (%)
ChargeSwitch® gDNA Plant Kit	21.09	16.02	9.10	15.40	6.02	39.07
DNA IQ™ System Kit	8.58	5.29	2.75	5.54	2.92	52.76
DNeasy® Blood & Tissue Kit	21.49	17.72	15.78	18.33	2.90	15.84
Phenol-chloroform-isoamyl alcohol	74.51	60.14	47.22	60.62	13.65	22.52
PrepFiler® BTA Forensic DNA Extraction Kit	11.77	8.06	3.04	7.62	4.38	57.47

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation.



Note: P1: 6 weeks, P2: 6 months, P3: 1year, CS.: ChargeSwitch, DN: DNA IQ, DNe: DNeasy, PC: Phenol, PF: Prepfiler.

Figure 5.11: Boxplots generated from the concentrations of DNA extracted from preserved degraded bone samples after 6 weeks, 6 months and 1 year using different extraction methods. Each boxplot represent 30 samples which were preserved in 5 different preservation methods.

5.5.5 Pairwise comparison between extraction methods

The multiple pairwise analyses using the Tukey test on extraction methods was carried out to compare each extraction method with others (Table 5.15).

Table 5.15: Table below shows the statistical data of the comparison between different extraction methods on 6 weeks, 6 months and 1 year preserved bone samples.

Comparison between extraction method	p adj					
	Fresh bone sample			Degraded bone sample		
	6 weeks	6 months	1 year	6 weeks	6 months	1 year
DNA IQ : ChargeSwitch	0.876	0.263	0.953	0.611	0.584	0.876
DNeasy : ChargeSwitch	0.999	0.999	0.730	0.999	0.999	0.854
Phenol : ChargeSwitch	**$2.70e^{-06}$**	**$1.00e^{-07}$**	**$2.80e^{-06}$**	**$1.00e^{-07}$**	**$1.00e^{-07}$**	**$6.00e^{-07}$**
Prepfiler : ChargeSwitch	0.877	0.693	0.965	0.825	0.812	0.893
DNeasy : DNA IQ	0.963	0.373	0.295	0.581	0.436	0.293
Phenol : DNA IQ	**0.000**	**0.000**	**$1.00e^{-07}$**	**0.000**	**0.000**	**0.000**
Prepfiler : DNA IQ	1.000	0.952	0.999	0.996	0.996	0.999
Phenol : DNeasy	**$7.00e^{-07}$**	**0.000**	**0.001**	**$1.00e^{-07}$**	**$4.00e^{-07}$**	**$5.42e^{-05}$**
Prepfiler : DNeasy	0.963	0.813	0.325	0.801	0.678	0.315
Prepfiler : Phenol	**0.000**	**0.000**	**$1.00e^{-07}$**	**0.000**	**0.000**	**0.000**

The results show that phenol-chloroform-isoamyl alcohol extraction method is far superior to other extraction methods, in terms of DNA yield, with all pairwise comparisons having p-value below 0.05. The results also show that there is no significant difference among ChargeSwitch® gDNA Plant Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit extraction methods with all the p-values among pairwise analyses being above 0.05.

5.5.6 DNA amplification and analysis on extracted preserved bone samples

All the extracted preserved bone samples were amplified using the multiplex (4-plex & IACs). 1 µl of each extracts was used for amplification.

The electropherograms obtained from the DNA extracts of preserved fresh bone samples showed that good quality DNA obtained from fresh bone samples preserved for 6 weeks (Figure 5.12) but the DNA degradation observed with the time of preservation increased. This can be confirmed as the DNA degradation since the peaks of 4-plex showed degradation pattern with more preservation time but no PCR inhibitors were indicated with both the IACs peaks being balanced in all the profiles developed (Figures 5.13 and 5.14). The electropherograms of the DNA extracts from preserved degraded bone samples showed a DNA degradation pattern at 6 weeks preservation and this pattern increased with the preservation time (Figures 5.15, 5.16 and 5.17) but no inhibition was observed.

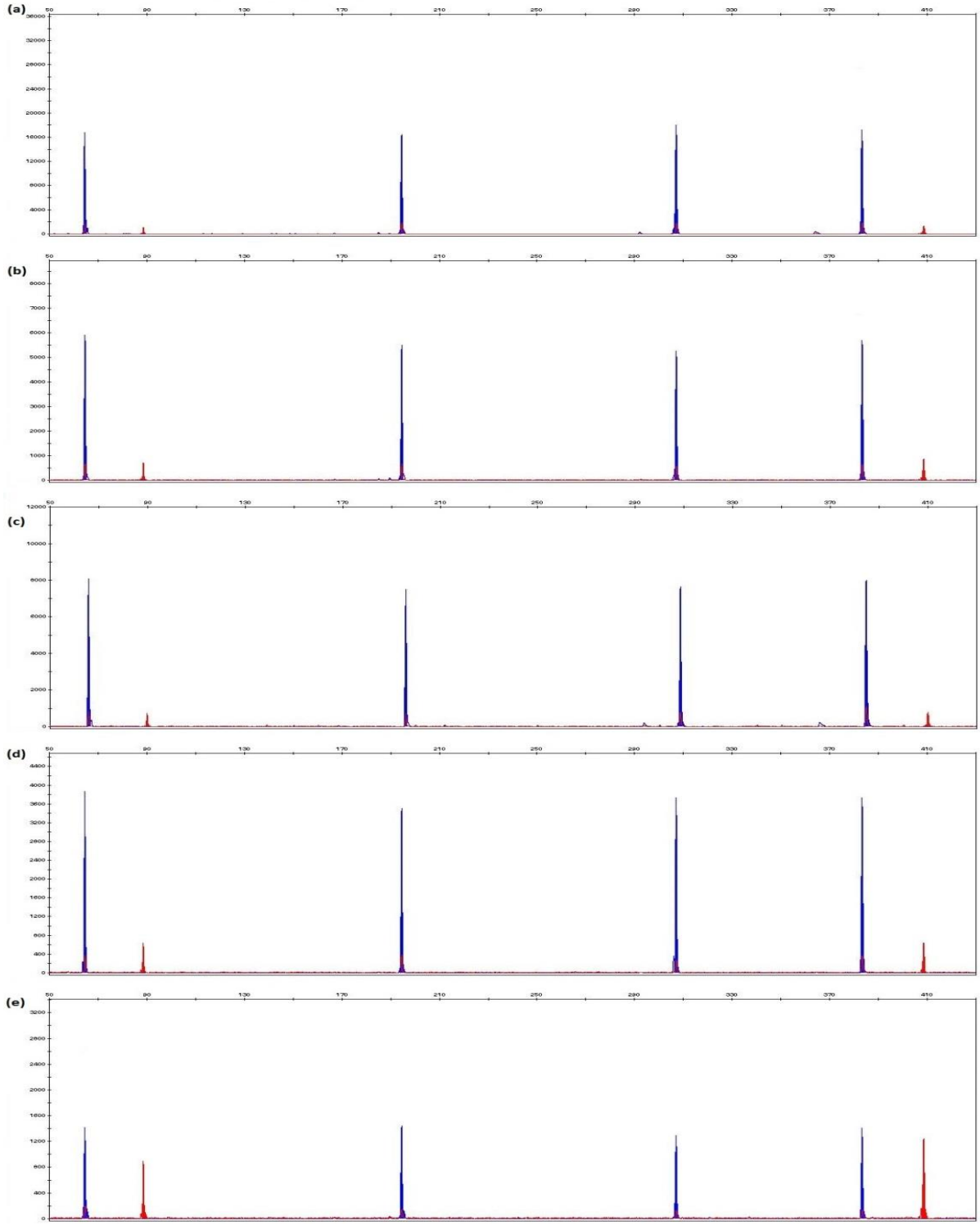


Figure 5.12: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 6 weeks using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

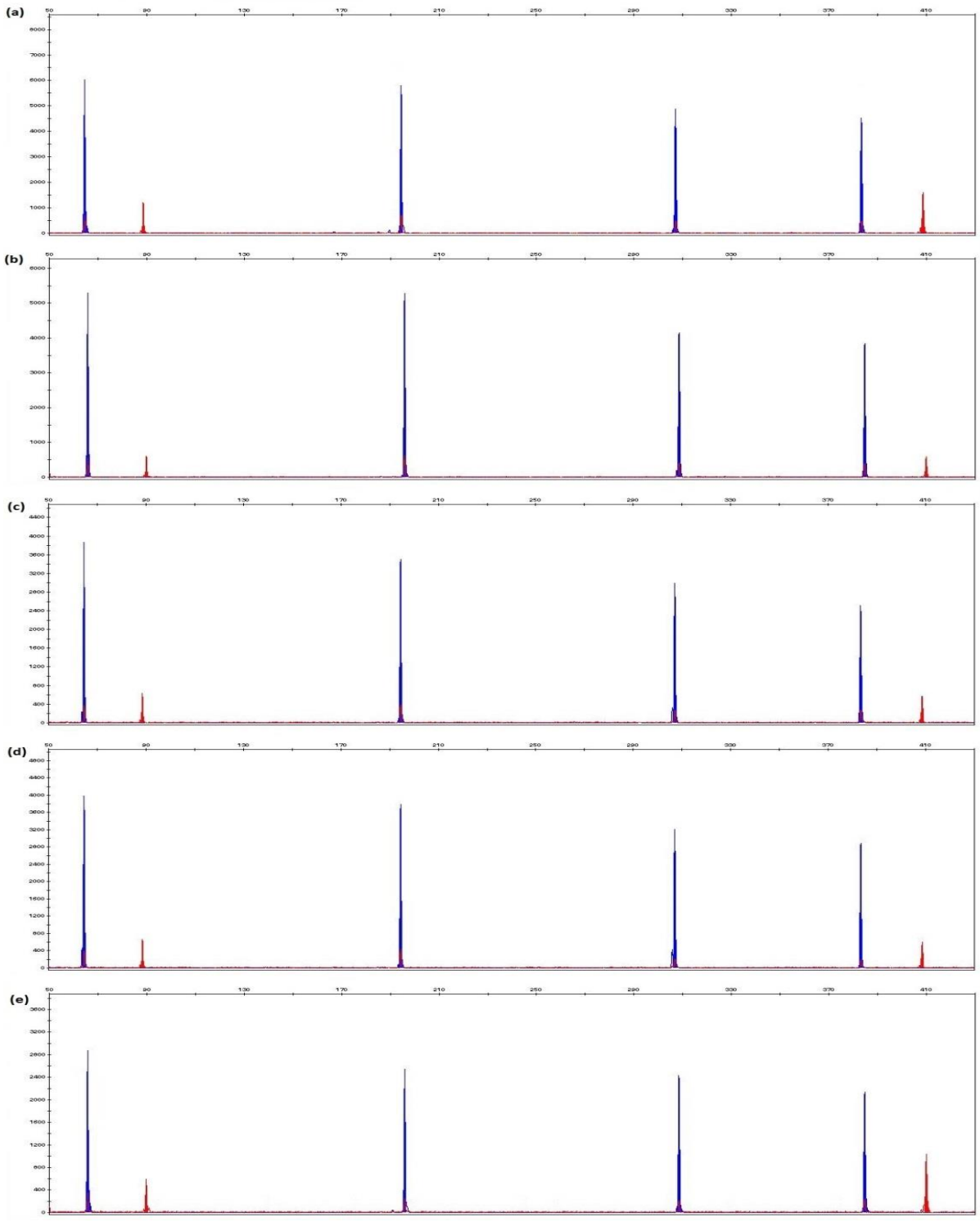


Figure 5.13: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 6 months using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

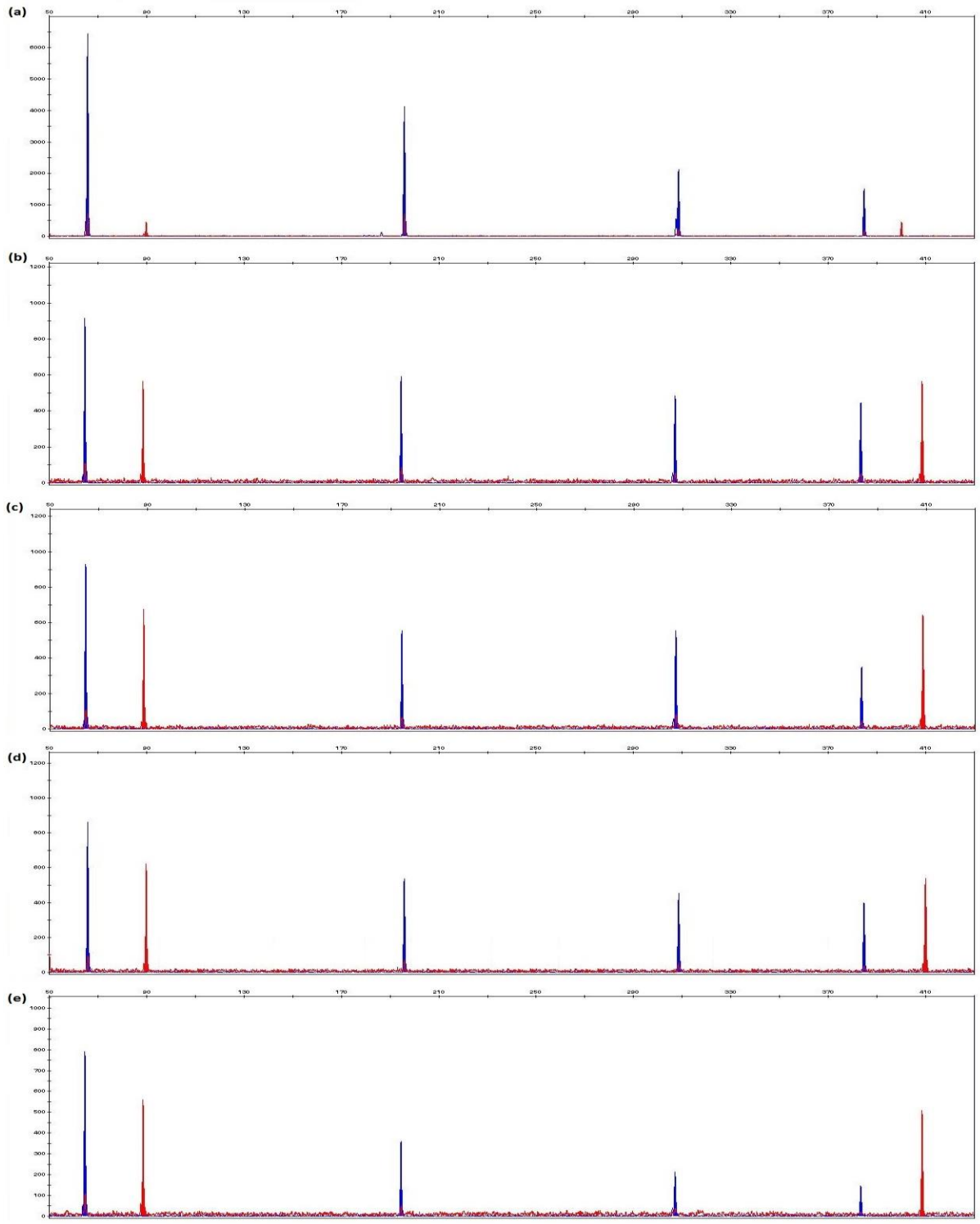


Figure 5.14: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 1 year using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

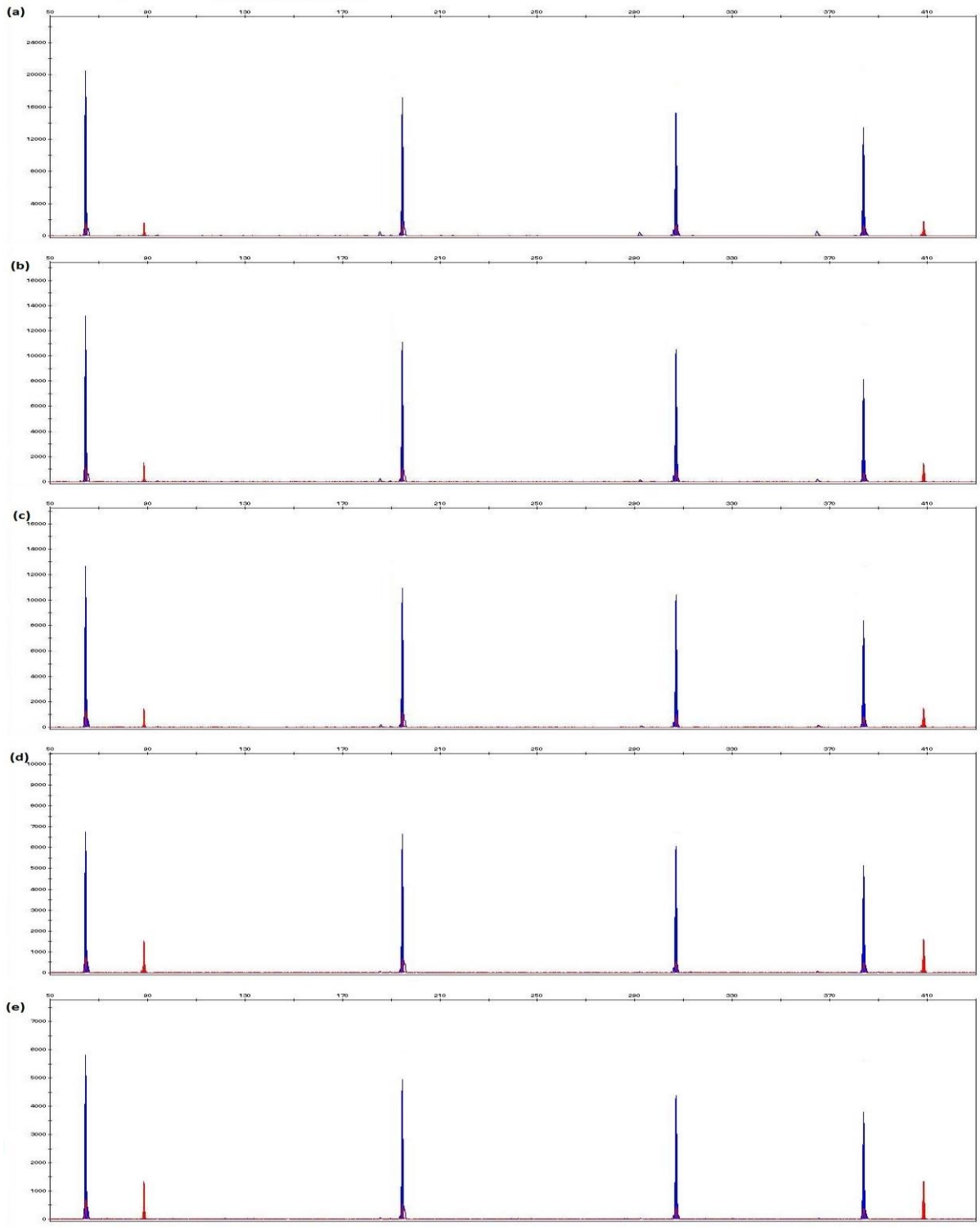


Figure 5.15: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 6 weeks using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

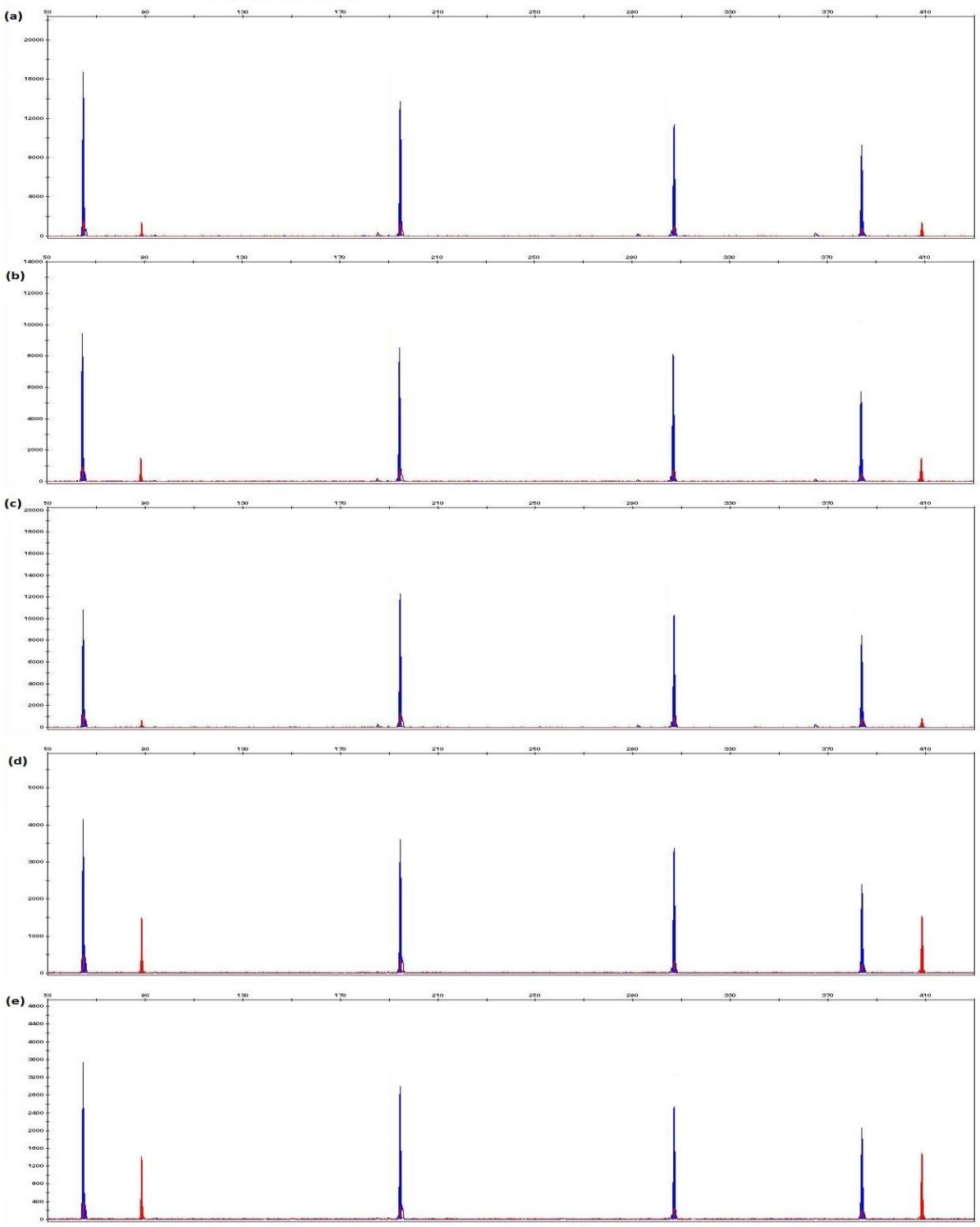


Figure 5.16: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 6 months using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

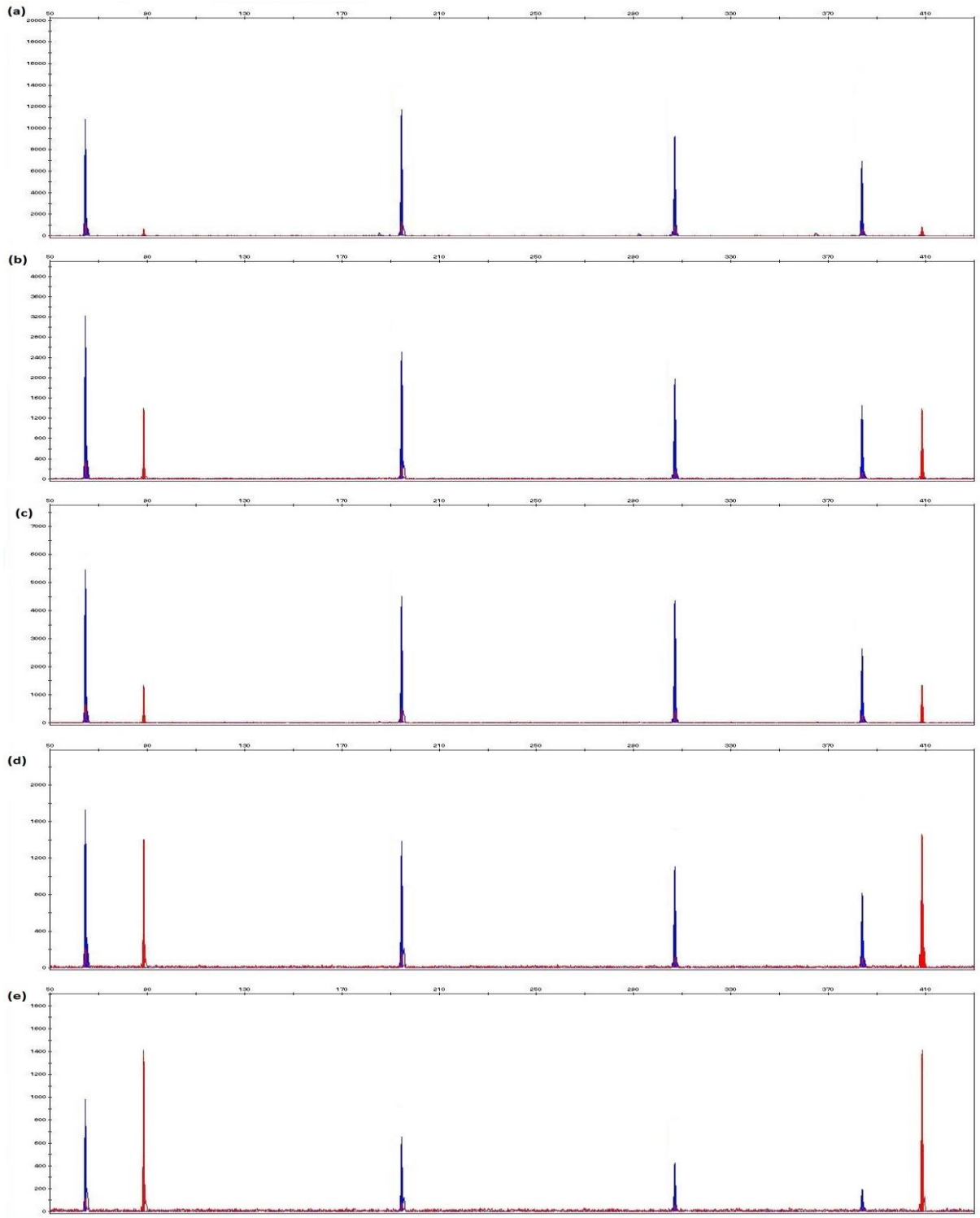


Figure 5.17: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 1 year using (a) phenol-chloroform-isoamyl alcohol, (b) ChargeSwitch® gDNA Plant Kit, (c) DNeasy® Blood & Tissue Kit, (d) PrepFiler® BTA Forensic DNA Extraction Kit and (e) DNA IQ™ System Kit extraction methods. The bone samples shown here were preserved by freezing and are indicative of all the other methods. 1 µl of each extract was used.

5.6 DISCUSSION

5.6.1 Decalcification of bone samples

Decalcification has been suggested as a step in the DNA extraction process since the DNA is tightly bound in dense crystalline and with decalcification, the DNA would be released (Huel et al. 2012). Even though there are studies showing good DNA recovery with decalcification (Jakubowska et al. 2012, Loreille et al. 2007), other studies have shown the reduction in the DNA concentration after decalcification (Pfeiffer et al. 1999, Fisher et al. 1993) and also that good DNA amounts can be extracted without decalcification (Parsons et al. 2007).

By increasing the time of incubation for decalcification, the quantity of DNA may increase but at the same time it can also damage the DNA and degrade the DNA by the time of analysis (Jakubowska et al. 2012, Rohland & Hofreiter 2007, Hagelberg & Clegg 1991). The decalcification process also adds more handling and pipetting steps which may cause contamination and also increase the extraction time.

The decalcification study on fresh bone samples prior to extraction showed that it is not a necessary step in the extraction process and the DNA yield is greater when the decalcification process is eliminated, presumably because free DNA is not being washed away. In addition, good quality electropherograms were produced from the extracted DNA samples without decalcification process, so in terms of this study decalcification was not determined to be a critical step.

5.6.2 Amicon 30kDa filters

The classic method of DNA purification and concentration in phenol-chloroform-isoamyl alcohol extraction is using alcohol precipitation (Gill et al. 1985). Using the alcohol precipitation process, the DNA is precipitated and formed into a pellet using centrifugation. Then, the solvent is removed and the pellet dried before being resuspended using TE buffer. This alcohol precipitation process is carried out to remove any residual phenol-chlorofrom-isoamyl alcohol which can inhibit PCR.

The cellulose filter has been used in place of precipitation to concentrate and purify the DNA from the aqueous extract of the phenol chloroform-isoamyl alcohol (Faber et al. 2013, Hudlow et al. 2011, Schiffner et al. 2005, Watanabe et al. 2003). Cellulose filters allow salts, phenol-chlorofrom-isoamyl alcohol, detergents and most of the proteins to pass through while retaining the DNA; the filter can be washed to remove any residual impurities that can inhibit the PCR.

The results obtained from comparison study between ethanol precipitation and Amicon 30kDa filter showed that ethanol precipitation gave higher DNA yields, but the use of Amicon 30kDa filters resulted in better DNA quality. This can be seen in the electropherograms produced from phenol-chlorofrom-isoamyl alcohol extractions using Amicon 30kDa filter (Figures 5.2). The use of Amicon 30kDa filter (Amicon ultra-0.5 centrifugal filter unit with ultracel-30 membrane) used in this study has been shown to give good results compared to Microcon YM-100 filter (Merck Millipore) when used on mock forensic samples prepared with small number of cells (Garvin & Fritsch 2013). It also reduced the sample processing time compare to ethanol precipitation technique. In this study the use of Amicon 30kDa filters (2 ml) was shown to be effective in pooling several digests of the same sample and helping to remove inhibitors; this removal of inhibitors has been previously reported (Noren et al. 2013).

5.6.3 Efficiency of extraction methods on bone samples

The evaluation of the extraction methods on fresh bone samples showed that all extraction methods are capable of extracting amplifiable DNA (Iyavoo et al. 2013). Phenol-chloroform-isoamyl alcohol extraction method consistently performed better than other extraction methods, followed by ChargeSwitch® gDNA Plant Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit. The same results were obtained when using degraded bone samples with the optimised technique.

Part 2 of this chapter, which looked at the effectiveness of the extraction methods using bone samples that had been preserved showed that the phenol-chloroform-isoamyl alcohol extraction method once again produced high and consistent DNA yield throughout the preservation period (6 weeks, 6 months and 1 year). Phenol-chloroform-isoamyl alcohol has been a proven extraction method on bone samples (Caputo et al. 2013, Kitayama et al. 2010, Gornik et al. 2002, Hochmeister et al. 1991). Also, phenol-chloroform-isoamyl alcohol extraction method produced higher DNA yields than the silica-based extraction methods. Although phenol-chloroform-isoamyl alcohol method produces high DNA yield, previous studies show that silica-based extraction methods perform better at removing PCR inhibitors from bone samples (Gupta et al. 2013, Davoren et al. 2007b). However, this problem was not faced in this study, where no PCR inhibition was detected. However, the silica-based methods do have the advantage of not needing to use such hazardous chemicals (Wang et al. 2011).

Among the silica-based extraction methods, DNeasy® Blood & Tissue Kit extraction method produced the highest DNA yields. The DNeasy® Blood & Tissue Kit was consistent in DNA yield at each preservation point like phenol-chloroform-isoamyl alcohol. This kit has been successfully used on both animal and human bone extraction (Rijks et al. 2011, Burjanivova et al. 2010). DNeasy® Blood & Tissue Kit uses a silica column while the other silica-based extraction methods use silica resins. This gives an advantage for DNeasy® Blood & Tissue Kit extraction method as the column captures the DNA and the cleaning processes are carried out by centrifugal technique while for silica resins, a magnetic stand

is used and the cleaning processes are carried out using pipetting, providing more points when the DNA can be lost. While using pipetting technique for cleaning processes, extra care has to be taken so that no disturbance happens to the bonding of DNA and silica resins by any contact with pipette tip. Any disturbance to the bonding may cause DNA loss and this mistake can be avoided in cleaning processes using centrifugal techniques. However, for automated extraction platforms, silica resin extraction methods are preferred (Chiou et al. 2013, Dundas et al. 2008, Greenspoon et al. 2004).

ChargeSwitch® gDNA Plant Kit extraction method showed marginally better DNA yield from preserved fresh bone samples at 6 weeks and 6 months preservation time compared to DNeasy® Blood & Tissue Kit and both extraction methods performed similarly in terms of DNA yield on preserved degraded bone samples at same preservation periods (Tables 5.14 and 5.15). But the efficiency of this extraction method declines dramatically at 1 year preservation point compared to DNeasy® Blood & Tissue Kit extraction method. All silica resin based extraction methods showed same pattern of DNA recovery as ChargeSwitch® gDNA Plant Kit extraction method. As the preservation time increases, more fragmented DNA will be produced because of the DNA degradation. As described in the previous paragraph, silica resin based extraction methods use pipetting for washing steps, thus providing more points for fragmented DNA lost. Vigorous pipetting technique also could cause in more DNA lost unlike the DNeasy® Blood & Tissue Kit extraction method which uses centrifugal techniques for washing steps with medium speed (6,000 x g) thus might have prevented DNA lost. Even though there is no publication on bone extraction using ChargeSwitch® gDNA Plant Kit, a similar extraction method: ChargeSwitch® Forensic DNA Purification Kit (Life Technologies™) has been used on human bone and teeth samples (Barbaro et al. 2008). The results of that study concluded that the ChargeSwitch® Forensic DNA Purification Kit extraction method greatly improves the ability to positively identify skeletal remains and can be used routinely since it is fast and avoids the use of toxic reagents. ChargeSwitch® gDNA Plant Kit was used in this thesis project since most of the samples were bones and they have complex cell structure as plant cells.

The PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit extraction methods produced similar DNA yields compared to other silica-based methods. However, DNA yields of both extraction methods dramatically declined with each preservation point. PrepFiler® BTA Forensic DNA Extraction Kit has been used on human bone extraction (Debska et al. 2013). A comparison study between QIAamp® DNA Investigator Kit (Qiagen) which is similar to DNeasy® Blood & Tissue Kit (Qiagen) and PrepFiler® Forensic DNA Extraction Kit (Life Technologies™) which is similar to PrepFiler® BTA Forensic DNA Extraction Kit has been carried out by Ludwikowska-Pawlowska et al. (2009). The results showed that better results were obtained from QIAamp® DNA Investigator Kit compare to PrepFiler® Forensic DNA Extraction Kit. This is similar with the results obtained in this chapter which showed that the DNeasy® Blood & Tissue Kit is better than the PrepFiler® BTA Forensic DNA Extraction Kit in terms of DNA yield and consistency.

The DNA IQ™ System also has been used on the human bone samples. Greenspoon et al. (2004) used the DNA IQ™ System on bone samples using an automated extraction platform and obtained DNA extracts without contamination. While Ye et al. (2014) used the DNA IQ™ System as an additional method for phenol-chloroform-isoamyl alcohol extraction on old and burned bones. The results obtained were compared with the QIAquick® PCR Purification Kit (Qiagen) which was also used as an additional method for phenol-chloroform-isoamyl alcohol extraction. The results showed that both methods are useful to obtain high quality DNA templates from aged bones but the DNA IQ™ System method is easier and faster to implement.

5.6.4 Conclusion

Overall, phenol-chloroform-isoamyl alcohol extraction method performed the best DNA yields on both preserved fresh and degraded bone samples, followed by DNeasy® Blood & Tissue Kit, ChargeSwitch® gDNA Plant Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit.

However, the performance of these extraction methods decreased more rapidly on preserved fresh bone samples compared to degraded bone samples as the time of preservation increases. This is likely to be caused by soft cancellous bone in fresh bone samples degrading faster than the cortical bone thus reducing the DNA yield in the preserved fresh bone samples tremendously compared to skeletonised bone samples which were used in degraded bone samples preservation. Previous studies have found that biological material degrade rapidly in soft tissue than in bone due to the more resilient structure of bone which acts as a physical barrier (Imaizumi et al. 2004, Ye et al. 2004, Grawet al. 2000). Also studies on mass disaster bone samples indicate that more intact elements, as well as elements encased in soft tissue, produced slightly higher identification rates than more fragmented remains (Mundorff et al. 2009).

Current DNA profiling on bone samples is preferred to be carried out using dense cortical bones such as femur (Ambers et al. 2014, Vanek et al. 2009, Kapinska & Szczerkowska 2008, Davoren et al. 2007a, Prinz et al. 2007). However, Mundoff and Davoren (2014) found that small cancellous bones on average had much higher amounts of DNA per unit mass than dense cortical bones. This can be clearly observed in the phenol-chloroform-isoamyl alcohol extraction method where DNA extraction from rib bone samples gave higher DNA yields compare to femur bone samples. But it was not very significant in the silica-based extraction methods.

CHAPTER 6

DNA PRESERVATION OF BONE SAMPLES

6.1 OVERVIEW

Preservation of the biological evidences plays paramount importance especially in cases with limited DNA from materials such as hairs, bones, teeth and other degraded samples. Successful bone preservation will enable forensic and medical personnel to confidently store bone until it is analysed. Also, in the case of mass disasters, the identification of human remains may take a longer time, thus preservation of those remains to prevent the DNA degradation is very important.

In this chapter, the effectiveness of five preservation methods: cell lysis solution (with 1% sodium azide), dehydration / freeze drying, ethanol (96%), freezing and room temperature storage, on bone samples which would substantially improve the ability to obtain amplifiable DNA were studied. These preserved bone samples were extracted using these five optimised extraction methods as described in the previous Chapter.

This builds on a previous study looking at the preservation of DNA in muscle tissue where ethanol (96%) and cell lysis solution (with 1% sodium azide) worked well (Nazir 2012). Additional methods such as freezing, dehydration / freeze drying and room temperature storage were studied on bone preservation.

6.1.1 Objectives

- Evaluate cell lysis solution (with 1% sodium azide), dehydration/freeze drying, ethanol (96%), freezing and room temperature storage for their capability for preserving DNA in bone samples.
- Evaluate the above preservation methods for short (6 weeks), intermediate (6 months) and longer (1 year) preservation time.

6.2 RESULTS

A total of 180 bone pieces (90 fresh and 90 degraded) which were preserved using cell lysis solution (with 1% sodium azide), dehydration / freeze drying, ethanol (96%), freezing and room temperature storage were tested. Femur and rib bones were preserved; they were either fresh or had been exposed to the environment for 3 months. The details of methods and materials used for bone preservations are as described in Chapter 2 (Section 2.4)

These preserved bone samples were extracted using optimised extraction methods after 6 weeks, 6 months and 1 year of preservation respectively. The final volume was standardized at 100 µl for comparison. The details of methods and materials used for each extraction method are as described in Chapter 2 (Section 2.6). The pre-process technique was used for degraded bone samples as described in Chapter 2 (Section 2.9).

After the extraction, quantification was carried out on all extracted samples using GoTaq® qPCR Master Mix quantification method.

These are the same samples which were extracted and used for extraction methods comparison study in Chapter 5 (Part 2: DNA extraction from preserved bone samples). In this chapter the statistical assessments were only carried out on the preservation methods to evaluate their capability to preserve DNA from the bone samples. The extraction methods were not taken into consideration.

6.2.1 Comparison using ANOVA

ANOVA followed by the Tukey test was carried out to identify any significant difference when same preservation method was applied to different bone types (femur and rib) (Table 6.1).

Table 6.1: Table below shows the statistical data of the comparison between different bone types which were preserved in the same preservation method.

Bone type with same preservation comparison	p adj					
	Fresh bone			Degraded bone		
	6 weeks	6 months	1 year	6 weeks	6 months	1 year
Rib – Cell lysis : Femur – Cell lysis	1.000	1.000	1.000	1.000	1.000	1.000
Rib – Dehydration : Femur – Dehydration	0.173	0.139	0.855	0.234	0.377	0.509
Rib – Ethanol : Femur – Ethanol	**0.015**	0.121	0.066	**0.027**	**0.021**	0.097
Rib – Freezing : Femur – Freezing	0.956	0.671	0.218	0.856	0.484	0.283
Rib – Room temp : Femur – Room temp	0.999	0.980	1.000	0.999	0.998	1.000

The result shows that there is no significant different between bone types when the same preservation method was applied except in the case of ethanol (96%) preservation method with p-value below 0.05 for 6 weeks fresh bone samples and 6 weeks and 6 months degraded bone samples. Further statistical calculations on the preservation methods were carried out together on both bone types using the extracted DNA concentrations.

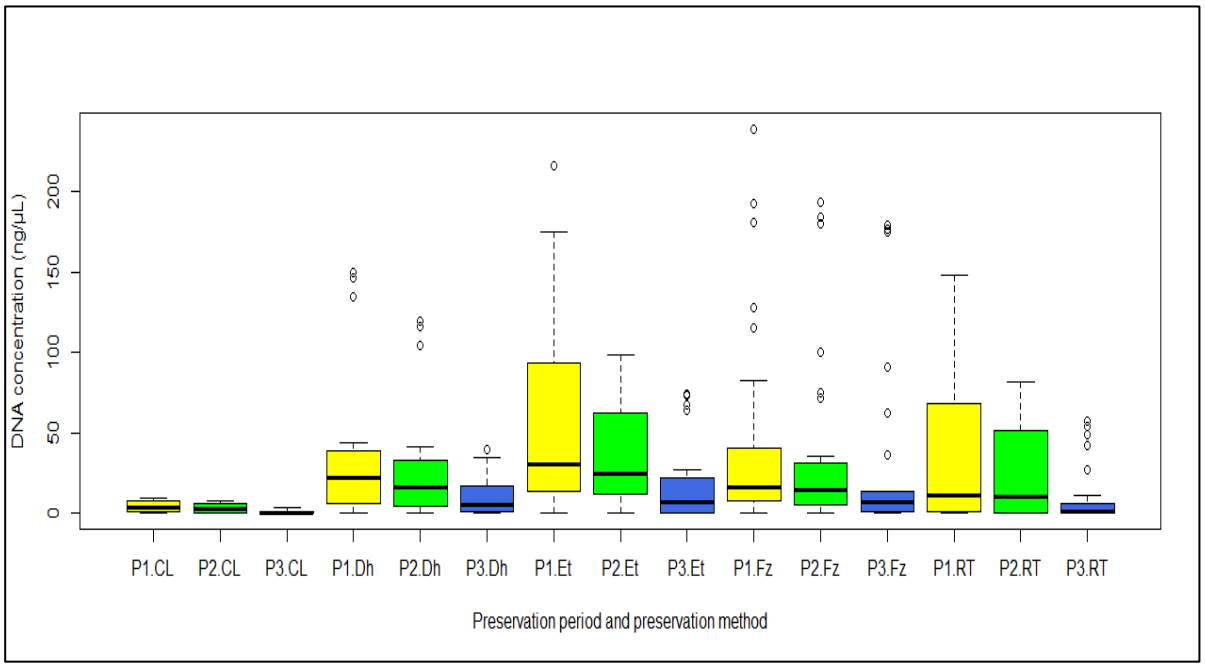
6.2.2 Preservation method comparison for fresh bone samples

The mean DNA concentration for each preservation method was calculated without considering the bone type, since there no significant difference between bones type when same preservation method was applied, other than with ethanol (96%). The results are as shown in Table 6.2 and Figure 6.1.

Table 6.2: Table below shows the mean DNA concentrations extracted from preserved fresh bone samples after 6 weeks, 6 months and 1 year on different preservation methods. Each mean value represents 30 samples which were extracted using 5 different extraction methods.

Preservation method	Mean DNA concentration (ng/µL)					
	6 weeks	6 months	1 year	Avg.	S.d.	R.S.D. (%)
Cell lysis solution (with 1% sodium azide)	3.91	3.03	0.88	2.61	1.56	59.80
Dehydration	31.75	25.27	10.75	22.59	10.75	47.60
Ethanol (96%)	51.30	35.37	18.70	35.12	16.30	46.41
Freezing	42.94	36.07	28.54	35.85	7.20	20.09
Room temperature	30.93	22.77	9.24	20.98	10.96	52.22

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation



Note: P1: 6 weeks, P2: 6 months, P3: 1year, CL.: Cell lysis solution, Dh: Dehydration, Et: Ethanol, Fz: Freezing, RT: Room temperature.

Figure 6.1: Boxplots generated from the concentrations of DNA extracted after 6 weeks, 6 months and 1 year preserved fresh bone samples. Each boxplot represent 30 samples which were extracted using 5 different extraction methods.

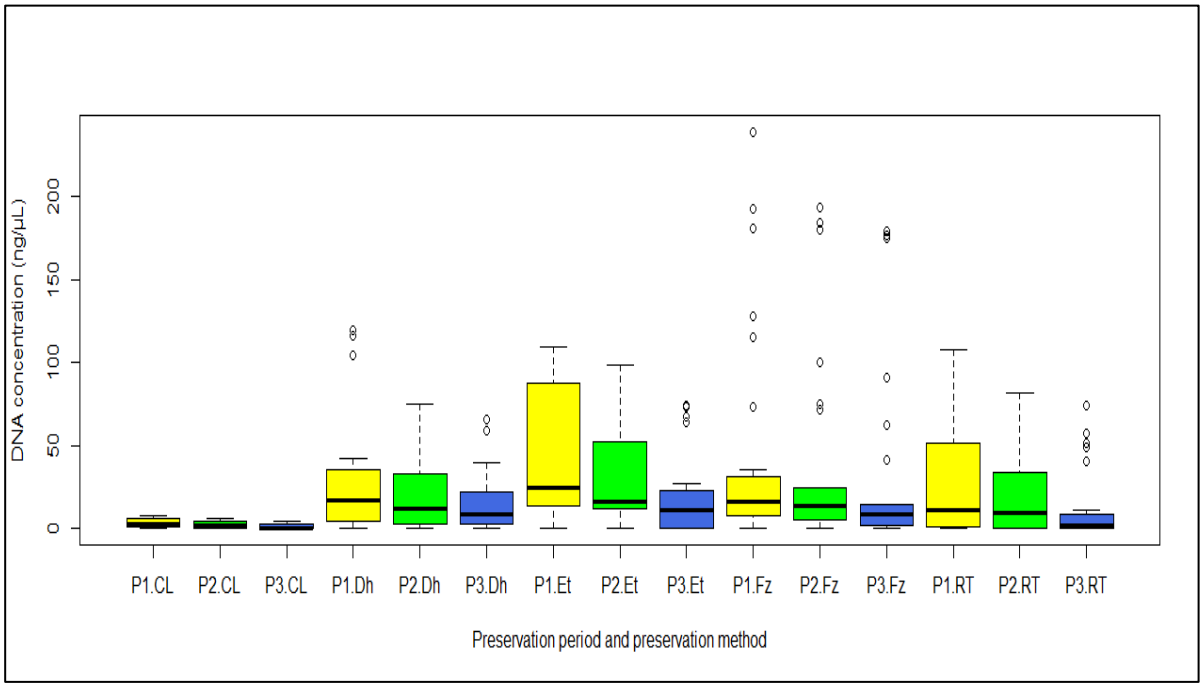
6.2.3 Preservation methods comparison for degraded bone samples

As with the fresh bone preservation methods comparison, the mean DNA concentration for each preservation method used to preserve the degraded bone samples was calculated without considering the bone type. The results are as shown in Table 6.3 and Figure 6.2.

Table 6.3: Table below shows the mean DNA concentrations extracted from preserved degraded bone samples after 6 weeks, 6 months and 1 year on different preservation methods. Each mean value represent 30 samples which were preserved in 5 different preservation methods.

Preservation method	Mean DNA concentration (ng/µL)					
	6 weeks	6 months	1 year	Avg.	S.d.	R.S.D. (%)
Cell lysis solution (with 1% sodium azide)	3.17	2.21	1.30	2.23	0.94	42.00
Dehydration	26.39	19.51	14.70	20.20	5.88	29.09
Ethanol (96%)	41.54	31.69	21.05	31.43	10.25	32.61
Freezing	40.85	34.83	29.50	35.06	5.68	16.20
Room temperature	25.49	18.98	11.35	18.61	7.08	38.04

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation



Note: P1: 6 weeks, P2: 6 months, P3: 1year, CL.: Cell lysis solution, Dh: Dehydration, Et: Ethanol, Fz: Freezing, RT: Room temperature.

Figure 6.2: Boxplot generated from the concentrations of DNA extracted after 6 weeks, 6 months and 1 year preserved degraded bone samples. Each boxplot represent 30 samples which were extracted using 5 different extraction methods.

6.2.4 Pairwise comparison between preservation methods

The multiple pairwise analyses using the Tukey test on extraction methods was carried out to compare each preservation method with others (Table 6.4).

Table 6.4: Table below shows the statistical data of the comparison between different preservation methods on 6 weeks, 6 months and 1 year preserved bone samples.

Comparison between preservation methods	p adj					
	Fresh bone sample			Degraded bone sample		
	6 weeks	6 months	1 year	6 weeks	6 months	1 year
Dehydration : Cell lysis solution	0.123	0.994	0.669	0.155	0.250	0.393
Ethanol : Cell lysis solution	**0.001**	**0.004**	0.117	**0.002**	**0.006**	0.073
Freezing : Cell lysis solution	**0.009**	**0.003**	**0.002**	**0.003**	**0.002**	**0.002**
Room temperature : Cell lysis solution	0.144	0.183	0.789	0.187	0.279	0.673
Ethanol : Dehydration	0.450	0.790	0.818	0.570	0.602	0.918
Freezing : Dehydration	0.872	0.747	0.119	0.614	0.371	0.291
Room temperature : Dehydration	0.999	0.999	0.999	0.999	0.999	0.992
Freezing : Ethanol	0.952	0.999	0.672	0.999	0.996	0.796
Room temperature : Ethanol	0.407	0.623	0.703	0.513	0.562	0.702
Room temperature : Freezing	0.840	0.573	0.073	0.557	0.336	0.120

The results show that there is a significant difference between cell lysis solution (with 1% sodium azide) preservation method with the freezing preservation method at all preservation periods in both fresh and degraded bone samples. A significant difference was also observed between cell lysis solution (with 1% sodium azide) preservation method with the ethanol (96%) preservation method at 6 weeks and 6 months preservation periods but not at 1 year preservation in both fresh and degraded bone samples. Other than that, no significant differences were found between other preservation methods.

6.2.5 DNA amplification and analysis on preserved fresh bone samples extracts

All the extracted preserved bone samples were amplified using the multiplex (4-plex & IACs). 1 µl of each extract was used for amplification.

Comparison of same preservation method with different preservation periods showed that DNA extracts of 6 months preserved fresh bone samples from freezing preservation method have less DNA degradation. This can be observed as both the electropherograms of 6 weeks and 6 months look similar (Figure 6.3). Cell lysis solution (with 1% sodium azide) extracts produced electropherograms with low peak height, concordant with their DNA concentration. Other than that, DNA degradation was higher in the bone samples preserved with this method. This can be seen as the 384 bp amplicon was not amplified in the 1 year preserved bone samples (Figure 6.4).

The electropherogms produced from the DNA extracts of preserved fresh bone samples also showed that good quality DNA profiles were obtained from 6 weeks preservation (Figure 6.5) but DNA degradation occurred as the time of preservation increases. This can be observed as the peaks of 4-plex show degradation pattern with more preservation time but no PCR inhibitors indicated with both the IAC peaks balanced in all the profiles (Figures 6.6 and 6.7).

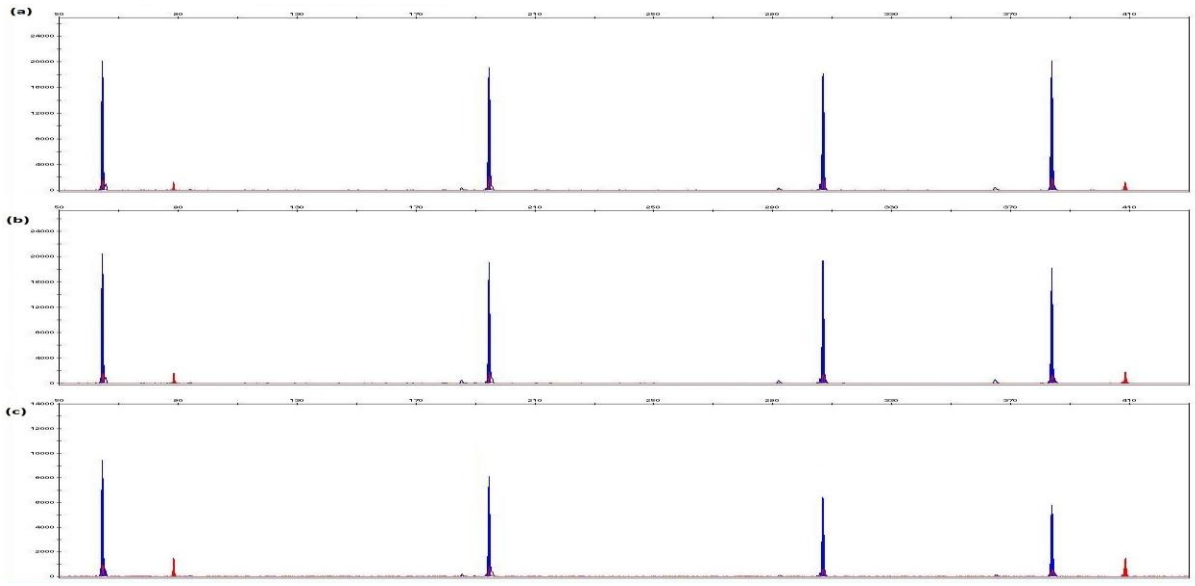


Figure 6.3: Showing summary of results generated from DNA extracts of fresh rib bone samples preserved using freezing method for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

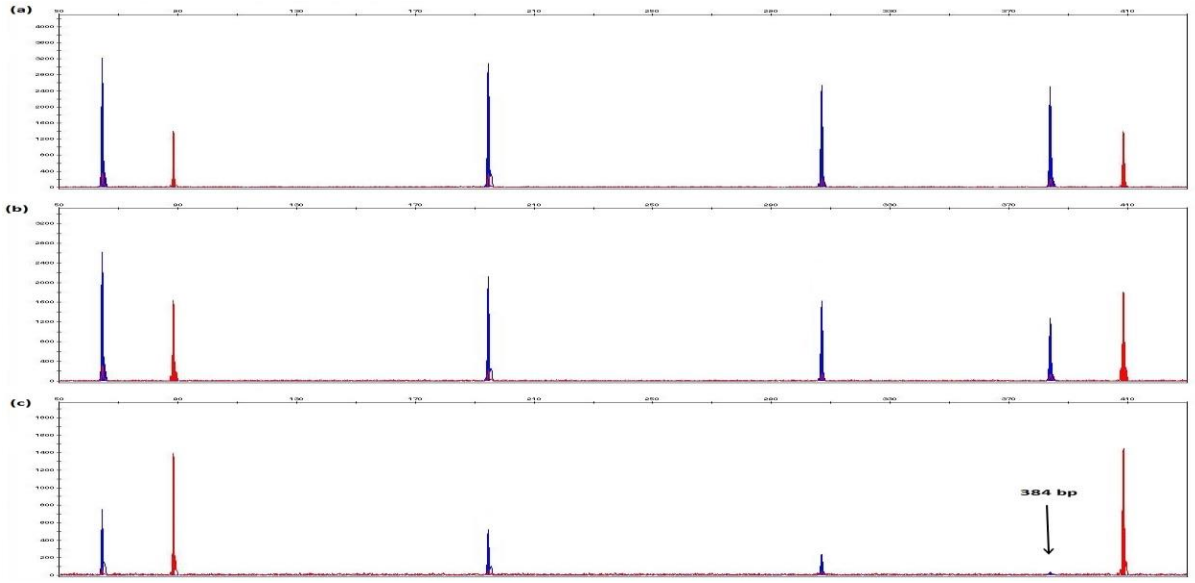


Figure 6.4: Showing summary of results generated from DNA extracts of fresh rib bone samples preserved using cell lysis solution (with 1% sodium azide) for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

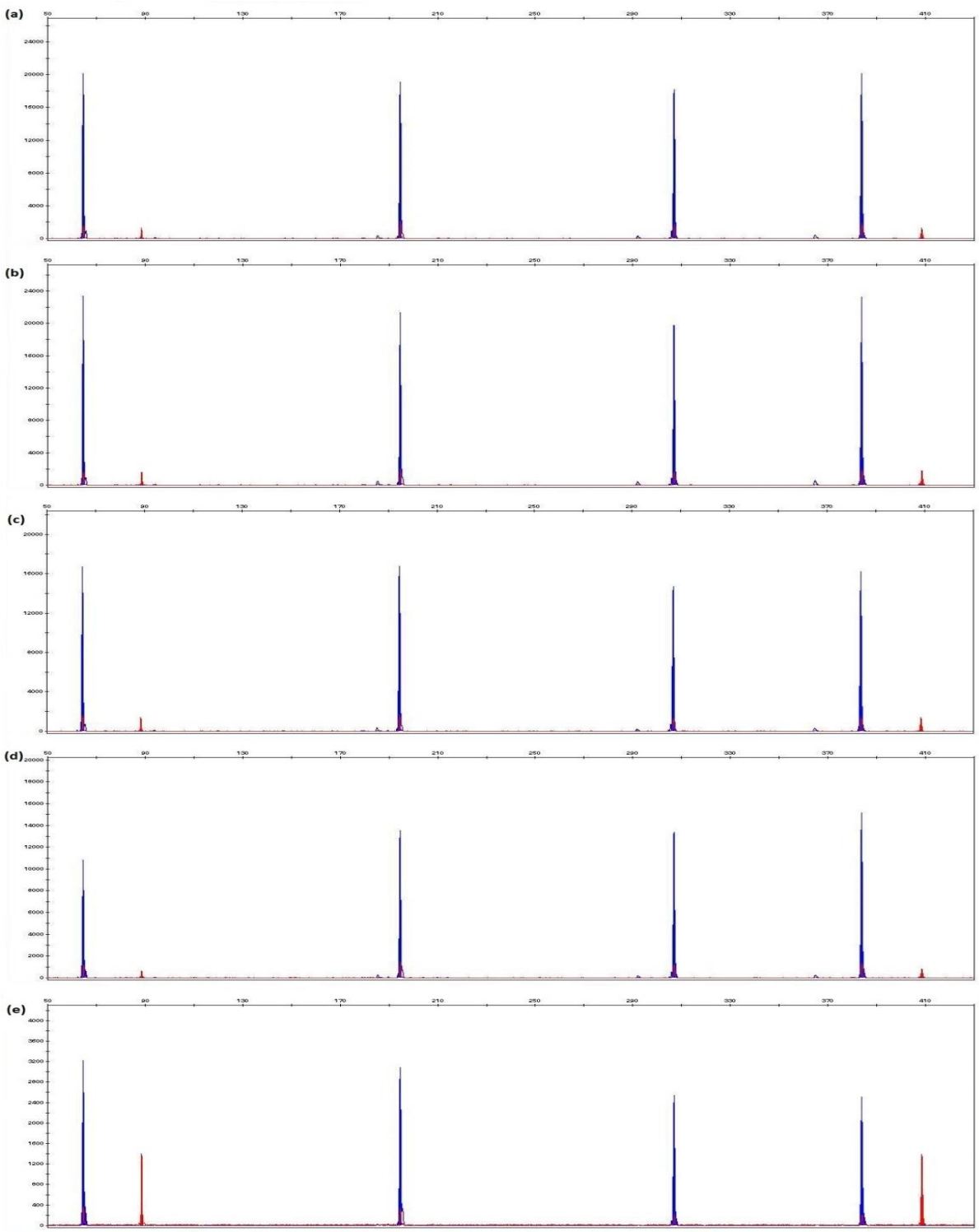


Figure 6.5: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 6 weeks using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

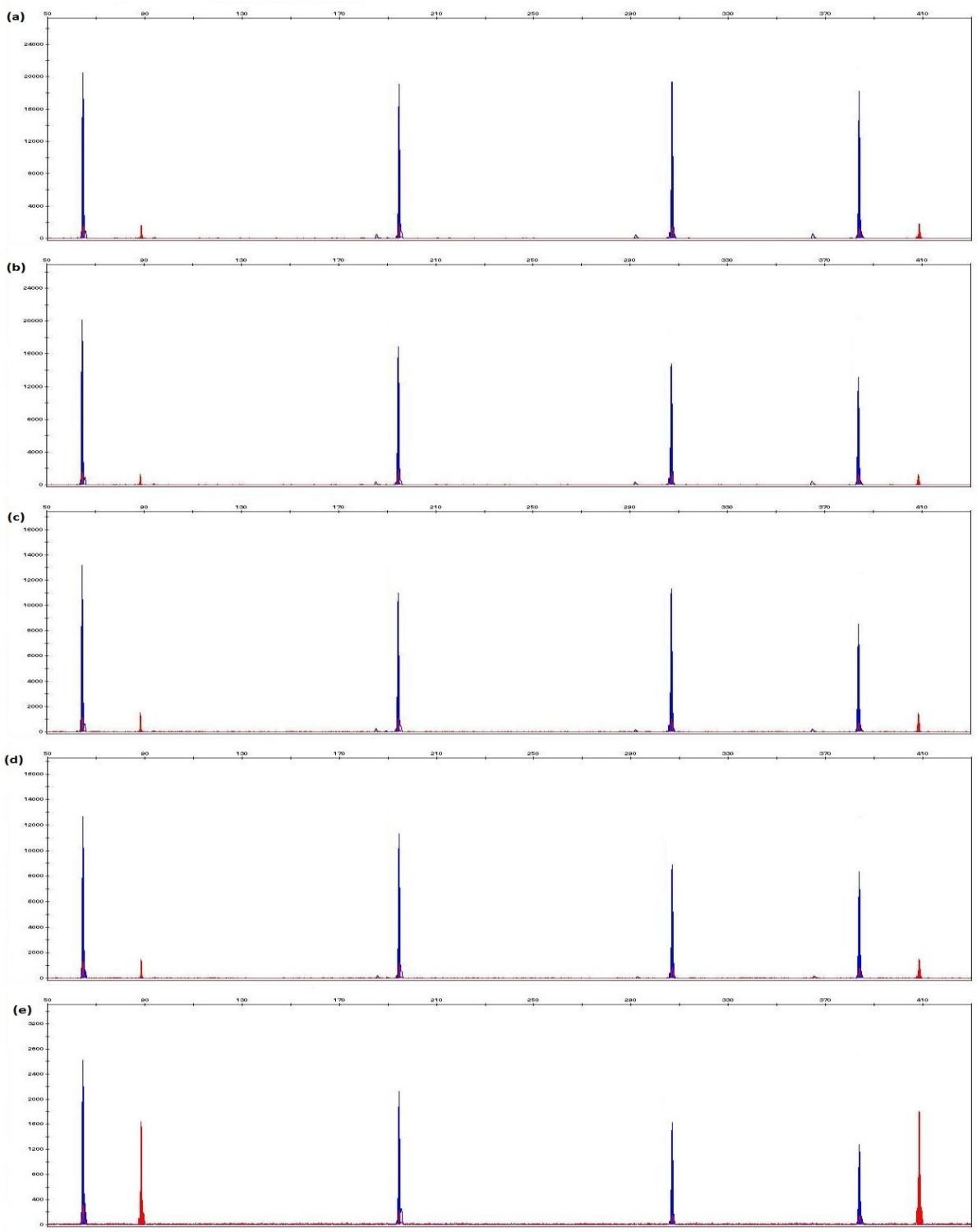


Figure 6.6: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 6 months using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

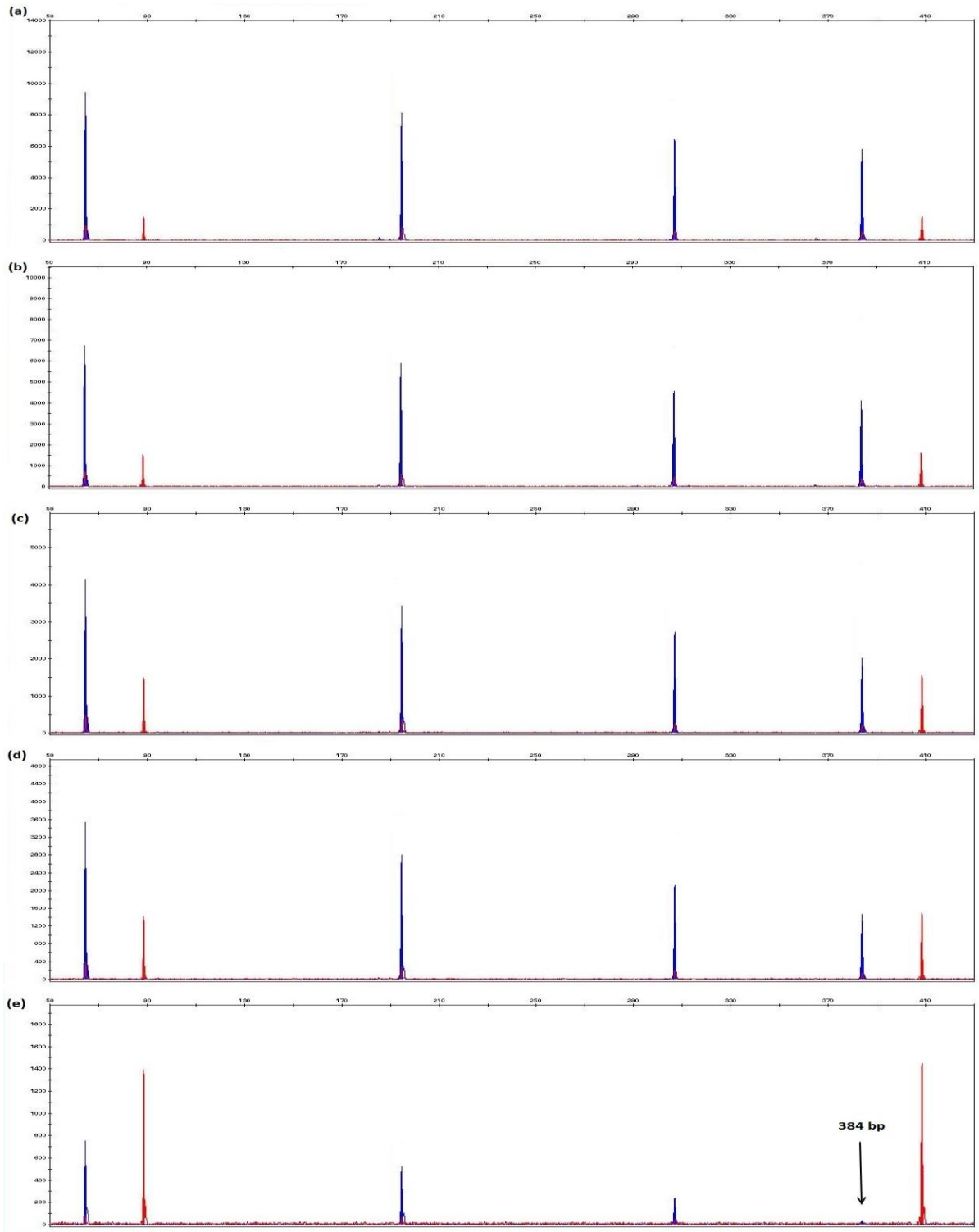


Figure 6.7: Examples of electropherograms generated from DNA extracts of fresh rib bone samples preserved for 1 year using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

6.2.6 DNA amplification and analysis on preserved degraded bone samples extracts

All the extracted preserved bone samples were amplified using the multiplex (4-plex & IACs). 1 µl of each extracts was used for amplification.

The pattern of preservation is very similar to that seen with fresh bone samples (Figure 6.8). Cell lysis solution (with 1% sodium azide) extracts also produced electrophrograms with no 384 bp amplicon amplified at the 1 year preserved bone samples (Figure 6.9) showing that DNA degradation was higher in the bone samples preserved with this method.

The electropherograms produced from DNA extracts of preserved degraded bone samples also showed DNA degradation pattern at 6 weeks preservation and this pattern increased with the preservation time (Figures 6.10, 6.11 and 6.12).

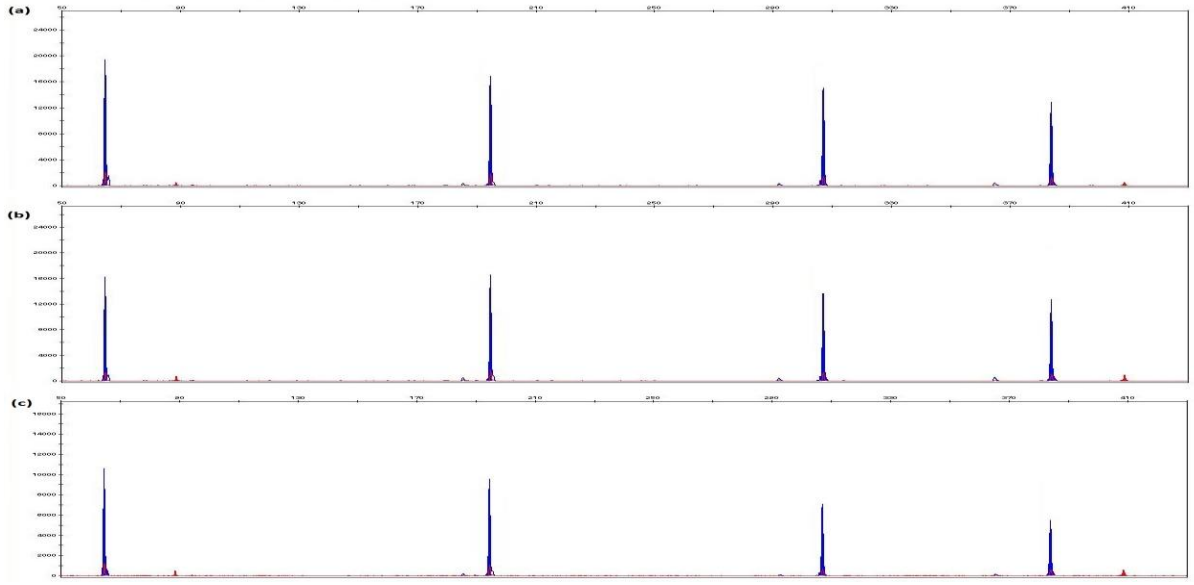


Figure 6.8: Showing summary of results generated from DNA extracts of degraded rib bone samples preserved using freezing method for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

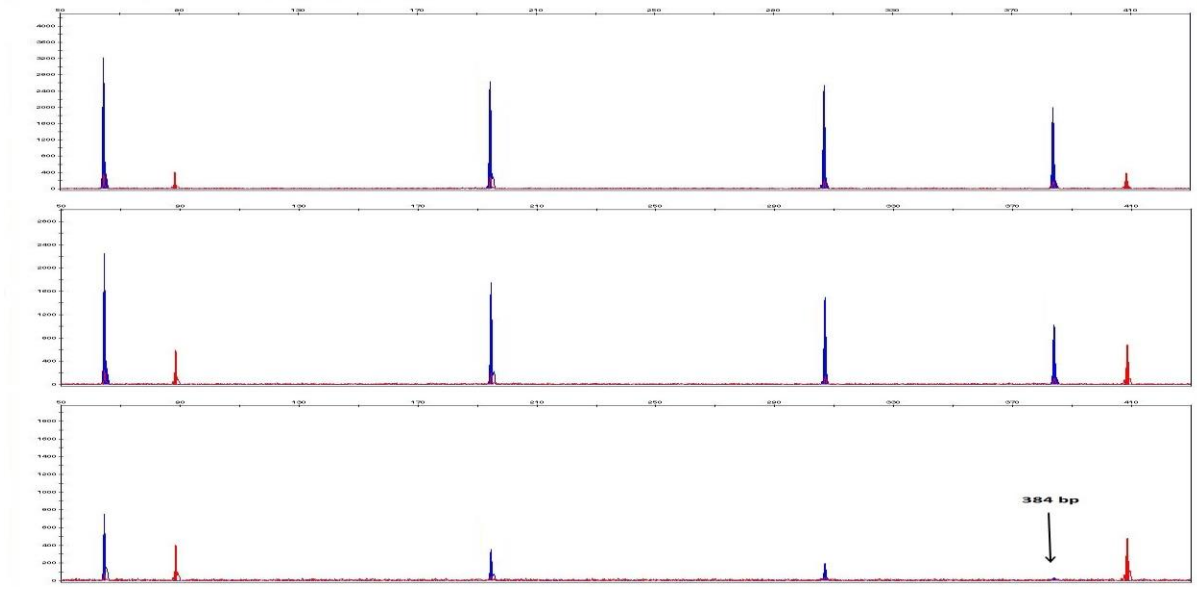


Figure 6.9: Showing summary of results generated from DNA extracts of degraded rib bone samples preserved using cell lysis solution (with 1% sodium azide) for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

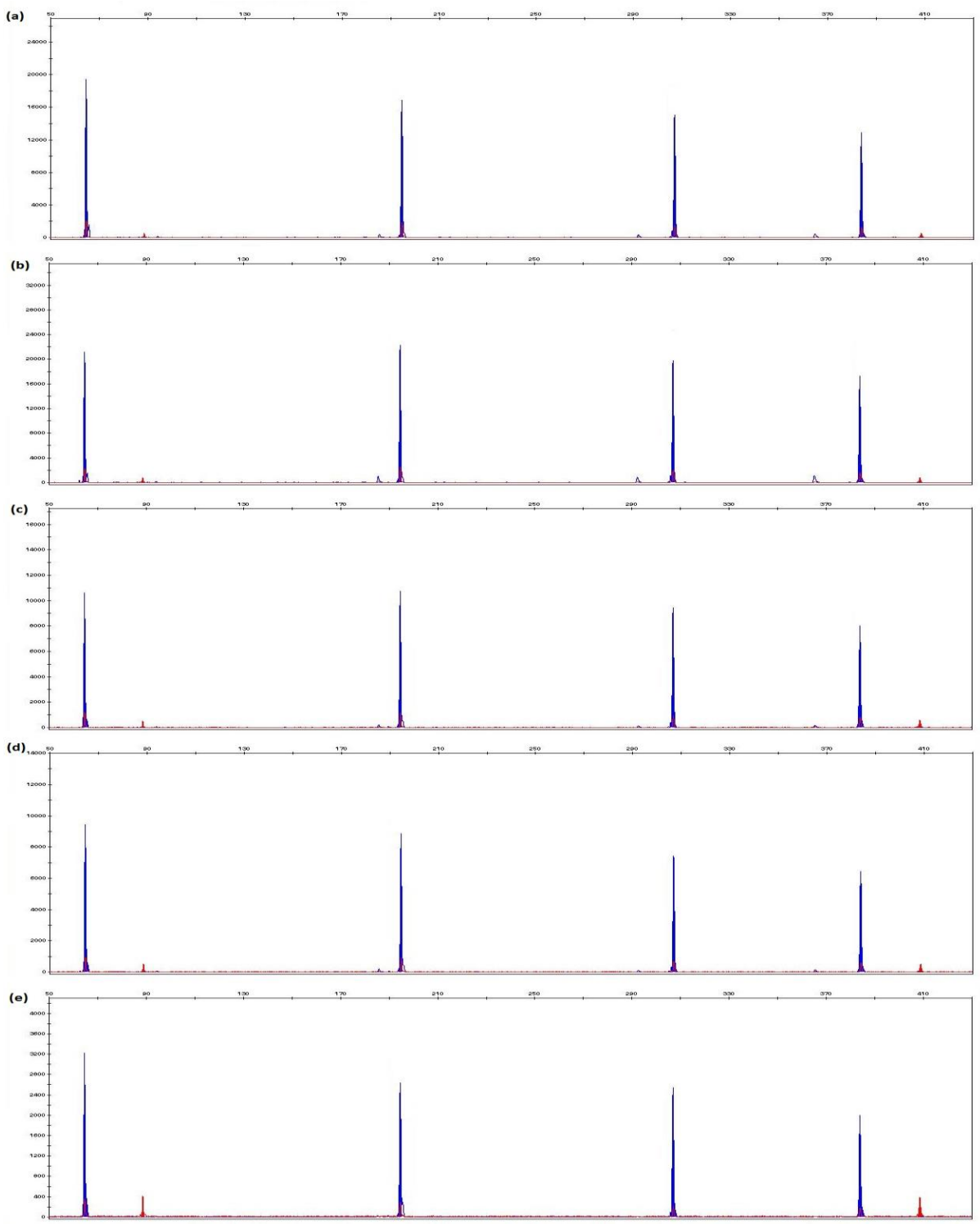


Figure 6.10: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 6 weeks using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

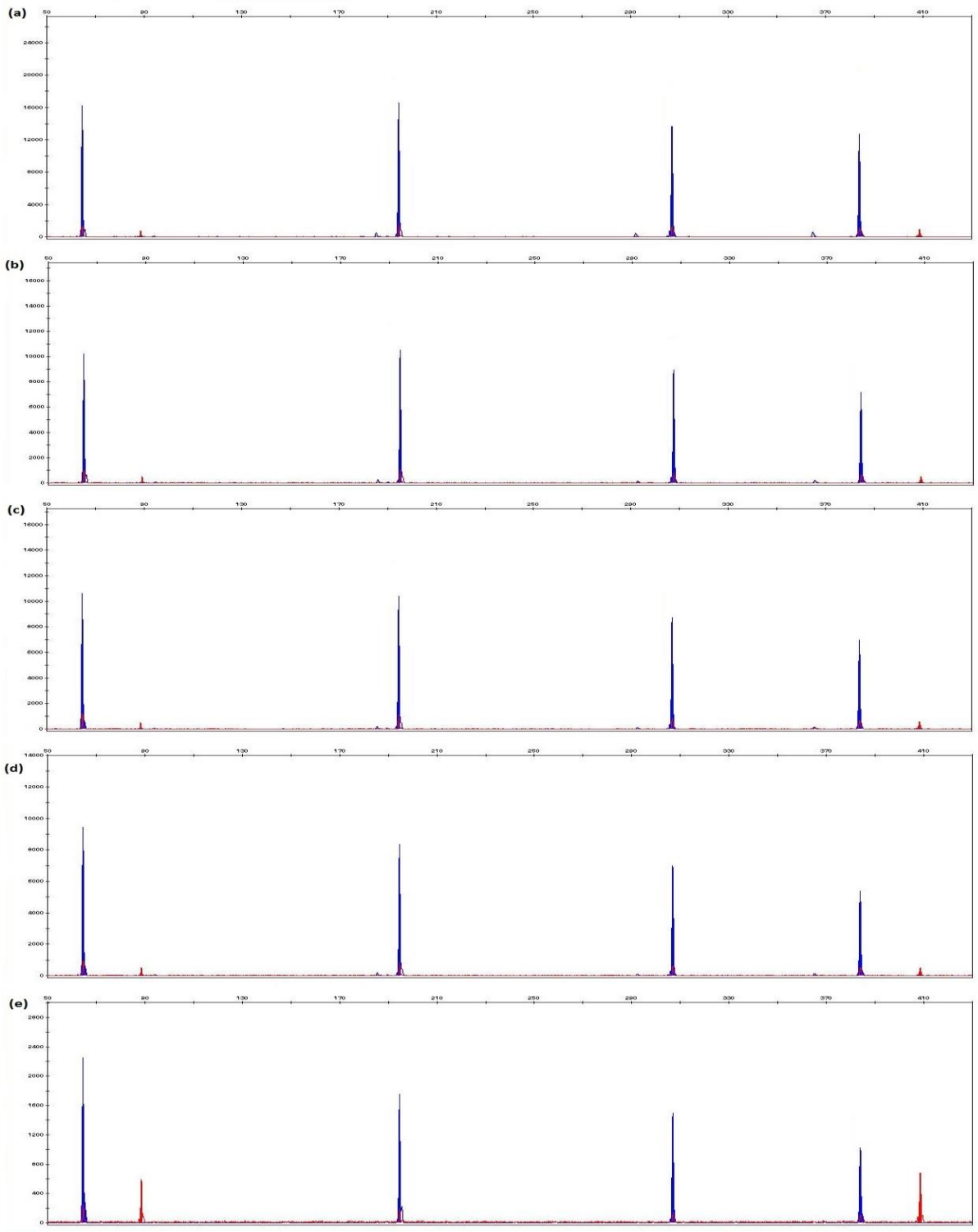


Figure 6.11: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 6 months using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

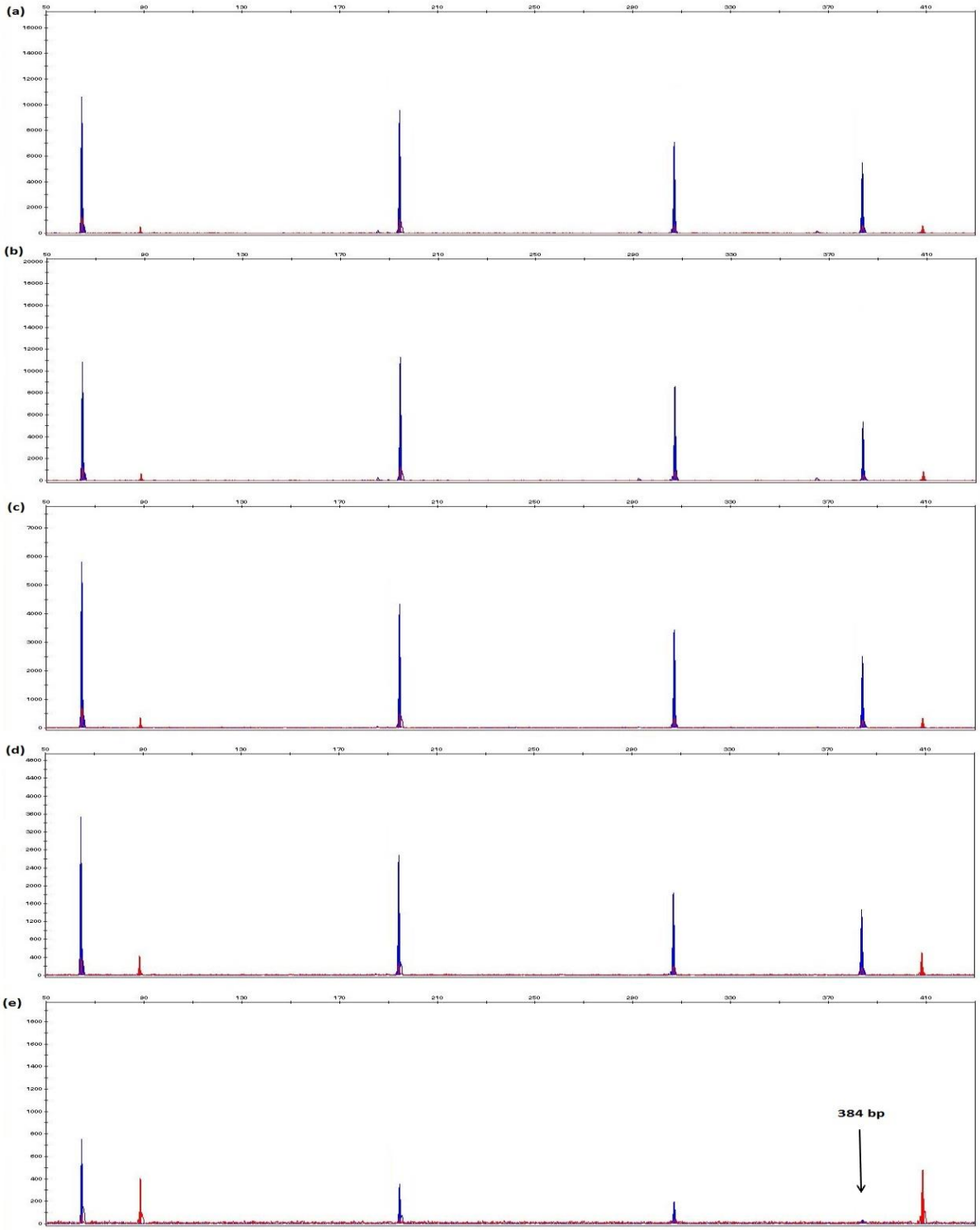


Figure 6.12: Examples of electropherograms generated from DNA extracts of degraded rib bone samples preserved for 1 year using (a) freezing, (b) ethanol (96%), (c) dehydration, (d) room temperature and (e) cell lysis solution (with 1% sodium azide) preservation methods. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

6.2.7 Analysis on lysate of cell lysis solution (with 1% sodium azide)

6.2.7.1 DNA extraction

Since the results obtained from DNA extraction of bone samples preserved in cell lysis solution (with 1% sodium azide) were not satisfactory, the lysates were extracted using phenol-chloroform-isoamyl alcohol extraction method. The additional pre-process technique using Amicon 30kDa filter (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration, Merck Millipore) was carried out on the lysates. 400 µl of each lysate were processed in accordance with the phenol-chloroform method. The final extraction volume was standardized at 100 µl for comparison study.

After the extraction, quantification was carried out on all extracted samples using GoTaq® qPCR Master Mix quantification method. The mean DNA concentration for each lysate was calculated based on the preserved bone types and preservation periods. The results are as shown in Table 6.5.

Table 6.5: Table below shows the mean DNA concentrations extracted from lysate of cell lysis solution after 6 weeks, 6 months and 1 year preservation fresh and degraded bone samples.

Lysate type	Mean DNA concentration (ng/µL)					
	6 weeks	6 months	1 year	Avg.	S.d.	R.S.D. (%)
Fresh bone samples preserved lysate	5.55	3.86	2.26	3.89	1.65	42.29
Degraded bone samples preserved lysate	1.92	1.14	1.03	1.36	0.49	35.59

Note: Avg.: Average, S.d.: Standard deviation, R.S.D.: Relative Standard Deviation

The results obtained show that more DNA was extracted from lysates of preserved fresh bone samples compare to the preserved fresh bone samples itself (please refer to Table 6.2). This can be observed at all preservation periods (6 weeks, 6 months and 1 year). However, the DNA extracted from lysates of preserved degraded bone samples were

below the DNA yields of preserved degraded bone samples itself at all preservation periods (please refer to Table 6.3).

6.2.7.2 DNA amplification and analysis on extracted lysate

All the extracted lysate samples were amplified using the multiplex (4-plex & IACs). 1 µl of each extracts was used for amplification.

The electropherograms produced from the extracted DNA samples show that degradation occured in the lysates of the cell lysis solution (with 1% soium azide) at all preservation periods in both fresh and degraded bone samples (Figures 6.13 and 6.14). However, the degradation is very obvious at 1 year preservation period with the drop-out of 384 bp amplicons in both the fresh and degraded bone samples preservation. No inhibitors were found since both the IACs peaks are present and also balanced.

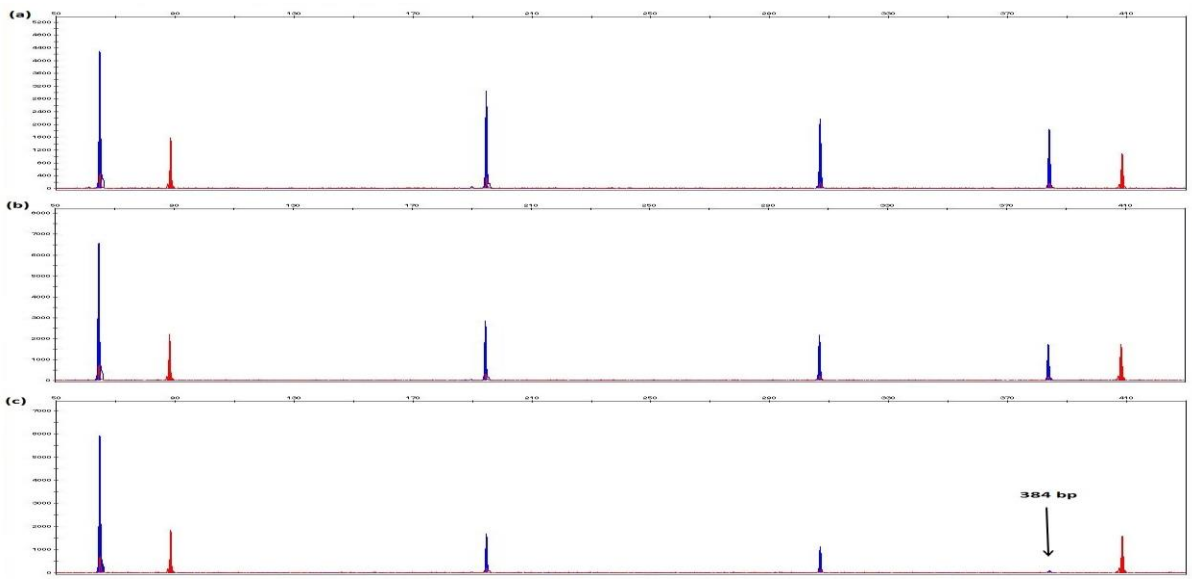


Figure 6.13: Examples of electropherograms generated using DNA from lysate of cell lysis solution (with 1% sodium azide) which was used for fresh rib bone samples preservation for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

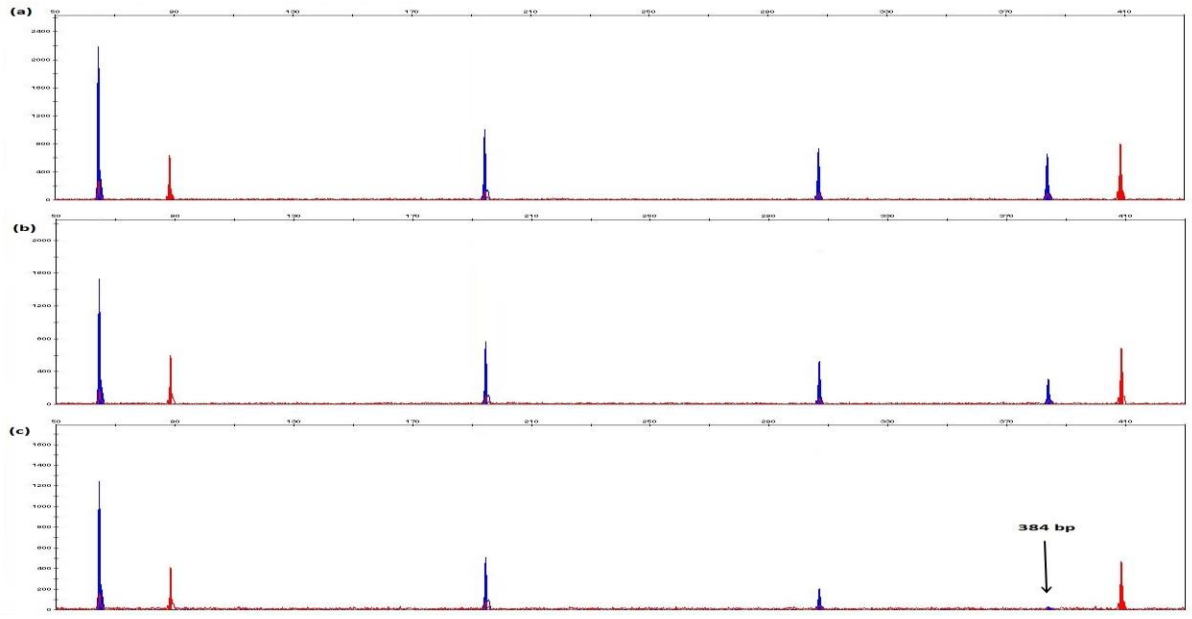


Figure 6.14: Examples of electropherograms generated using DNA from lysate of cell lysis solution (with 1% sodium azide) which was used for degraded rib bone samples preservation for (a) 6 weeks, (b) 6 months, and (c) 1 year. The bone samples were extracted using phenol-chlororom-isoamyl alcohol extraction method. 1 µl of each extract was used.

6.3 DISCUSSION

Five preservation techniques were examined; cell lysis solution (with 1% sodium azide), dehydration / freeze drying, ethanol (96%), freezing and room temperature storage. In this research, these preservation methods were tested for their efficiency for storage of fresh and degraded bone samples for 6 weeks, 6 months and 1 year. DNA extraction was carried out using 50 mg of each pulverized bone samples for preserved fresh bone samples and total of 250 mg for preserved degraded bone samples together with Amicon 30kDa filters (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration, Merck Millipore) after digestion. Extractions were carried out in triplicate and the final elution volume was set at 100 µl in each extraction for a comparable study. The extracts were quantified using GoTaq® qPCR Master Mix (Promega) quantification method and the DNA concentrations were used for this comparison study.

Most of the previous preservation studies have been done on tissues compared to bones for subsequent DNA analyses (Allen-Hall & McNevin 2012, Michaud & Foran 2011, Nagy 2010). But when human remains are degraded or damaged, bones and teeth are the only remaining DNA sources. Thus, identifications have been done using DNA from bones and teeth on bodies from mass graves of war and armed conflicts (Gojanovic & Sutlovic 2007, Andelinovic et al. 2005). Freezing is seen as the best method for the preservation of biological material (Nagy 2010); however, following mass disasters and in post-conflict environments electricity supply may not be reliable and it may be advantageous to use some form of preservative method on the bone samples.

The results obtained in this chapter support the concept that, freezing is the best method for the bone preservation prior to DNA analysis. Even though ethanol (96%) preservation method performs marginally better than freezing on shorter preservation time (6 weeks), the freezing preservation method is the best for longer preservation (1 year). In this study

freezing was at -20 ^{o}C, thus highly specialised equipment is not required. Another advantage of freezing is that no other substances need to be added to the samples. However, at this temperature, there is still enzymatic activity and degradation. Therefore freezing alone is not suitable for long-term storage (Todorova et al. 2012) and ultra-low temperature freezing has been recommended for longer storage (Nagy 2010) until DNA profiling could be carried out.

Ethanol (96%) has also been widely used for preserving tissue samples (Nagy 2010, Nietfeldt & Ballinger 1989). Ethanol works as preservative by removing water from the sample and denaturing proteins and nucleases (Flournoy et al. 1996, Seutin et al. 1991) and also inhibits the cellular enzymes (Penna et al. 2001). Ethanol also acts as antimicrobial agent by protecting the biological samples against bacterial degradation (Seutin et al. 1991). This antimicrobial advantage of ethanol has the added benefit of 'sterilising' the samples, which may be important when considering shipments. Ethanol (96%) was the best preservation method for short-term storage (6 weeks) but its performance dropped slightly with long-term storage (6 months and 1 year) compared to freezing preservation method which is more consistent throughout the preservation periods. Potentially this situation could be alleviated by changing the ethanol for continuous freshness and effect of the ethanol or by adding more ethanol to be at actual level since ethanol easily evaporates (Nagy 2010). A similar result was reported by Michaud and Foran (2011) where they studied several preservation methods on pig tissues for 6 months and they found that alcohol was not suitable for long-term storage compare to other preservation methods, although work by Nazir (2012), demonstrated that 96% ethanol worked effectively on muscle tissue preserved for a period of one year at room temperature. This could be because the ethanol absorbed into the muscle tissue and preserved them from inside while in the bone preservation, the bone's solid structure could have prevented the ethanol from penetrating into the bones, thus DNA degradation still occurred in the soft cancellous bone in the preserved bone samples. Also only 96% ethanol were tested in this study, however Michaud and Foran (2011) found that the

percentage of alcohol tested (40%, 70% and 90%) did not show any noticeable different in DNA preservation.

Dehydration / freeze-drying has been recommended to replace standard freezing, which is costly and also subject to mechanical failure of the freezer (Anchordoquy & Molina 2007). Dehydration is based on the assumption that DNA is more stable when dry. By freeze-drying, the moisture in the biological samples is frozen by reducing the surrounding pressure and then sublimated directly from solid phase to gas phase, thus drying them for storage. However, degradation still occurs during storage of the dehydrated samples (Anchordoquy & Molina 2007). From the results obtained in this chapter, dehydration preservation methods did not perform as well as standard freezing and 96% ethanol, but better that room temperature and cell lysis solution (with 1% sodium azide). Like the ethanol (96%) preservation method, its performance also declines with the length of the preservation.

Room temperature storage has been preferred as the easiest way to store the biological samples. Room temperature storage of bone samples have showed successful DNA profiling on exhumed bone (Caputo et al. 2013) and it has been suggested that in some circumstances freezing is not possible.

Other variations on room temperature storage have also been described. Geovani et al. (2006) studied the storage of bone samples at room temperature by adding 98% glycerol solution and found no bacterial and fungal growth. Lee et al. (2012) showed that room temperature storage of biological samples using DNA storage medium, SampleMatrix™ (Biomatrica®, US). The medium allows for dry storage and also stabilizes the DNA during room temperature storage and the results showed a 2 to 10-fold higher DNA recovery compared to freezing. However, the room temperature preservation study in this chapter was carried out without any solution or medium. The results showed medium DNA recovery was obtained at short-term storage but the DNA yield dropped significantly

for long-term storage compare to freezing, ethanol (96%) and dehydration preservation methods. These results showed that without any solution or medium, the degradation of DNA in room temperature stored bone samples is high. This pattern was observed in both fresh and degraded preserved bone samples. A previous study also showed that brief storage of the bone samples at room temperature does not affect the amount of amplifiable DNA but long-term storage does reduces the DNA and reproducibility of the DNA amplification (Burger et al. 1999).

Cell lysis solution readily permeates tissues, lyses the cell and binds the divalent cations which are require for nuclease activity (Caputo et al. 2011, Graham et al. 2008). Sodium azide was added in an attempt to inhibit any bacterial growth (Sadiq 1995). Results from the study in this chapter showed that bone samples preserved in cell lysis solution (with 1% sodium azide) showed very low DNA yield compared to other preservation methods. The recovery of DNA in preserved fresh bone samples was low compared to preserved degraded bone samples. This could be because the soft cancellous bone in fresh bone samples degrading faster than the cortical bone thus reducing the DNA yield in the preserved fresh bone samples tremendously compared to skeletonised bone samples which were used in degraded bone samples preservation. Since the DNA is released into the cell lysis solution (Abolmaaty et al. 2000), the lysate also was extracted using phenol-chloroform-isoamyl alcohol extraction method with the additional pre-process technique using Amicon 30kDa filter (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration) to test to see if DNA was present in them. The results showed more DNA present in the lysate of preserved fresh bone samples compared to preserved degraded bone samples. This finding supports the above statement where fresh bones contain more soft tissues / soft cancellous bone than the degraded samples, thus the recovery of DNA from fresh bone samples decreased with the length of the preservation since more cells from soft tissue / soft cancellous bone lysed and the DNA is released into the lysate. In addition, the cell lysis solution also resulted in DNA degradation compared to other preservation methods. This can be seen from the electropherograms produced where both the bone samples and the lysates produced degradation pattern with the increase of

preservation time. Cell lysis which lyse the cells and release the DNA into the solution also capture the capture the divalent cations which are required for the nuclease activity (Caputo et al. 2011, Graham et al. 2008). However, if not adequate amount of cell lysis buffer used, this could result in DNA degradation since more free divalent cations available for the nuclease activity; this situation most likely happened in this bone preservation study since bone samples contained high amount of DNA. By adding chelating agents like EDTA which bind to metal ions such as Mg^{2+} and Ca^{2+}, which are required for normal function of nuclease activity on DNA, nuclease activity also can be reduced (Giannakis et al. 1991, Seutin et al. 1991).

Overall, freezing was found to be the best preservation method for both fresh and degraded bone samples for long-term storage. This is followed by ethanol (96%), dehydration / freeze drying and room temperature preservation methods where good DNA yields observed up to 6 months preservation but dropped at 1 year preservation. Ethanol (96%) was found to be the best preservation method for short-term storage (6 weeks). However, full profiles still obtained from both fresh and degraded bone samples from all these preservation methods.

Cell lysis solution (with 1% sodium azide) preservation method tended to be good for short-term storage but with the long-term preservation, less DNA yield was obtained and also the electrophrograms developed showing the DNA degradation. Thus this preservation method should be the last choice compared to other preservation methods for fresh and degraded bone sample storage.

CHAPTER 7

APPLICATION OF MULTIPLEX (4-PLEX & IACS) PCR ASSAY ON FORENSIC SAMPLES

7.1 OVERVIEW

To further test the effectiveness of the in-house multiplex and the five extraction techniques, 30 bone samples from casework in Malaysia were processed. The usefulness of the in-house multiplex was further assessed by examining simulated forensic evidence that had been exposed to environmental insult (in the United Arab Emirates – these samples were provided by Bushra Idris, PhD student, UCLan).

7.1.1 Objectives

- Extract forensic bone samples from Malaysia using the five extraction methods: ChargeSwitch® gDNA Plant kit, DNA IQ™ System kit, DNeasy® Blood & Tissue kit and PrepFiler® BTA Forensic DNA Extraction kit and Phenol-chloroform-isoamyl alcohol to identify the best extraction method based on the DNA yields.
- Evaluate the data obtained from the electropherograms to assess the capability of the multiplex (4-plex & IACs) to differentiate between degraded DNA and PCR inhibition.
- Amplify and analyse the simulated forensic samples of body fluids (blood, semen and saliva) to further evaluate the usage of multiplex (4-plex & IACs) on DNA degradation and PCR inhibition.

PART 1: BONE SAMPLES FROM MALAYSIA

The forensic samples which were collected from Malaysia consisted of 30 bones which are as shown in Table 7.1.

Table 7.1: Table below shows the bone types, year found and the numbers assigned to the bone samples collected from Malaysia.

Number	Bone type	Year found	Number	Bone type	Year found
1	Unknown	2012	16	Rib	Unknown
2	Unknown	2012	17	Rib	1997
3	Unknown	2012	18	Rib	2002
4	Unknown	2012	19	Rib	2002
5	Unknown	2012	20	Rib	Unknown
6	Unknown	2012	21	Rib	Unknown
7	Unknown	2012	22	Rib	Unknown
8	Unknown	2012	23	Rib	2002
9	Unknown	2012	24	Rib	2010
10	Femur	Unknown	25	Rib	1997
11	Clavicle	Unknown	26	Rib	2004
12	Spine	Unknown	27	Rib	Unknown
13	Spine	1998	28	Rib	1997
14	Rib	1998	29	Rib	1998
15	Rib	Unknown	30	Rib	2001

Nine bone samples (1-9) were collected from Forensic DNA Laboratory of Department of Chemistry Malaysia while the remaining 21 bone samples were collected from Forensic Medicine Department of Penang General Hospital. From the 9 bone samples which were collected from Department of Chemistry Malaysia, 6 were cut into small pieces (1-5 and 8) and 3 bones were pulverised, therefore the bone types are unknown, but all the bone samples (1-30) are of human origin based on DNA profiles. The photographs of the bone samples are as shown in the Figure 7.1.

Figure 7.1: Photographs of the bone samples collected from Malaysia with their numbering.

7.2 RESULTS

7.2.1 DNA extraction of forensic bone samples

All the 30 bone samples were extracted using ChargeSwitch® gDNA Plant kit, DNA IQ™ System kit, DNeasy® Blood & Tissue kit and PrepFiler® BTA Forensic DNA Extraction kit and phenol-chloroform-isoamyl alcohol extraction methods. Since these bone samples were considered to be degraded, a starting amount of 250 mg pulverised bone sample was used in each extraction. The pre-process technique of concentrating and cleaning-up the digested bone samples was carried out using Amicon 30kDa filters (Amicon ultra-2 ml centrifugal filters for DNA purification and concentration, Merck Millipore) prior to DNA extraction as described in Chapter 2 (Section 2.9).

A total of 150 extracts were obtained, consisting of 30 extracts with each extraction method. Only 1 extract per extraction method was carried out on each of the 30 bone samples (i.e. not in triplicate as with earlier experiments). The final elution volume was 100 µl for each sample with all extraction methods.

7.2.1.1 Extraction method comparison using DNA concentration

DNA quantification on all the extracted bone samples was carried out using the Quantifiler® Human DNA Quantification kit on ABI 3500 Prism® Genetic Analyzer. The mean DNA concentration was calculated based on the DNA quantification results (Table 7.2) and comparison boxplot between the extractions methods was plotted (Figure 7.2).

Table 7.2: Table below shows the mean DNA concentrations extracted from different extraction methods.

Extraction method	Mean DNA concentration (ng/µl)	Standard deviation
ChargeSwitch® gDNA Plant kit	0.05	0.15
DNA IQ™ System kit	0.24	0.96
DNeasy® Blood & Tissue kit	0.06	0.15
Phenol-chloroform-isoamyl alcohol	0.59	1.21
PrepFiler® BTA Forensic DNA Extraction kit	0.30	0.99

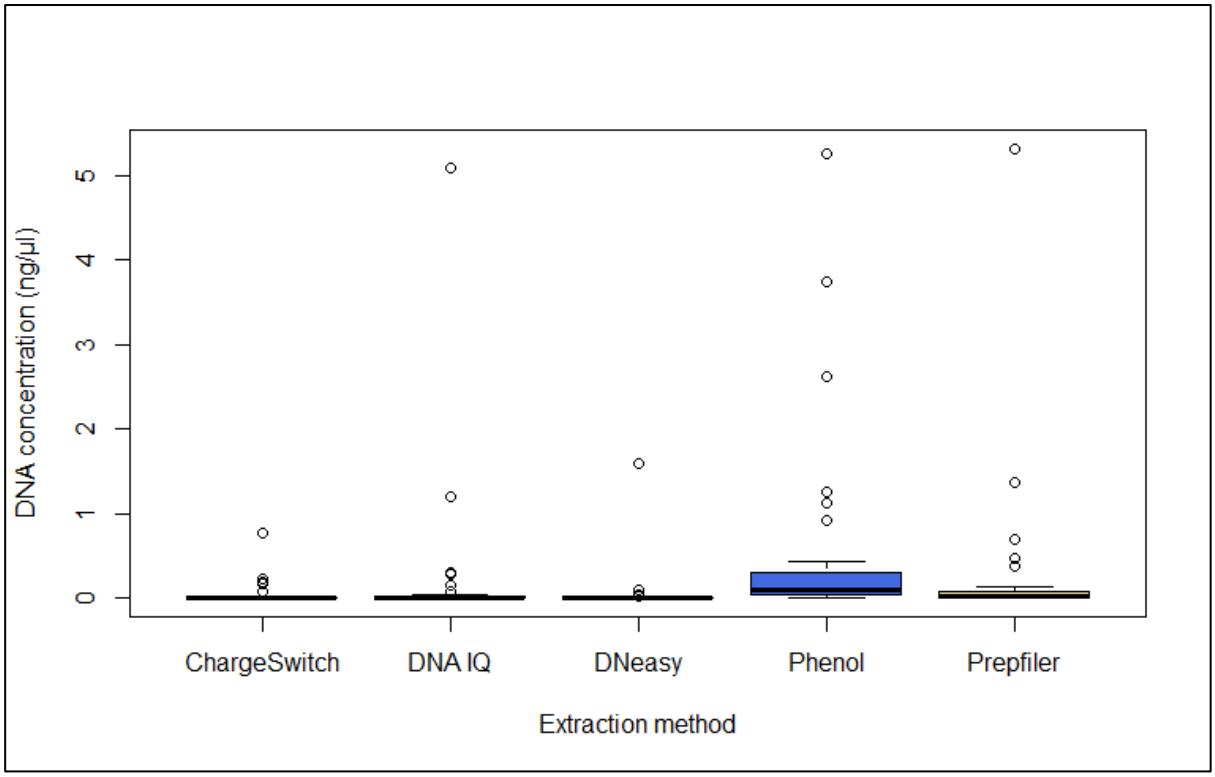


Figure 7.2: Boxplots generated from the concentrations of DNA extracted using different extraction methods.

The ANOVA showed there was no significant difference between the extraction methods ($F_{4,145}$ = 2.12, p = 0.08). However, many outliers were observed from the boxplot obtained. Thus, several bone samples with high DNA yields were selected for further study. Bone samples 3, 5, 7 and 9 were selected.

7.2.1.2 Extraction method comparison using DNA concentration of selected bone samples

The concentration of selected bone samples are as shown in the Table 7.3. From these DNA concentrations, the means were calculated for each extraction method. A boxplot also was plotted (Figure 7.3).

Table 7.3: Table below shows the DNA concentrations of selected bone samples which were extracted from different extraction methods.

Extraction method	DNA concentration (ng/µl)				
	Bone 3	Bone 5	Bone 7	Bone 9	Mean
ChargeSwitch® gDNA Plant kit	0.77	0.23	0.18	0.19	0.34
DNA IQ™ System kit	5.09	1.21	0.29	0.14	1.68
DNeasy® Blood & Tissue kit	1.59	0.05	0.00	0.04	0.42
Phenol-chloroform-isoamyl alcohol	1.13	3.74	1.25	5.27	2.85
PrepFiler® BTA Forensic DNA Extraction kit	5.32	0.70	0.14	1.37	1.88

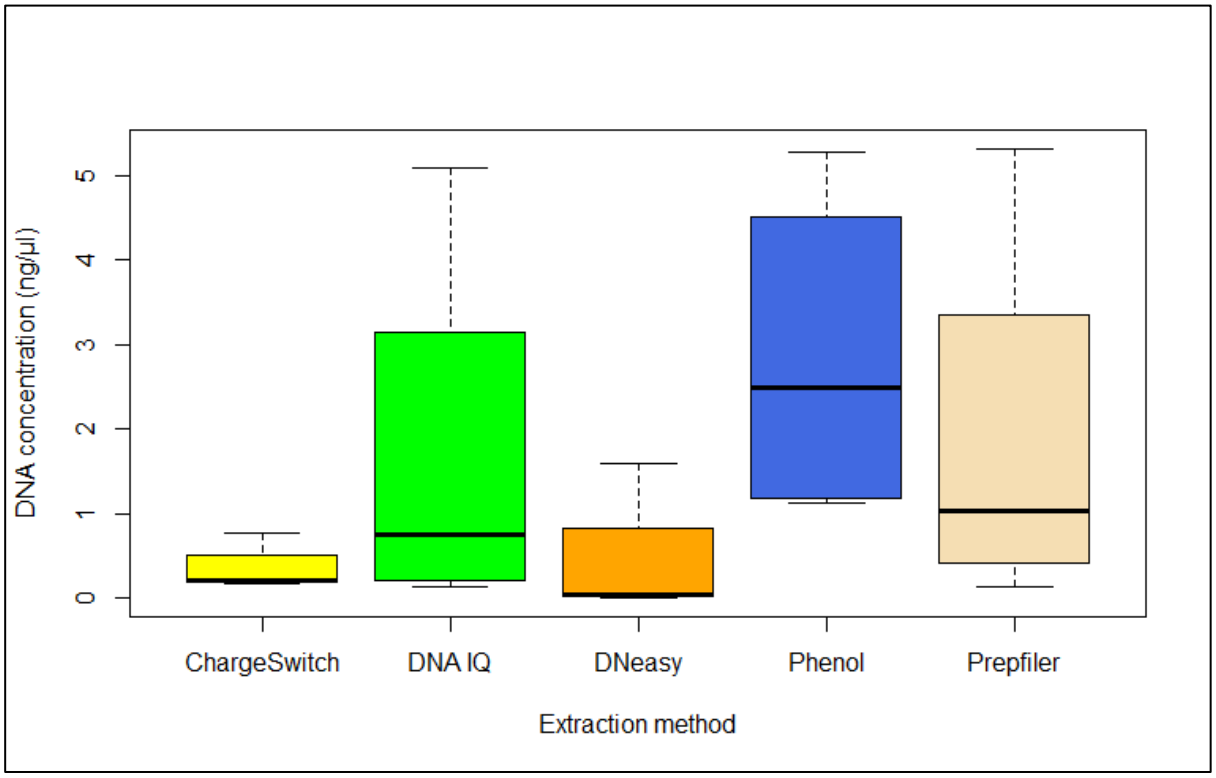


Figure 7.3: Boxplots generated from the concentrations of DNA extracted using different extraction methods.

The statistical data on DNA concentrations obtained from the selected bone samples (3, 5, 7 and 9) showed similar results to those with the 30 bone samples when assessing the efficiency of the extraction methods. Even though ANOVA did not detect a significant difference between the extraction methods ($F_{4,15}$ = 1.43, p = 0.27), phenol-chloroform-isoamyl alcohol appeared to be the best extraction method for forensic bone samples based on the mean DNA concentration of 2.85 ng/µl, followed by PrepFiler® BTA Forensic DNA Extraction kit (1.88 ng/µl), DNA IQ™ System kit (1.68 ng/µl), DNeasy® Blood & Tissue kit (0.42 ng/µl) and ChargeSwitch® gDNA Plant kit (0.34 ng/µl).

7.2.2 Analysis on extracted forensic bone samples using Multiplex (4-plex & IACs)

All 150 samples which were extracted from 30 forensic bone samples were amplified using the multiplex (4-plex & IACs). 1 µl of each sample was used to carry out the amplification. The electropherograms produced are as shown in the Figures 7.4, 7.5, 7.6 and 7.7. Electropherograms of bone samples 3, 5, 7 and 9 were selected since these bone samples produced high DNA yields.

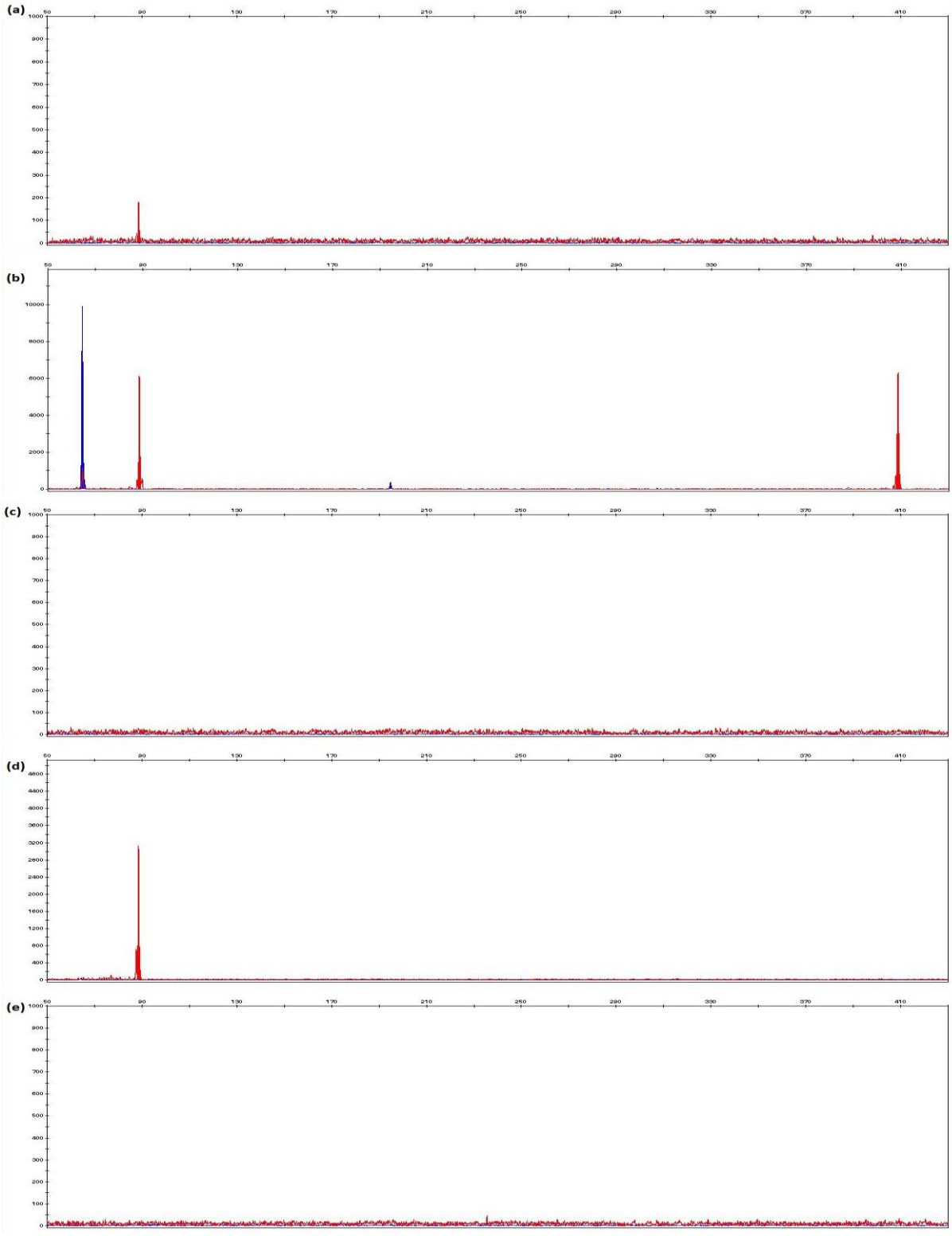


Figure 7.4: Electropherograms generated from bone sample 3 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) PrepFiler® BTA Forensic DNA Extraction kit, (c) DNA IQ™ System kit, (d) DNeasy® Blood & Tissue kit and (e) ChargeSwitch® gDNA Plant kit. 1 µl of each sample was used for amplification.

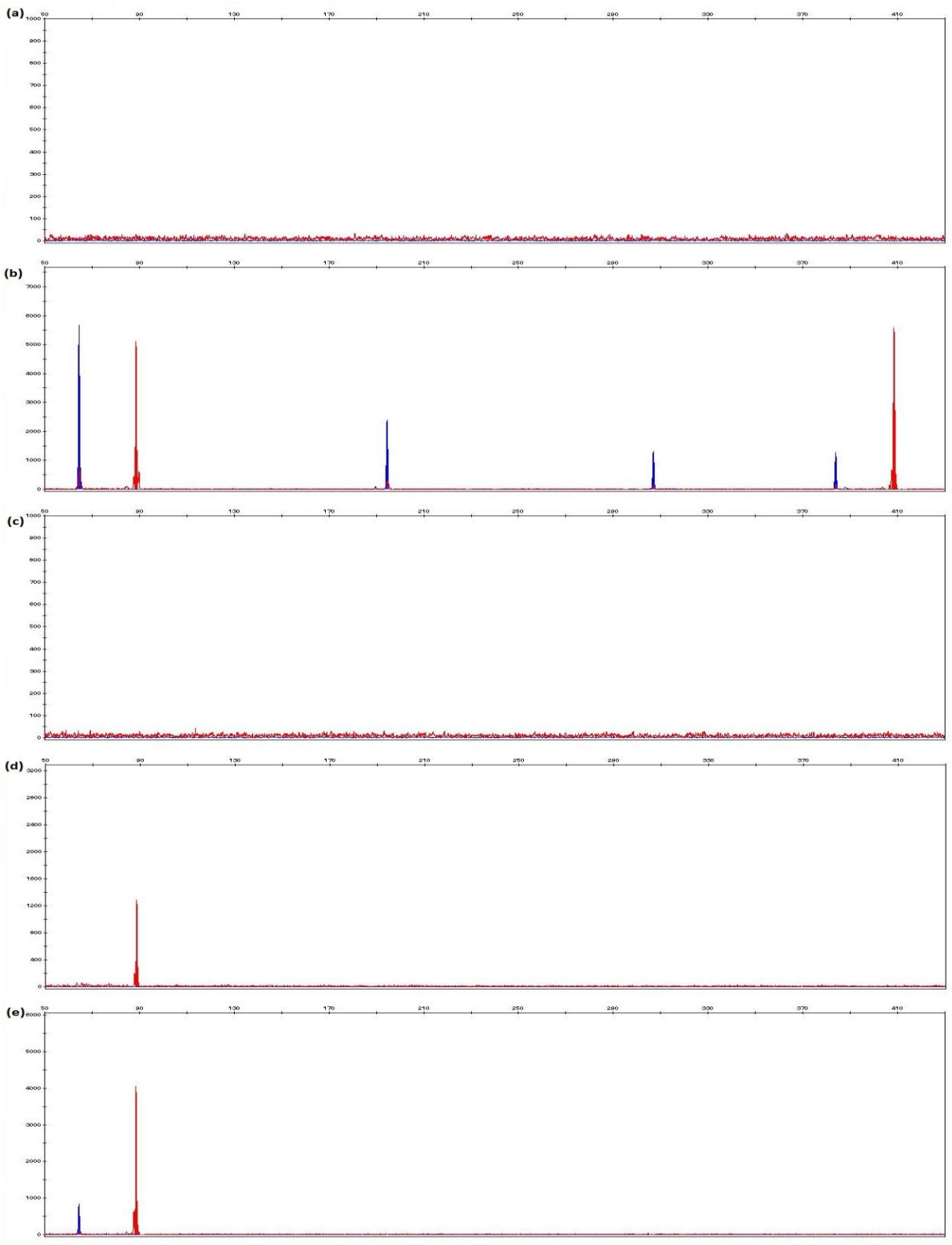


Figure 7.5: Electropherograms generated from bone sample 5 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) PrepFiler® BTA Forensic DNA Extraction kit, (c) DNA IQ™ System kit, (d) DNeasy® Blood & Tissue kit and (e) ChargeSwitch® gDNA Plant kit. 1 µl of each sample was used for amplification.

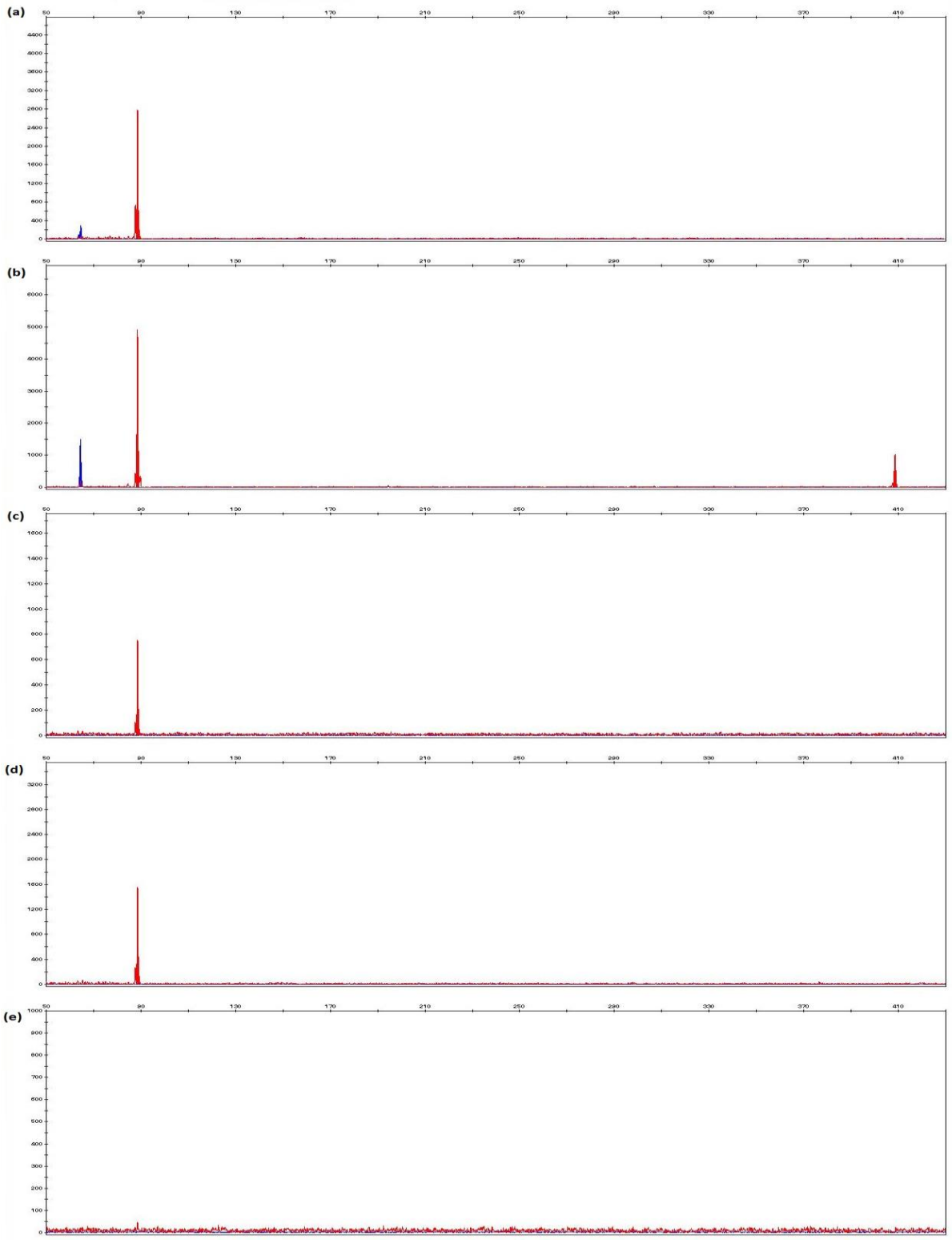


Figure 7.6: Electropherograms generated from bone sample 7 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) PrepFiler® BTA Forensic DNA Extraction kit, (c) DNA IQ™ System kit, (d) DNeasy® Blood & Tissue kit and (e) ChargeSwitch® gDNA Plant kit. 1 µl of each sample was used for amplification.

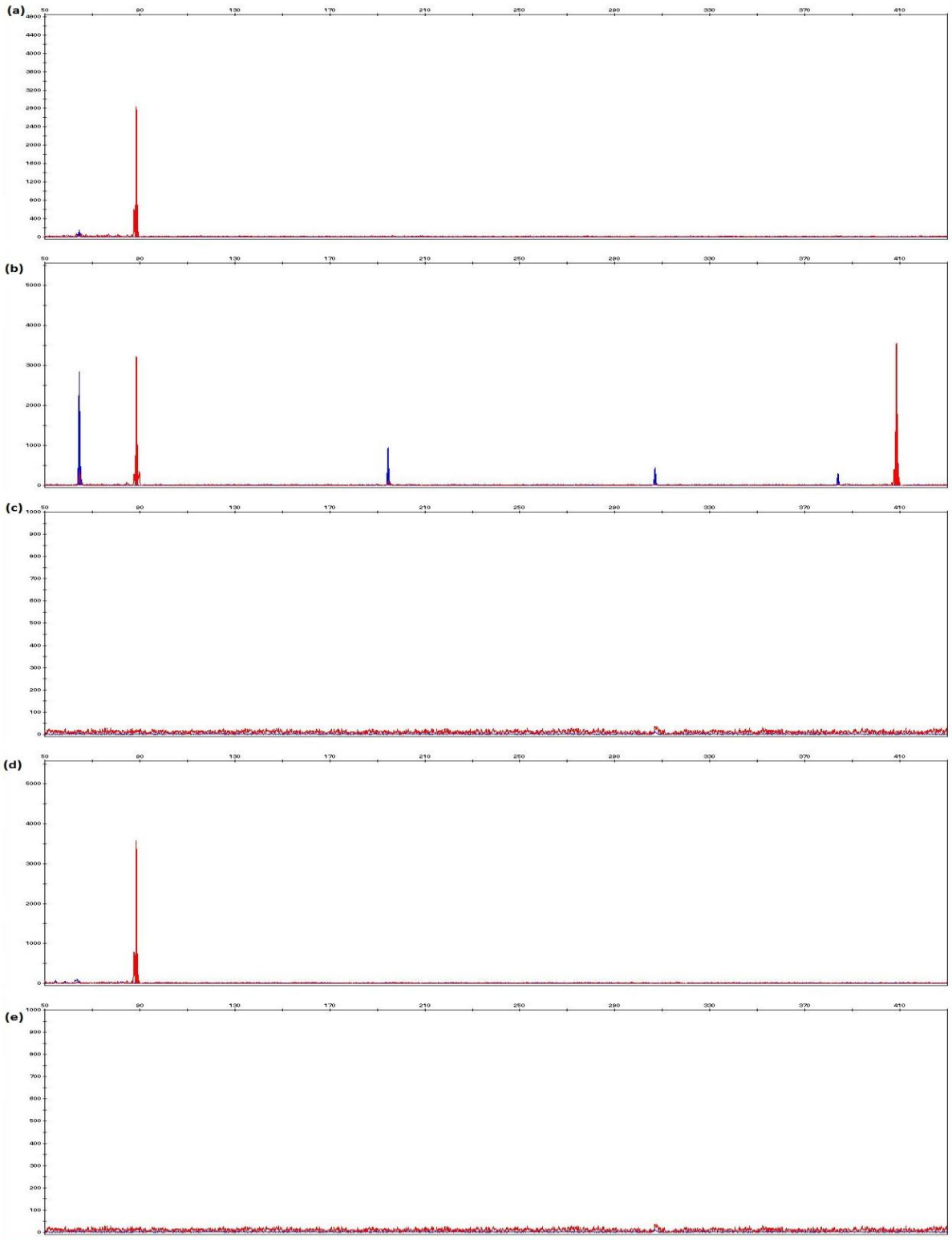


Figure 7.7: Electropherograms generated from bone sample 9 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) PrepFiler® BTA Forensic DNA Extraction kit, (c) DNA IQ™ System kit, (d) DNeasy® Blood & Tissue kit and (e) ChargeSwitch® gDNA Plant kit. 1 µl of each sample was used for amplification.

The IAC peak height details of the electropherograms were tabled for better comparison and also for ratio calculation (Tables 7.4 and 7.5).

Table 7.4: Table below shows the ratio of IACs peaks with samples extracted using different extraction methods.

Bone sample	Extraction method	IACs peak height (RFU)		IAC_{410}/IAC_{90}
		90	410	
3	Phenol-chloroform-isoamyl alcohol	182	n.d.	N/A
	PrepFiler® BTA Forensic DNA Extraction kit	6132	6304	1.03
	DNA IQ™ System kit	n.d.	n.d.	N/A
	DNeasy® Blood & Tissue kit	3129	n.d.	N/A
	ChargeSwitch® gDNA Plant kit	n.d.	n.d.	N/A
5	Phenol-chloroform-isoamyl alcohol	n.d.	n.d.	N/A
	PrepFiler® BTA Forensic DNA Extraction kit	5104	5578	1.09
	DNA IQ™ System kit	n.d.	n.d.	N/A
	DNeasy® Blood & Tissue kit	1290	n.d.	N/A
	ChargeSwitch® gDNA Plant kit	4061	n.d.	N/A
7	Phenol-chloroform-isoamyl alcohol	2780	n.d.	N/A
	PrepFiler® BTA Forensic DNA Extraction kit	4917	1021	0.21
	DNA IQ™ System kit	754	n.d.	N/A
	DNeasy® Blood & Tissue kit	1550	n.d.	N/A
	ChargeSwitch® gDNA Plant kit	n.d.	n.d.	N/A
9	Phenol-chloroform-isoamyl alcohol	2845	n.d.	N/A
	PrepFiler® BTA Forensic DNA Extraction kit	3221	3560	1.11
	DNA IQ™ System kit	n.d.	n.d.	N/A
	DNeasy® Blood & Tissue kit	3576	n.d.	N/A
	ChargeSwitch® gDNA Plant kit	n.d.	n.d.	N/A

Note: n.d.: not detected, N/A: Not Applicable.

Table 7.5: Table below shows the ratio of IAC peaks with a positive control sample (fresh human bone) extracted using different extraction methods.

Bone sample	Extraction method	IACs peak height (RFU)		IAC_{410}/IAC_{90}
		90	410	
Control	Phenol-chloroform-isoamyl alcohol	4069	4551	1.12
	PrepFiler® BTA Forensic DNA Extraction kit	4632	4786	1.03
	DNA IQ™ System kit	4552	5009	1.10
	DNeasy® Blood & Tissue kit	4760	5113	1.07
	ChargeSwitch® gDNA Plant kit	4145	4448	1.07

The results of the ratio of IAC_{410}/IAC_{90} show that DNA extracts without PCR inhibitors were obtained from bone samples 3, 5 and 9 which were extracted using PrepFiler® BTA Forensic DNA Extraction kit. This can be confirmed since both the IAC peaks are balanced (ratio of IAC_{410}/IAC_{90} are around 1.00). However, an unbalanced ratio of 0.21 was also obtained from bone sample 7 using this extraction kit.

However, all the DNA extracts obtained from other extraction methods showed PCR inhibitors to be present in the samples since IAC_{410} was not amplified. High amount of inhibitors were obtained in the DNA IQ™ System kit and ChargeSwitch® gDNA Plant kit extracts since both IACs peak were not amplified in almost all the samples. The same pattern of inhibition was observed in all the 150 samples which were extracted.

From the electropherograms obtained, the PrepFiler® BTA Forensic DNA Extraction kit extracted samples (bone samples 3, 5 and 9) (Figure 7.4, 7.5 and 7.7) displayed high levels of DNA degradation. Since no inhibition was observed in these profiles, the results obtained are very reliable. Even though high concentrations of DNA were obtained based on Quantifiler® Human DNA Quantification kit results, these bone samples are highly degraded.

7.2.3 Re-analysis on extracted forensic bone samples using multiplex (4-plex & IACs)

Since high DNA concentrations were obtained from the bone samples 3, 5, 7 and 9 but unsatisfactory elecropherograms were obtained, these samples were diluted using distilled water 10-fold to reduce the PCR inhibitors. Only samples extracted using ChargeSwitch® gDNA Plant kit, DNA IQ™ System kit, DNeasy® Blood & Tissue kit and phenol-chloroform-isoamyl alcohol extraction methods were re-analysed since PrepFiler® BTA Forensic DNA Extraction kit gave reliable results from the previous analysis.

The diluted samples were amplified using the multiplex (4-plex & IACs). The electropherograms produced are as shown in the Figures 7.8, 7.9, 7.10 and 7.11.

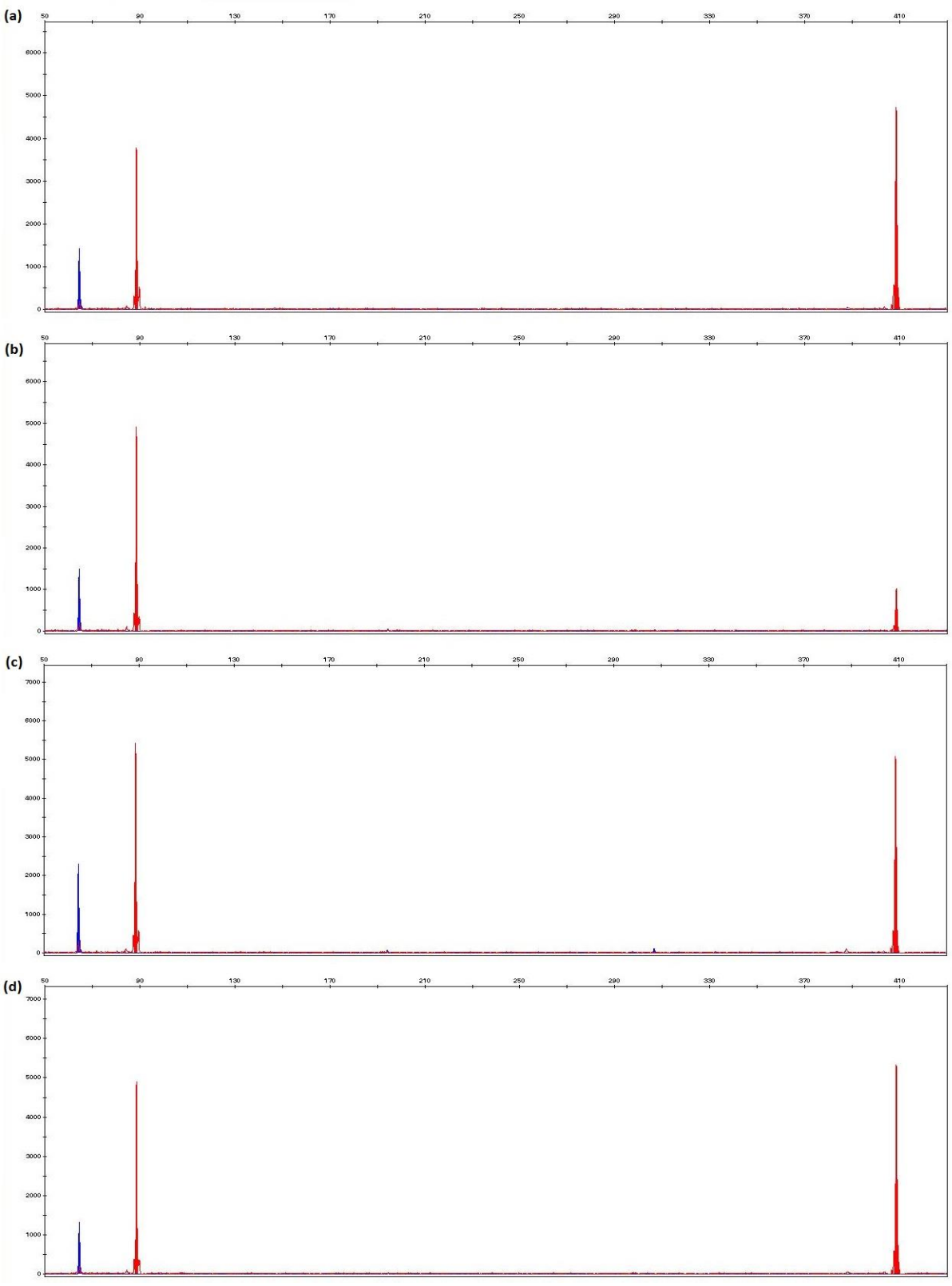


Figure 7.8: Electropherograms generated from bone sample 3 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) DNA IQ™ System kit, (c) DNeasy® Blood & Tissue kit and (d) ChargeSwitch® gDNA Plant kit. 1 µl of each diluted sample was used for amplification.

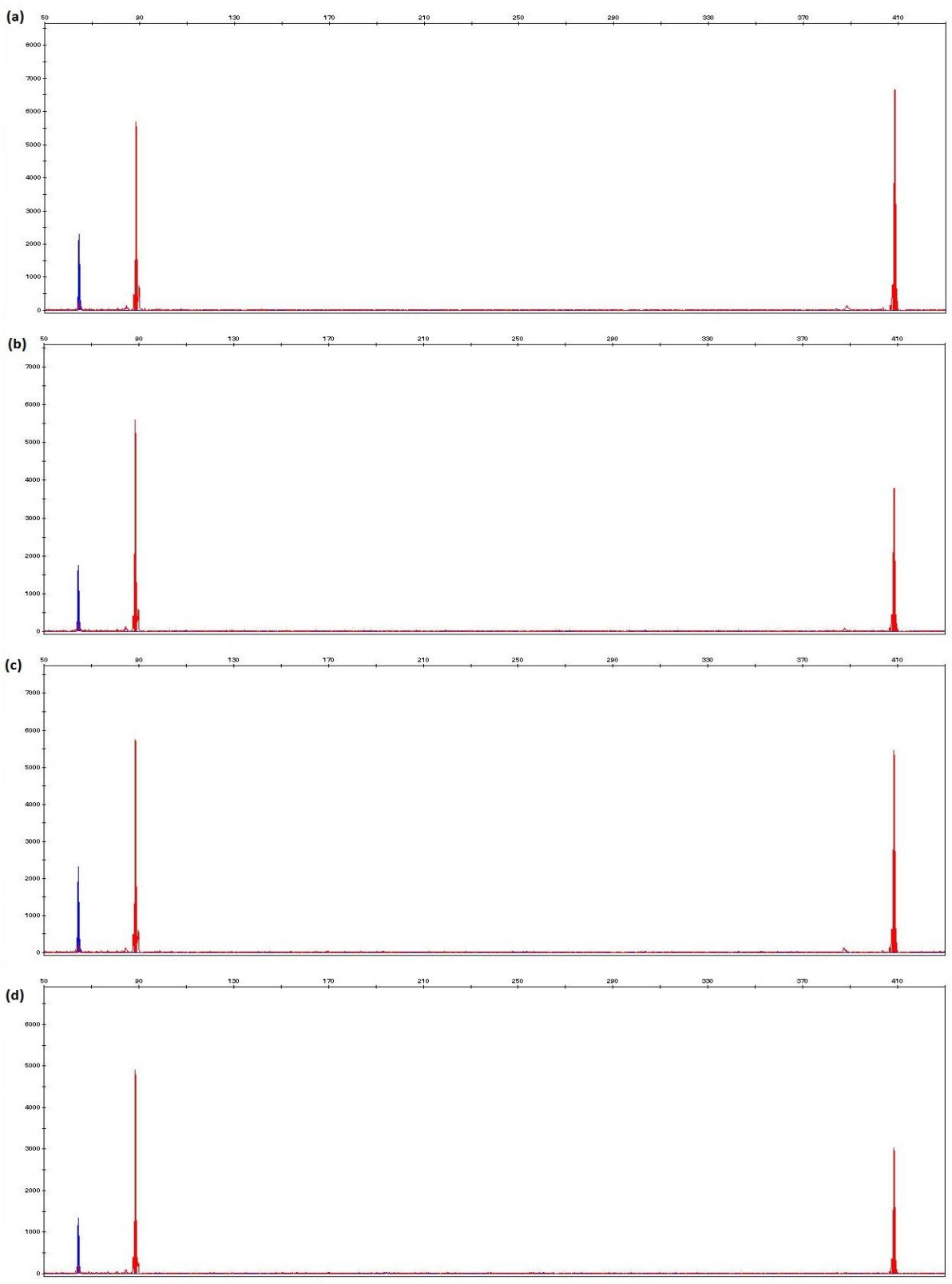


Figure 7.9: Electropherograms generated from bone sample 5 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) DNA IQ™ System kit, (c) DNeasy® Blood & Tissue kit and (d) ChargeSwitch® gDNA Plant kit. 1 µl of each diluted sample was used for amplification.

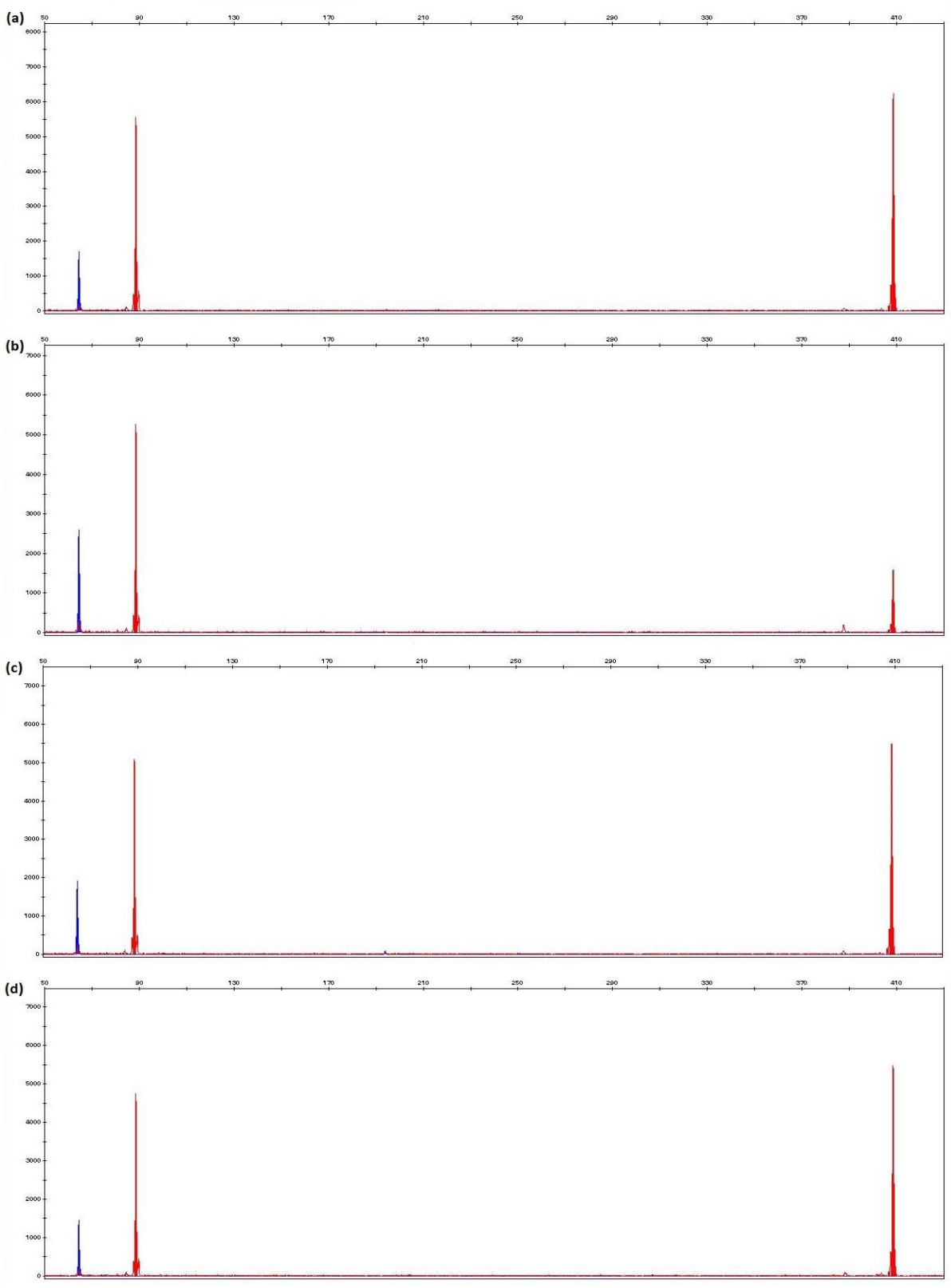


Figure 7.10: Electropherograms generated from bone sample 7 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) DNA IQ™ System kit, (c) DNeasy® Blood & Tissue kit and (d) ChargeSwitch® gDNA Plant kit. 1 µl of each diluted sample was used for amplification.

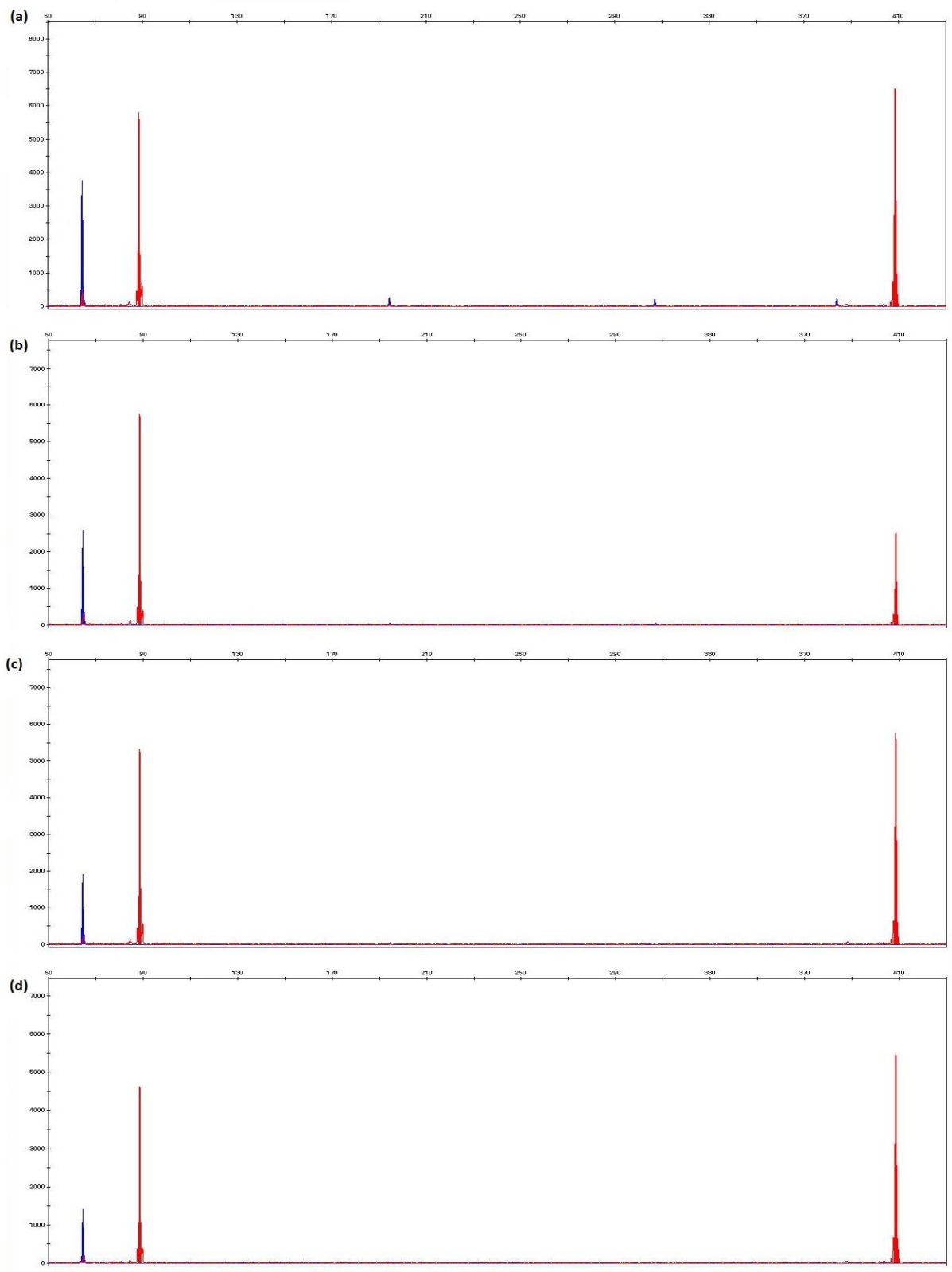


Figure 7.11: Electropherograms generated from bone sample 9 with extraction using (a) phenol-chloroform-isoamyl alcohol, (b) DNA IQ™ System kit, (c) DNeasy® Blood & Tissue kit and (d) ChargeSwitch® gDNA Plant kit. 1 µl of each diluted sample was used for amplification.

The electropherograms of the re-analysed bone samples (3, 5, 7 and 9) showed that with a 10-fold dilution the PCR inhibitors were either eliminated or reduced. No inhibition was observed in the diluted samples which were extracted using phenol-chloroform-isoamyl alcohol and DNeasy® Blood & Tissue kit extraction methods indicated less inhibitors were presented in the extracted samples and with dilution, they were eliminated. Inhibition was still observed in the diluted samples which were extracted by ChargeSwitch® gDNA Plant kit and DNA IQ™ System kit extraction methods. This can be observed with the imbalance of the IACs peaks. The imbalance of the IACs peaks was more pronounced in the DNA IQ™ System kit extracted samples and indicated that high levels of PCR inhibitors were present in the extracts, thus even after dilution, the inhibitors could not be eliminated. These results showed improvement in the same quality and also IAC peaks where both IAC peaks were present after sample dilution. For more balanced IAC peaks, different dilution could be carried out on different extracted sample and also by not reducing the DNA amount added to the PCR reaction.

The electropherograms also showed that the dilution brought down the DNA concentration. This can be seen in the electropherograms from all direct amplifications where the IAC peaks were not balanced. However, with PrepFiler® BTA Forensic DNA Extraction kit, direct amplification resulted in more balanced IAC peaks.

PART 2: SAMPLES FROM THE U.A.E.

Three types of body fluids: blood, semen and saliva, which subjected to environmental insult in the U.A.E to create simulated forensic samples. These samples were extracted using the Chelex-100 extraction method with the final elution volume of 50 µl. A total of 120 samples were extracted from all three body fluids.

7.3 RESULTS

7.3.1 Analysis on simulated forensic samples of body fluids using multiplex (4-plex & IACs)

A total of 120 simulated forensic samples which consist of blood, semen and saliva samples were also analysed using the multiplex (4-plex & IACs) to further test the capability of this multiplex to differentiate between DNA degradation and PCR inhibition.

These samples which were subjected to environmental insult in the U.A.E. were extracted using the Chelex-100. The final volume of each extract was 50 µl. 1 µl of each sample was used to carry out the amplification. Then electrophoresis was carried out on all the amplified samples.

The electropherograms produced showed that semen samples produced good DNA profiles compared to other body fluids. Semen samples produced full profiles from samples with shorter exposure to environmental insult while partial profiles were developed from saliva extracts from the same period while almost no DNA profile was developed from blood samples.

Even though semen produced good profiles, saliva extracts are of better quality than semen and blood. This can be seen as both the IAC peaks were produced in most of the saliva samples but this quality was missing from the samples of semen and blood. Since the saliva samples produced good quality profiles, the degradation of DNA also can be observed from these samples as the days of exposure increased.

Blood tended to produce high inhibition since blood samples were the worst in amplifying both IACs. Thus, several selected blood samples were diluted using distilled water 10-fold to reduce PCR inhibitors. The diluted samples were amplified using the multiplex (4-plex

& IACs). Then electrophoresis was carried out on all the amplified samples. The electropherograms produced were compared with the previous electropherograms of the same samples. An example of a comparison of electropherograms is as shown in Figure 7.12.

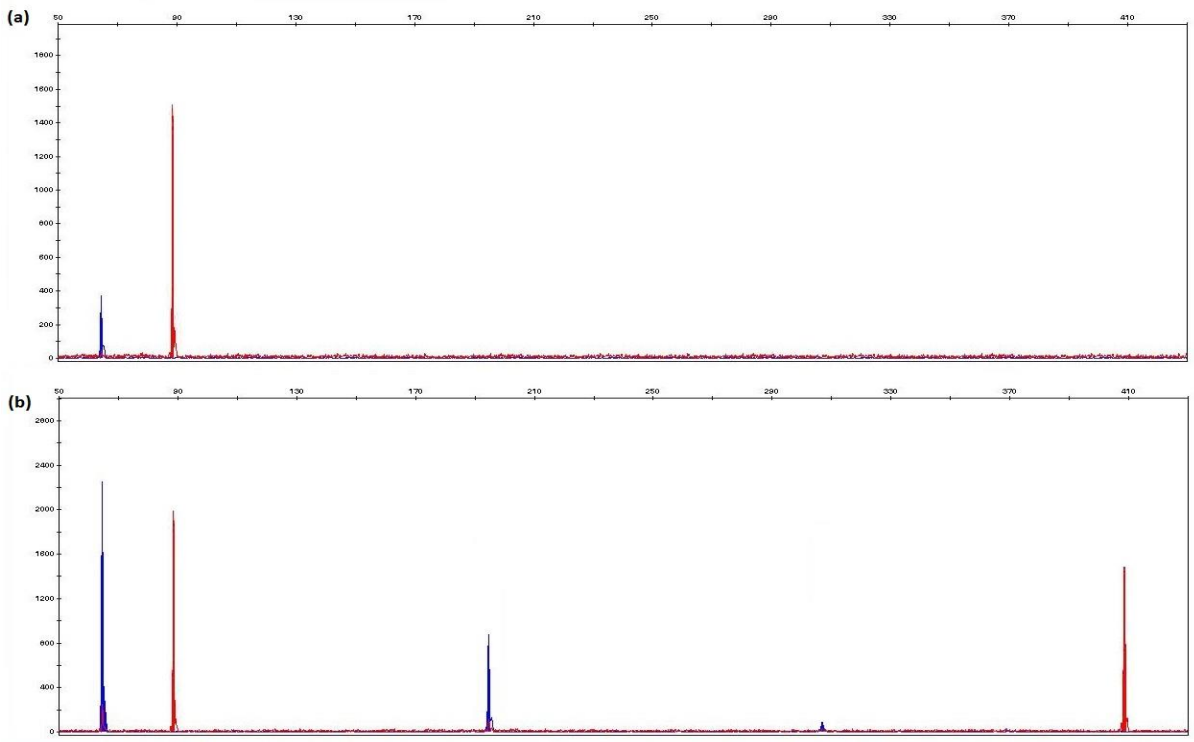


Figure 7.12: Electropherograms generated from extract of blood on cloth using (a) direct amplification and (b) 10-fold diluted sample amplification. 1 µl of each sample was used for amplification.

The direct amplification from the extracts of blood on cloth showing the presence of inhibition with the IAC_{410} not produced. Only one of the 4-plex peaks was produced which was unexpected and either this sample was badly degraded or this is caused by inhibition. With the dilution of 10-fold on the sample, more balanced IAC peaks were produced indicating the amount of inhibitor added to the PCR reaction was very small or nothing at all. Since the amplified sample was free of inhibitor or with very small amount of inhibitor, it can be clearly observed that the sample amplified was degraded since degradation pattern was observed in the 4-plex with smaller amplicons amplified better than the bigger amplicons.

7.4 DISCUSSION

Optimal DNA extraction of forensic samples is essential since problematic samples from crime scenes contain only small amount of DNA, which may be heavily degraded or highly inhibited. Thus successful DNA profiling of forensic samples is largely dependent on the quality and the amount of the DNA recovered during the extraction process (Alaeddini 2012, Koechl et al. 2005).

Five extraction methods: ChargeSwitch® gDNA Plant kit, DNA IQ™ System kit, DNeasy® Blood & Tissue kit and PrepFiler® BTA Forensic DNA Extraction kit and phenol-chloroform-isoamyl alcohol were assessed for their efficiency to extracted quality DNA from bone samples. For this purpose, 30 forensic bone samples were collected from Malaysia. The weather in Malaysia is generally humid with high temperature. Previous studies have shown that heat can degrade the DNA in bone samples (Arismendi et al. 2004) but bone DNA also can survive for long periods (Allentoft et al. 2012, Hagelberg & Clegg 1991). In addition, since these bones were from unidentified persons and exposed to the environment for longer time before collection, there is a high possibility that these bones were exposed to the environment and were possibly in contact with soil and plant materials, thus PCR inhibitors in the form of humic acid could have contaminated the bones (Seo et al. 2013, Kreader 1996). Thus these bone samples from Malaysia were selected for this study. Several types of bones were collected since the DNA yields from bones differ between the bone types (Mundorff & Davoren 2014).

The extraction results did not show much difference between these five extractions methods in DNA recovery from forensic bone samples. Even though no significant differences were observed, based on the mean DNA recovery values, phenol-chloroform-isoamyl alcohol extraction method tended to be the best followed by PrepFiler® BTA Forensic DNA Extraction kit, DNA IQ™ System kit, DNeasy® Blood & Tissue kit and ChargeSwitch® gDNA Plant kit. In previous studies as well, phenol-chloroform-isoamyl alcohol was shown to be the best extraction method for bone (Caputo et al. 2013, Jakubowska et al. 2012, Kitayama et al. 2010, Gornik et al. 2002). Among the silica-based

extraction methods, PrepFiler® BTA Forensic DNA Extraction kit produced high amount of DNA yields from bone samples. A study has shown that DNA was successfully extracted using this kit on excavated human remains (Debska et al. 2013). BTA stands for "Bone, Tooth and Adhesives" and this extraction kit was specifically developed for better DNA extraction from forensic bone samples.

Inadequate sample quality can commonly result in poor DNA amplification success rates for forensic case samples (Ballantyne et al. 2011). Thus, the extracted samples were amplified using the multiplex (4-plex & IACs) to assess the quality of extracted DNA. The presence of Internal Amplification Controls (IACs) in this multiplex makes it a useful tool to identify PCR inhibition (Sachadyn & Kur 1998). The results showed that PrepFiler® BTA Forensic DNA Extraction kit was the best extraction method among these five methods in removing PCR inhibitors. This can be seen as both the IAC peaks were produced in all the amplified samples. Phenol-chloroform-isoamyl alcohol extraction method which is the best in DNA recovery was not good enough to remove PCR inhibitors. High amount of inhibition was observed in DNA IQ™ System kit and ChargeSwitch® gDNA Plant kit with both IAC peaks almost always not produced.

Other than indicating the presence of PCR inhibition, this multiplex also was useful to identify degradation in the samples. When both the IAC peaks are balanced, DNA degradation can be observed using the 4-plex bands. This can be seen in the samples extracted using PrepFiler® BTA Forensic DNA Extraction kit where less or no PCR inhibition was detected (Figures 7.4 to 7.7). This confirms that most of the tested bone samples were highly degraded since most of the bone samples were quite old based on the time they were found (Table 7.1).

To have more assessment on the capability of this multiplex (4-plex & IACs), simulated bone samples using body fluids: blood, semen and saliva were also tested. These samples were exposed to environmental assault in U.A.E. The electropherograms produced from these samples show that saliva samples produced good quality profiles with both the IACs produced in almost all the samples. Saliva is a common source for DNA in property crime

(Bond & Hammond 2008). Objects used for forensic sampling include bottles, cans, cigarette butts, foodstuffs and bite marks (Abaz et al. 2002, Sweet & Hildebrand 1999, Sweet et al. 1997). However, most of the time, presumption being made that saliva presents on these samples but they do not produce DNA profiles (Bond & Hammond 2008).

Even though saliva samples produced good quality DNA as observed in the electropherograms produced using the multiplex (4-plex & IACs), semen samples produced more full profiles than all the samples tested. Seminal stains are normally encountered in sexual assault cases (De Moors et al. 2013, Hulme et al. 2013, Garvin et al. 2012). The high degree of nuclear compaction in sperm makes the DNA hard to degrade unlike in other body fluid samples (Griffin 2013). However, in sexual assault cases, seminal fluid is mostly found on clothing such as denim which may lead to carry over of PCR inhibitors (Mulero et al. 2008, Larkin & Harbison 1999).

Blood samples which were analysed using the multiplex (4-plex & IACs) showed high level of inhibition. Heme which present in the blood is well known for PCR inhibition (Kermekchiev et al. 2009, Al-Soud & Radstrom 2001, Akane et al. 1994). Blood samples showed high level of inhibition since both IACs were not produced together in almost all samples. When several blood samples which showed high level of inhibition were diluted and amplified, a reduction in PCR inhibition was observed since better IAC peaks were produced (Figure 7.12). This confirmed that most of the blood samples were affected by the PCR inhibitors. With more dilution, more balanced IACs peaks could be possibly obtained but it may cause the reduction in the DNA concentration of the sample thus inconclusive result with the 4-plex peaks.

The application of multiplex (4-plex & IACs) showed that it is useful tool to differentiate DNA degradation and PCR inhibition which were observed in the forensic samples. Its capability to detect the PCR inhibition was really high on the tested forensic samples of bone, blood, semen and saliva.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE WORK

The main aim of this project was to develop a new multiplex (4-plex & IACs) that can be used to assess the quality and quantity of the DNA extracts. The 4-plex multiplex without the IACs was developed for the degradation study in tissue samples (Nazir et al. 2012). But, problems arise when inhibited samples were being analysed using this 4-plex multiplex. This is because highly degraded DNA samples produced either partial DNA profiles or negative results and similar results also produced from inhibited samples which could be mistakenly assumed to be degraded DNA (Kontanis & Reed 2006). Thus two IACs were included to detect the presence of PCR inhibitors.

As shown in *Chapter 3*, this multiplex has a good range of DNA concentrations for successful amplification. The minimum amount of DNA required for this multiplex to generate full profile was 0.10 ng and partial profiles were obtained down to 0.02 ng. Identifiler® Plus kit (Life Technologies™) gives full profile with minimum of 0.125 ng DNA (Wang et al. 2012) and PowerPlex® 16 HS System (Promega) also gives full profile with minimum of 0.125 ng DNA (Ensenberger et al. 2010). Thus, this new multiplex system shows that it has a sensitivity level comparable to the leading commercial kits in the market. On the sensitivity of this multiplex to detect the PCR inhibition, this multiplex system failed to produce acceptable DNA profiles when 25 ng/µl human collagen type 1; 1.25 ng/µl humic acid; 5 ng/µl tannic acid; 5 ng/µl hematin; 2.5% ethanol; 1.25% phenol and 0.625X TE buffer were present in a PCR reaction. Compared to AmpFlSTR® SGM Plus® kit which has humic acid acceptable limit of 8 ng/µl (Zahra et al. 2011), phenol acceptable limit of 15% for Tth DNA polymerase to mediate reverse transcriptase activity (Katcher & Schwartz 1994) and real-time PCR acceptable limit of tannic acid at 0.4 ng in a 25 µl PCR

reaction (Kontanis & Reed 2006) and humic acid at 4.8 ng/µl (Seo et al. 2012), this multiplex has more resistance to the PCR inhibitors. The value of the IAC markers became clear when studies were carried out with this range of PCR inhibitors. The robustness and sensitivity of the IAC markers allowed these markers to maintain PCR quality control and aided interpretation of DNA profiles in identifying false negatives and partial profiles caused by PCR inhibition. In addition, the 4-plex was useful to identify DNA degradation. With the range of 70 bp to 384 bp, the 4-plex can cover the target sizes of DNA fragments essential for forensic DNA profiling.

Also, PCR inhibition can be detected through the use of an internal PCR control (IPC) during real-time quantification (Seo et al. 2012, Kontanis & Reed 2006). This technique could save more time and cost since less expensive reagents are involved while the 4-plex & IACs multiplex has to be analyzed on a Genetic Analyzer to evaluate the quality of a sample which will caused more time and cost. Accurate quantification result also could be obtained from the real-time quantification, while the estimation of DNA quantification using this multiplex was very poor. As described in *Chapter 4*, a correlation graph was plotted based on the average peak heights of 4-plex and DNA concentrations from the serial diluted control DNA samples. This correlation graph has coefficient of determination (R^2) of 0.8308 and can only be used when the sample concentrations are between 12.5 – 100 ng. Also, only 30% of tested samples produced relative standard deviations below 10% indicating inaccuracy of this correlation graph. However, there are limitations with real-time quantification approach where the amplicon in the PCR reactions is typically short, and so does not necessarily reveal the full extent of PCR inhibition. But with the 4-plex & IACs results, full extend of the sample quality could be revealed including the DNA degradation and the presence of PCR inhibition.

Other than the development and the validation of this multiplex, the efficiency of five extraction methods (ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit and phenol-chloroform-isoamyl alcohol) on DNA extraction from bone samples was assessed. The ultimate aim of the DNA extraction process is to obtain maximum amounts of DNA from the samples

submitted. At the same time, it is also important to get a pure DNA extraction by eliminating inhibitors which can reduce the efficiency of the amplification process, thus selection of extraction techniques is very important for samples containing PCR inhibitors. As described in *Chapter 5,* all these five extraction methods were found to be effective on both fresh and degraded bone samples. Phenol-chloroform-isoamyl alcohol extraction method gave the highest DNA yields on both preserved fresh and degraded bone samples, followed by DNeasy® Blood & Tissue Kit, ChargeSwitch® gDNA Plant Kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit. DNA concentration technique using Amicon 30kDa filter (Amicon ultra-0.5 centrifugal filter unit with ultracel-30 membrane) was used instead of conventional technique of ethanol precipitation for Phenol-chloroform-isoamyl alcohol extraction method (Gill et al. 1985). This not only improved the purity of the extracted DNA but also reduced the sample processing time. Even though phenol-chloroform-isoamyl alcohol extraction method produced better DNA yield compared to other extraction methods, the silica based extraction methods were developed to eliminate the exposure to the hazardous chemical from phenol-chloroform-isoamyl alcohol extraction (Wang et al. 2011). Also a pre-process technique also was used prior to the extraction. This technique was developed using Amicon 30kDa filters (Amicon ultra-2 mL centrifugal filters for DNA purification and concentration) which concentrated the samples before main extraction steps carried out. The application of this technique was found to produce higher DNA yields and at the same time produce DNA profiles without any inhibition. This is the first attempt in trying this technique and it produced satisfactory results. New and better extraction methods arise every day, thus it is essential to continue seeking for new methods of DNA extraction and purification as well as to improve the existing ones, in order to recover even the smallest amount of DNA present.

As shown in *Chapter 6*, five preservation methods; cell lysis solution (with 1% sodium azide), dehydration/freeze drying, ethanol (96%), freezing and room temperature storage methods were studied for their capability to preserve bone samples for DNA analysis. Freezing is the best preservation technique for long-term storage (Todorova et al. 2012) however ethanol (96%) has also been widely used for preserving tissue samples (Nagy

2010, Nietfeldt & Ballinger 1989). In this study, freezing was found to be the best preservation method for both fresh and degraded bone samples for long-term storage. This was followed by ethanol (96%), dehydration / freeze drying and room temperature preservation methods. Cell lysis solution (with 1% sodium azide) preservation method tended to be good for short-term storage but with the long-term preservation, less DNA yield was obtained. Preservation for bone samples is not a necessity since skeletonised bone samples preserve the DNA for longer time (Rohland & Hofreiter 2007). The 4-plex & IACs multiplex which was used to assess the DNA degradation in this preserved bone samples gave satisfactory result. However, the gap between each markers in 4-plex is quite high, thus more information of DNA degradation could not be gathered. Most of the degraded samples only produced a 70 bp peak, thus inclusion of another smaller marker (between the 70 bp and 194 bp) will aid in better understanding of the level of DNA degradation.

As described in *Chapter 7*, the multiplex (4-plex & IACs) was applied on the forensic samples to evaluate its usage on different types of biological samples. Also, the extraction methods were tested on bone samples from crime scenes to further evaluate their efficiency. Degraded bone samples were collected from Malaysia, while simulated crime samples of blood, semen and saliva were generated from environmental insult in the U.A.E. Even though phenol-chloroform-isoamyl alcohol extraction method produced higher DNA yields, extracts with less PCR inhibitors were obtained from PrepFiler® BTA Forensic DNA Extraction Kit on crime scene bone samples. High amount of inhibition was observed in DNA IQ™ System kit and ChargeSwitch® gDNA Plant kit. PCR inhibiton is a main problem encountered in the forensic samples (Kelly et al. 2012, Bright et al. 2010, Petricevic et al. 2010). Several improved extraction kits specifically for forensic samples are available in the market such as ChargeSwitch® Forensic DNA Purification Kit (Barbaro et al. 2008). PrepFiler® BTA Forensic DNA Extraction Kit where the BTA stands for “Bone, Tooth and Adhesives” was specifically developed for better DNA extraction from forensic bone samples, thus produced best result in forensic samples extraction in this study. Thus, more sample specific forensic extraction kits should be developed to counter

samples like blood, semen and saliva to produce higher DNA yield with lesser or no PCR inhibitors. Blood from simulated forensic sample showed high level of inhibition when tested with the multiplex while semen found to produce stronger DNA profiles even though saliva produced good quality profiles. The application of multiplex on these forensic samples showed that it is useful tool to differentiate DNA degradation and PCR inhibition which were observed in the samples. Its capability to detect the PCR inhibition was really high on the tested forensic samples of bone, blood, semen and saliva. However, wide range of forensic samples types should be tested with this multiple to assess its full capability on evaluating degraded and inhibited forensic samples. With addition of more markers it also can help in other applications of forensic analysis such as single nucleotide polymorphism analysis.

Overall, the studies carried out showed in practical terms the applicability of this multiplex (4-plex and IACs) assay within the DNA profiling framework, giving confidence in the results obtained particularly with respect to challenging samples such as degraded and inhibited samples.

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APPENDIX

The following Appendices are available in this section:

Appendix 1: COSHH assessment training certificate.

Appendix 2: Ethical approval letter from the University of Central Lancashire's Health, Safety, and Ethics Committee.

Appendix 3: Approval letter of sample collection from Forensic Division, Department of Chemistry Malaysia.

Appendix 4: Reagent preparation.

Appendix 5: Conferences / proceedings.

Appendix 6: Publications.

Appendix 1: COSHH assessment training certificate

Certificate of Training

This is to certify that

Sasitaran Iyavoo

Has attended a course on

COSHH Assessment

The Course covered the following topics;
Legal Requirements, Identification of hazardous substances, the COSHH Assessor, Information sources, Hierarchy of control, Completing the COSHH Assessment record

Date
11 November 2011

Steve Whittle
Course Tutor

C. M. Edwards

Christine Edwards
University Health, Safety and Environment Manager

uclan
University of Central Lancashire

Appendix 2: Ethical approval letter from the University of Central Lancashire's Health, Safety, and Ethics Committee

4 April 2012

William Goodwin / Sasitaran Iyavoo
School of Forensic and Investigative Sciences
University of Central Lancashire

Dear William / Sasitaran

Re: STEM Ethics Committee Application
Unique Reference Number: STEM 035

The STEM ethics committee has granted approval of your proposal application '**DNA extraction using silica-coated magnetic beads for the recovery of DNA from degraded and inhibited samples**'.

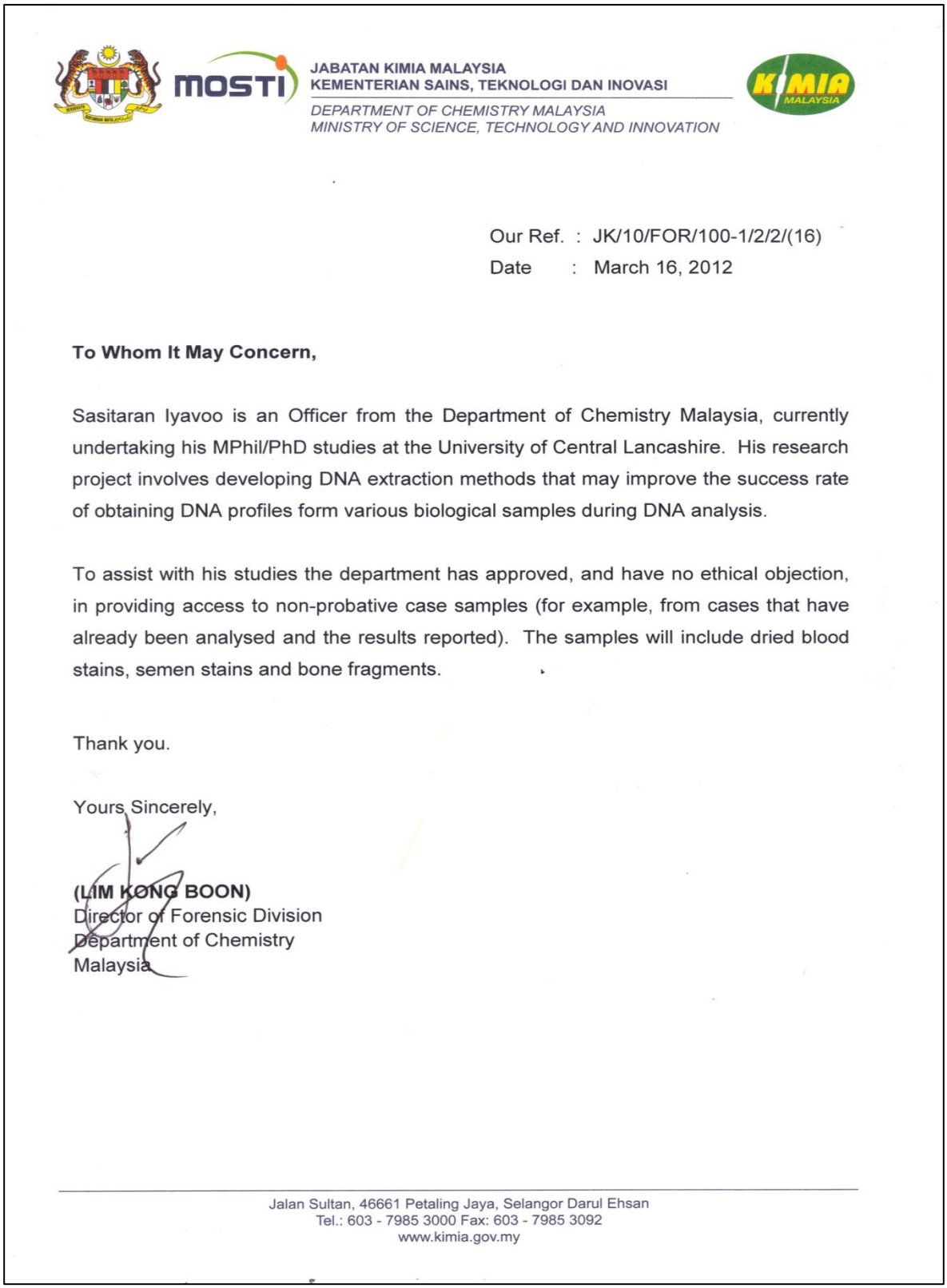
Please note that approval is granted up to the end of project date or for 5 years, whichever is the longer. This is on the assumption that the project does not significantly change, in which case, you should check whether further ethical clearance is required.

We shall e-mail you a copy of the end-of-project report form to complete within a month of the anticipated date of project completion you specified on your application form. This should be completed, within 3 months, to complete the ethics governance procedures or, alternatively, an amended end-of-project date forwarded to roffice@uclan.ac.uk quoting your unique reference number.

Yours sincerely

Tal Simmons
Chair
STEM Ethics Committee

Appendix 3: Approval letter of sample collection from Forensic Division, Department of Chemistry Malaysia



JABATAN KIMIA MALAYSIA
KEMENTERIAN SAINS, TEKNOLOGI DAN INOVASI
DEPARTMENT OF CHEMISTRY MALAYSIA
MINISTRY OF SCIENCE, TECHNOLOGY AND INNOVATION

Our Ref. : JK/10/FOR/100-1/2/2/(16)
Date : March 16, 2012

To Whom It May Concern,

Sasitaran Iyavoo is an Officer from the Department of Chemistry Malaysia, currently undertaking his MPhil/PhD studies at the University of Central Lancashire. His research project involves developing DNA extraction methods that may improve the success rate of obtaining DNA profiles form various biological samples during DNA analysis.

To assist with his studies the department has approved, and have no ethical objection, in providing access to non-probative case samples (for example, from cases that have already been analysed and the results reported). The samples will include dried blood stains, semen stains and bone fragments.

Thank you.

Yours Sincerely,

(LIM KONG BOON)
Director of Forensic Division
Department of Chemistry
Malaysia

Jalan Sultan, 46661 Petaling Jaya, Selangor Darul Ehsan
Tel.: 603 - 7985 3000 Fax: 603 - 7985 3092
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Appendix 4: Reagent preparation

The following section describes the preparation of the reagents used during the various studies. Their use is indicated in *Chapter 2: General Methods and Materials*.

Preparation of digestion buffer

For the preparation of 100 ml, 1 ml of 1 M Tris-HCl, 2 ml of 0.5 M EDTA pH 8, 1 ml of 5 M NaCl and 20 ml of 10% (w/v) SDS were mixed with 76 ml of distilled water.

Preparation of 1 X TE buffer

For the preparation of 100 ml, 1 ml of 1 M Tris-HCl and 20 ml of 0.5 M EDTA pH 8 were mixed with 79 ml of distilled water.

Appendix 5: Conferences / proceedings

Poster presentation: *Use of silica-coated magnetic nanoparticles for the recovery of DNA from degraded and inhibited samples* at Postgraduate Research Conference, University of Central Lancashire, Preston, 2 – 5 July 2012.

Poster presentation: *Evaluation of five DNA extraction systems for recovery of DNA from bone* at International Society for Forensic Genetics Congress, Melbourne, Australia, 2 – 6 September 2013**.**

Appendix 6: Publications

Development of a multiplex system to assess DNA persistence in taphonomic studies (2013), Electrophoresis, Volume 34, Issue 24, Pages 3352-3360.

Evaluation of five DNA extraction systems for recovery of DNA from bone (2013), Forensic Science International: Genetics Supplement Series, Volume 4, Issue 1, Pages e174-e175.

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Sharizah Alimat[1]
Nathalie Zahra[1]
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Received May 22, 2013
Revised September 15, 2013
Accepted September 16, 2013

Research Article

Development of a multiplex system to assess DNA persistence in taphonomic studies

In this study, we have developed a PCR multiplex that can be used to assess DNA degradation and at the same time monitor for inhibition: primers have been designed to amplify human, pig, and rabbit DNA, allowing pig and rabbit to be used as experimental models for taphonomic research, but also enabling studies on human DNA persistence in forensic evidence. Internal amplified controls have been added to monitor for inhibition, allowing the effects of degradation and inhibition to be differentiated. Sequence data for single-copy nuclear recombination activation gene (RAG-1) from human, pig, and rabbit were aligned to identify conserved regions and primers were designed that targeted amplicons of 70, 194, 305, and 384 bp. Robust amplification in all three species was possible using as little as 0.3 ng of template DNA. These have been combined with primers that will amplify a bacterial DNA template within the PCR. The multiplex has been evaluated in a series of experiments to gain more knowledge of DNA persistence in soft tissues, which can be important when assessing what material to collect following events such as mass disasters or conflict, when muscle or bone material can be used to aid with the identification of human remains. The experiments used pigs as a model species. When whole pig bodies were exposed to the environment in Northwest England, DNA in muscle tissue persisted for over 24 days in the summer and over 77 days in the winter, with full profiles generated from these samples. In addition to time, accumulated degree days (ADD) were also used as a measure that combines both time and temperature—24 days was in summer equivalent to 295 ADD whereas 77 days in winter was equivalent to 494 ADD.

Keywords:
DNA degradation / DNA persistence / DNA profiling / Forensic genetics
DOI 10.1002/elps.201300240

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Different experimental approaches have been utilized to estimate the level of DNA degradation in postmortem tissue and body fluids. These include comet assays [1], the development of species-specific PCR primers and agarose gel electrophoresis [2–5], species-specific primers and real-time PCR [6–8], Southern blotting and hybridization [9, 10], competitive PCR [11], and profiling of DNA extracts with polymorphic markers systems [12–14].

Understanding DNA degradation is important when undertaking forensic analysis [15]. For example, the level of degradation dictates which tissues to sample from human remains when using DNA to assist with the identification [16, 17]. Bone and teeth are preferred over soft tissues when dealing with highly degraded remains; however, processing of hard tissues takes longer and is more expensive than when using muscle tissues. Assessing the level of degradation can also be important in deciding which DNA profiling systems are most appropriate for the analysis of different sample types. While most laboratories will have a standard STR-based profiling system that will typically amplify DNA up to 400–500 bp, highly degraded samples may benefit from analysis using specialized assays that either amplify STR markers using short amplicons [18–20], target SNPs [21–24], or insertion-deletion polymorphisms (indels) [25, 26].

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Abbreviations: ADD, accumulated degree days; **IAC**, internal amplification control; **RAG-1**, recombination activation gene

Colour Online: See the article online to view Figs. 1 and 2 in colour.

In previous studies, the reported persistence of DNA in soft muscle has varied: experiments in Western Australia using pig muscle from whole animals could amplify DNA after ten days in winter [3], in Thailand pig muscle yielded DNA for up to four days when exposed to the environment (eight days when immersed in water) [5], rat brain tissue yielded DNA when stored at 20°C for up to 42 days [8], human brain and thyroid tissue yielded DNA for over 100 days when stored in a container or immersed in water at 21°C [14]. Of the above studies, only Larkin et al. [3] took into account the level of DNA degradation in relation to both time and temperature (accumulated degree days (ADD)) for the purpose of estimating postmortem interval [3, 27–29].

Here we describe the development of a PCR multiplex that targets loci with amplicons over a similar range to that seen in many commercial PCR amplification kits used for forensic DNA profiling. We have combined the multiplex with previously described internal amplification controls (IACs) that can monitor for PCR inhibition [30, 31]. We have used the optimized multiplex to work with human, pig, and rabbit DNA, allowing it to be used as a tool to assess the persistence/degradation of DNA in soft muscle tissue using pigs or rabbits as an experimental model. We describe an application of the multiplex assessing the persistence of DNA in pig muscle tissue in a series of field experiments.

2 Materials and methods

2.1 Design of PCR primers

Sequence data for a nuclear recombination activation gene (RAG-1) from human, rabbit, and pig were downloaded from GenBank and aligned using Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to identify conserved regions: primers that would amplify 70, 194, 305, and 384 bp amplicons from the three species were identified (Table 1). These primers were designed using the publicly available software Primer3 [32, 33] and the oligonucleotide property calculator software [34].

Primer pairs with 5′ fluorescein-labeled or ROX-labeled forward primers were synthesized (Life Technologies™, UK) and were delivered desalted and lyophilized: 100 μM stock solutions were prepared by adding the appropriate volume of 1× TE buffer (0.1 M Tris HCl, 0.01 M EDTA, pH 8.0; Sigma, UK) and stored at −20°C, while an aliquot of a 10 μM working solution was kept at 4°C.

2.2 DNA samples

DNA extraction was carried out using DNeasy® Blood and Tissue kit (Qiagen, UK) according to the manufacturer's instructions using 25–30 mg of tissue. Pig and rabbit positive control DNA samples were extracted from soft muscle tissue samples collected at day 0.

2.3 DNA quantification

Extracts used in the sensitivity study were quantified in triplicate using real-time PCR of the 70 bp fragment with two unlabeled primers. GoTaq® qPCR Master Mix (Promega®, UK) was used following the manufacturer's recommended protocol with an ABI 7500 real-time PCR machine (Life Technologies™).

Field samples were extracted as above, but quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies™). Samples were prepared by mixing 3 μL DNA with 147 μL 1× TE and 150 μL PicoGreen and pipetted (300 μL) into microplate wells—all samples were prepared in triplicate. Fluorescence was determined using TECAN GENios Pro plate reader at absorption and emission wavelengths of 485 and 535 nm, respectively. Data were obtained in the form of relative fluorescent unit.

2.4 Multiplex PCR

The primer mix and thermal cycler conditions were prepared according to the optimized PCR condition (Table 1). In a total reaction volume of 10.0 μL, 5.0 μL 2× Platinum Multiplex PCR Master Mix (Applied Biosystems), 0.6 μL of primers mix, 2.5 μL of dH_2O, 1 μL of DNA template, and 1 μL of IAC template, prepared as described by Zahra et al. [30], were added. PCR was carried out in a thermal cycler GeneAmp® 2700 (Life Technologies™) using following conditions: 95°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

For sensitivity studies dilutions (2.80, 0.93, 0.31, 0.10, and 0.03 ng) were made for human, pig, and rabbit day DNA. Human genomic DNA G3041 (273 ng/μL) was obtained from Promega®.

2.5 Capillary electrophoresis

Each sample for fragment analysis was prepared by adding 1 μL of PCR product to 10 μL of Hi-Di™ formamide (Life Technologies™) containing 0.7 μL GS500 ROX size standard (Life Technologies™). The samples were heated at 95°C for 5 min and snap-cooled for at least 3 min.

Optimization of the multiplex PCR was performed using an ABI 310 Prism® Genetic Analyzer (Life Technologies™). DNA fragment analysis was carried out using a 47 cm-long capillary using POP-4™ polymer (Life Technologies™). Electrophoresis running buffer was used at 1× concentration. The GSPOP 4 (1 mL) F.md4 run module with dye set DS-32 (filter set F) was used with the following parameters: run temperature 60°C, syringe pump time 150 s, prerun voltage 15 kV, prerun time 120 s, injection time 5 s, injection voltage 15 kV, run voltage 15 kV, and run time 30 min.

Due to the ability to increase throughput DNA fragment, analysis for the field samples was performed using an ABI 3500 Genetic Analyzer (Life Technologies) using a 50 cm

Table 1. Shown below are four PCR primer sets for conserved regions of pig, rabbit, and human

Nuclear gene	Forward and reverse primers (5′-3′)	Primer conc. (μM)	Amplicon (bp)
RAG-1 NCBI reference M77666.1	CCT CAA AGT CAT GGG CAG C	0.08	70
	GAC TCT CCA GGT CAG TAG G	(0.05)	
	GCT GTT TGC TTG GCC ATC CG	0.19	194
	GTG CTG GAA GAC ACA TTC TTC	(0.1)	
	ATG AGG TCT GGC GTT CCA AC	0.15	305
	TGG TCA TGA GCT TCC TGG CA	(0.15)	
	GAG CAA TCT CCA GCA GTC CT	0.56	384
	GCT AAA CTT CCT GTG CAT GA	(0.4)	
IAC 90	CTG TCA AAT CTA AAC ACC CTG ATG CG[a)]	0.60[a)]	90
	GTC AGC TTG CAT AAT ATC GAG ATA ACG C	0.15	
IAC 410	CTG TCA AAT CTA AAC ACC CTG ATG CG[a)]	0.60[a)]	410
	GTA CAA TGT TGA CGT TCC TCG CTG	0.45	

The sequence and concentration of the IAC primers are also given. The primer concentrations shown in brackets are used when the IAC primers are not included.
a) The same forward primer is used for both the IAC 90 and 410; 0.6 μM is the total primer concentration.

capillary array and 3500 POP-6™ polymer. The FragmentAnalysis50_POP6 run module was used in combination with the dye set DS-32 (filter set F with the following parameters: run temperature 60°C, prerun voltage 15 kV, prerun time 180 s, injection time 10 s, injection voltage 1.6 kV, run voltage 15 kV, and run time 2700 s).

The data obtained from CE were analyzed using GeneMapper™ ID version 3.2 (ABI 310) and GeneMapper® Software v4.1 (ABI 3500) (both Life Technologies™).

2.6 Simulated DNA degradation

The exposure of forensic evidence samples to environmental insult, fragmentation of full-length genomic DNA, and the reduction of overall concentration of amplifiable DNA are observed. Therefore, the effect of DNA degradation on the amplification efficiency of the multiplex (4-plex) was examined according to the protocol described by Swango et al. [35]. A degradation series was prepared by digesting high molecular weight (177 μg/mL) DNA with DNase I (Applied Biosystems) for a progressive length of time. A reaction containing 15.7 μg DNA, 10× DNase buffer (100 mM Tris, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6), and nuclease-free water to bring the reaction volume to 110 μL, was prepared. Ten microliters were removed and used as a control sample. DNA in the remaining solution was digested by adding 1.25 μL of DNase I (2 U/μL), and then the mixture was incubated at 24°C. From this solution, 10 μL was removed at specific time points (5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min), DNase I activity was stopped by mixing 2 μL of DNase inactivation reagent, and incubating at 65°C for 15 min. After incubation, samples were centrifuged at 10 000 × *g* for 2 min and supernatant was transferred to a fresh tube. The degree of DNA degradation was assessed by running 2 μL of digested products on 2% agarose gel using 100 bp DNA ladder (BioLabs®, UK). DNA quantification was performed using Human Quantifiler® kit (Life Technologies™) according to the manufacturer's instruction.

2.7 Field studies

In order to assess the degradation of DNA in soft muscle tissue whole pig carcasses were laid out at the University of Central Lancashire's Taphonomic Research in Anthropology: Centre for Experimental Studies (TRACES), which comprises 13 acres of field facilities in the Northwest of England [36]; carcasses were covered by a wire mesh cage to prevent scavenging by large mammals. Field experiments were carried out in February–May 2010, May–June 2010, and September–October 2010. Muscle samples of approximately 1 g were taken from the upper hind limb at regular intervals until either no DNA could be recovered (this was based on an assessment of the DNA extract on an agarose gel and was only relevant for the February series) or no muscle tissue remained for analysis (as occurred with both the May and September experiments). The muscle tissues remain moist throughout all experiments described here and samples were taken from below the tissue surface, avoiding the complications that could be introduced by muscle tissues becoming desiccated, which would have enhanced the preservation of DNA. In a preliminary series of experiments related to this study, different regions of muscles were sampled: the hind and fore legs, both upper and lower (i.e. in contact with the ground) and the neck, no significant difference was seen in the rates of degradation [37]. Samples were collected in all cases from three separate pig carcasses and were transported on ice and frozen at −20°C until DNA was extracted. Ambient temperatures were measured at the field site using HDT-200 and HDT-250 USB data loggers (Thermosense, UK). When analyzing the field samples with the multiplex, 0.6 ng of DNA template were added to each PCR and the CE undertaken using a 3500 Genetic Analyzer.

3 Results

3.1 Selection of loci and primer design

Single-copy RAG-1 is involved in somatic (V(D)J) rearrangement of B- and T-cell lymphocytes, which is essential for the development of a normal immune system and its functions. The RAG-1 gene is found throughout higher vertebrates and comprises a 3.1 kb exon without introns. It evolves slowly, has minimal saturation at the third position of codons, and a low frequency of indels [38–40]. Multiple conserved regions could be identified in the alignment between human, pig, and rabbit sequence, allowing a range of amplicons sizes from the same gene (Supporting Information Fig. 1). Additional sites were available allowing amplicons be added at a later stage, if desired.

3.2 Multiplex sensitivity and specificity

The four primer pairs were found to be optimum at working concentrations of 0.05, 0.1, 0.15, and 0.4 μM for 70, 194, 305, and 384 bp, respectively (Table 1). The $MgCl_2$ concentration was kept at 2.5 mM for all PCR. The optimized 4-plex PCR was assessed for any nonspecific amplification that would lead to extra peaks and could interfere with target peaks. Positive control DNA samples of all three species were evaluated on ABI Prism® 310 and 3500 Genetic Analyzers.

DNA fragment analysis for multiplex PCR was carried out using serial dilutions of positive control DNA samples of human (Promega®), rabbit, and pig extracted from day 0 postmortem soft muscle tissues and quantified using real-time PCR. The 4-plex PCR was found to work efficiently in triplicate samples of all three species between 2.80 and 0.1 ng of DNA template (Supporting Information Fig. 2); no extra peaks were observed in any of the amplifications and full amplification was observed in all three species with template amounts as low as 0.3 ng; when the template amount dropped below 0.3 ng allele dropout, which is typical of low-template PCR [41, 42], was observed in some profiles. The multiplex was also tested with DNA from insects that would commonly be found on the carcasses [43]; no amplification was seen with DNA from *Calliphora vomitoria*, *Protophormia terraenovae*, *Lucilia sericata*, *Lucilia caesar*, or *Lucilia illustris*. Addition of the IAC template and primers did not alter the sensitivity of specificity of the multiplex.

To assess the ability of the multiplex (4-plex) assay to quantify DNA in degraded samples, a ten-point degradation series was prepared by treating aliquots of high molecular weight genomic DNA (Promega®) with DNase I for increasing periods of time ranging from 5 to 180 min. The result was, as expected, a degradation series exhibiting incremental increase in the extent of DNA degradation. Full 4-plex profiles were obtained reproducibly until 30 min of DNase I digestion, but with a decline in overall peak height and a relative decline in the amplification of large loci. There was complete failure of amplification in all DNA samples digested with DNase I for a period of 45 min or above (Supporting Information Fig. 3).

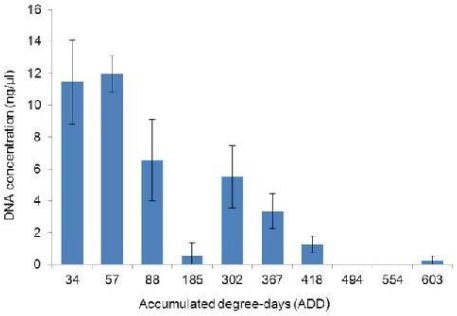

Figure 1. Shown above is the PicoGreen®-based DNA quantification of samples collected from pig carcasses in February 2010. The error bars indicate the standard error from three replicates. A total of 200 μL was extracted from each sample and 3 μL was used for quantification.

The standard deviation of the amplicon sizes for multiple runs of the same and different samples was below 1 bp for all amplicons. The sizing of fragments varied in accuracy: 70 bp (64.32 ± 0.69 bp); 194 bp (194.54 ± 0.04 bp), 305 bp (305 ± 0.5 bp), and 384 bp (383.9 ± 0.05 bp) on the ABI 310 Genetic Analyzer. Slightly different values were seen when using the 3500 Genetic Analyzer; 70 bp (65.17 ± 0.02 bp), 194 bp (195.66 ± 0.03 bp), 305 bp (307.38 ± 0.60 bp), and 384 bp (384.69 ± 0.15 bp).

3.3 Taphonomic study using field DNA samples

Three separate repeats of an experiment to assess the persistence of DNA in soft tissue were undertaken over the course of a year (2010), with experiments covering winter–spring, spring–summer, and autumn. Sampling time points varied depending on the season and the temperatures: in May samples were taken at between three and six day intervals whereas in February samples were only taken approximately once a week.

The breakdown of soft tissues was much more rapid, as would be expected, in summer and sampling was not possible after day 27 in the May–June experiment. Following collection of soft muscle tissue DNA was extracted and quantified using PicoGreen®. The values obtained from the quantification were used as a guide for adding DNA to the multiplex. An example of the quantification results from the February series is shown in Fig. 1. PicoGreen® is nonspecific and so will quantify bacterial and fungal DNA. However, this did not appear to confound the quantification of the endogenous DNA as the values went down to zero over time, before then increasing as bacterial and fungus colonized the remaining tissues. Based

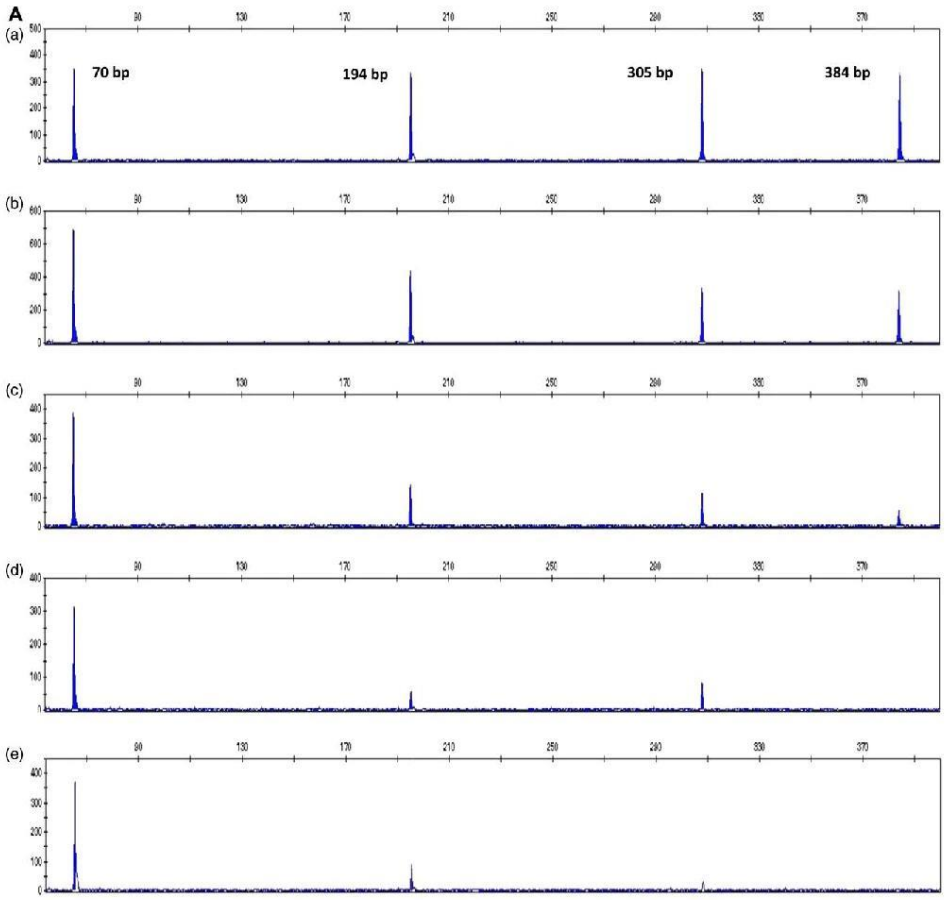

Figure 2. (A) Examples of electropherograms generated from 1 μL of DNA extract (from a total of 200 μL) extracted from pig soft muscle tissues collected during May 2010 project at (b) 159 ADD, (c) 203 ADD, (d) and (e) 338 ADD. Human positive control (a) is also shown (0.5 ng). The amount of DNA in 1 μL of each extract was quantified using real-time PCR: (b) 0.82 ng, (c) 0.73 ng, (d) 0.32 ng, (e) 0.59 ng. (B) Electropherograms with the same samples as in (A) with the addition of the IAC 90 and 410 internal amplification controls.

on the quantifications, 0.6 ng was added to the PCR multiplex and the absence or presence of each of the amplicons was noted. The PCRs were generally well balanced, which indicated that the quantification using PicoGreen® was reasonably accurate. An example of electropherograms taken from the May series is shown in Fig. 2 (February and September are shown in Supporting Information Figs. 4 and 5)—input DNA was kept constant for these using 1 μL of extract in each PCR (i.e. 0.5% of the total extract) and the amount of DNA added was assessed by real-time PCR. As time/ADD increase, the larger amplicons show a relative decline in abundance. This is expected, but somewhat surprisingly amplicons can still be amplified up to day 90/603 ADD in the February experiment. The IAC primers shown in Fig. 2B produce two peaks of 90 and 410 bp; these are well balanced in all of the electropherograms, indicating the absence of PCR inhibitors (had PCR inhibitors been present then we would expect the 410 bp IAC to decrease with respect to the 90 bp IAC). Table 2 contains the data from all three field experiments—0.6 ng was used as template in each of the reactions. As expected, DNA persists

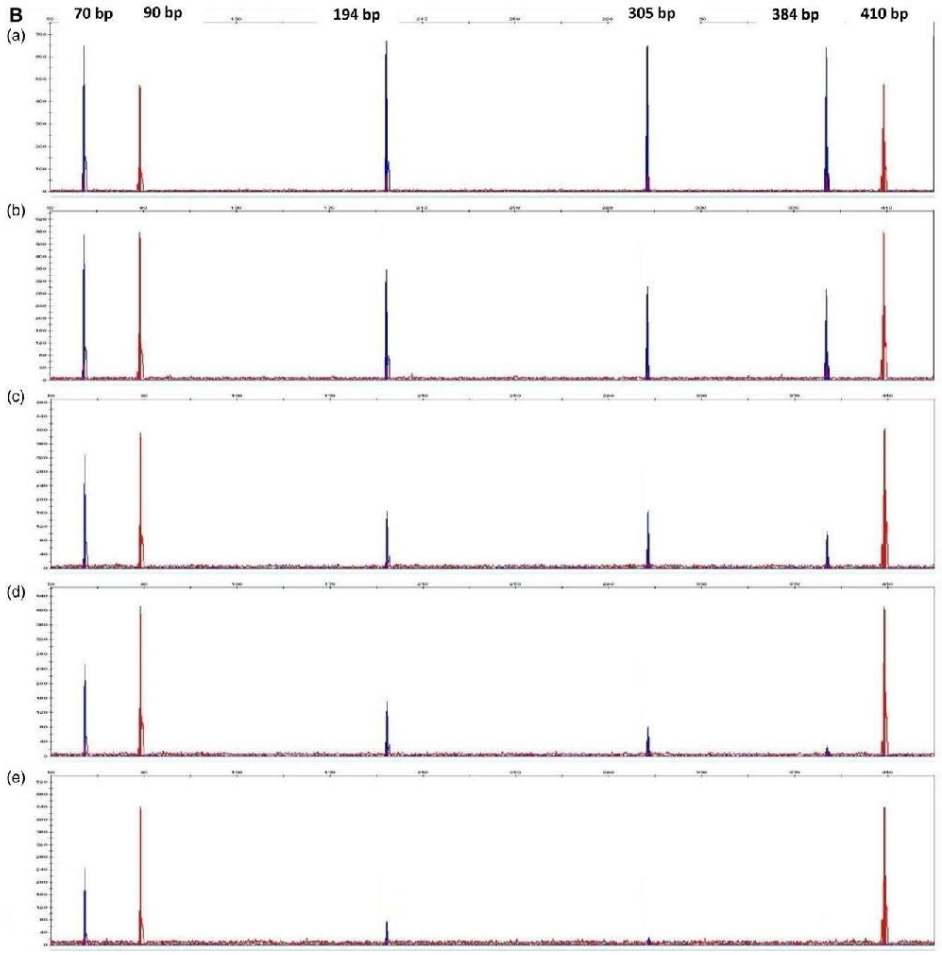

Figure 2. Continued.

for fewer days as the average temperature increases. However, full profiles could be obtained from all samples in the May series (average daily temperature 12.3°C) up to and including day 24 (ADD 295) and in September series (average daily temperature 11.4°C) up to and including day 30 (ADD 342). We could have potentially increased the number of loci amplified in some of the degraded samples by adding more template DNA, but in the context of this series of experiments were more interested in the general pattern of degradation and providing a conservative estimate of DNA persistence—with casework samples it may be appropriate to carry out further analysis.

The data were analyzed using the generalized linear mixed model fit by the Laplace approximation to measure analysis of deviance using the software R (R Development Core Team. http://www.R-project.org). The analysis demonstrated that the relationship between ADD and amplification success was not different for the 305 and 384 bp amplicons ($Z = 0.96$, $p = 0.76$), but was different for pairwise comparisons of all other amplicons. At time zero there was no difference in amplification success of the four amplicons, but with increased ADD the probability of getting the 194, 305, and 384 bp amplicons was 4, 6.15, and 5.92 times lower than getting the 70 bp amplicons ($p < 0.001$ for all permutations).

Table 2. Shown below are the results of 4-plex multiplex amplification of pig soft muscle tissues project

Days	ADD	Pig whole carcasses			
		70 bp	194 bp	305 bp	384 bp
(A) February 2010					
14	34	+++	+++	+++	+++
21	57	+++	+++	+++	+++
29	88	+++	+++	+++	+++
41	185	+++	+++	+++	+++
57	302	+++	+++	+++	+++
64	367	+++	+++	+++	+++
71	418	+++	+++	+++	+++
77	494	+++	+++	+++	+++
84	554	+++	++−	+−−	+−−
90	603	+++	++−	+−−	+−−
(B) May 2010 project					
6	77	+++	+++	+++	+++
13	159	+++	+++	+++	+++
16	203	+++	+++	+++	+++
24	295	+++	+++	+++	+++
27	338	+++	+++	+−−	+−−
(C) September 2010					
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	+++	+++
18	254	+++	+++	+++	+++
22	288	+++	+++	+++	+++
30	342	+++	+++	+++	+++
38	420	+++	−−−	−−−	−−−
44	490	+++	+−−	−−−	−−−

"+" denotes the presence and "−" represents the absence of a PCR amplicon.

The probability of getting 305 and 384 bp was 1.54 and 1.58 times less than the 194 bp amplicons ($p < 0.001$ for both permutations).

4 Discussion

A multiplex has been designed that will amplify four amplicons between 70 and 384 bp, which is a size range typically analyzed using commercial kits for forensic DNA profiling. The primers work robustly using between 2.80 and 0.3 ng of template DNA from humans, pigs, and rabbits, which gives the multiplex more utility than one that is specific to human DNA. In addition, the addition of the IACs to the multiplex allows for the simultaneous detection of PCR inhibitors. Similar multiplexes have been described in the literature previously for carrying out persistence studies, but to our knowledge this is the first system described that will work with humans and other mammalian species that can be used as model systems. The system was designed, in part, to be used for taphonomic studies at the University of Central Lancashire's TRACES facility, where pigs and rabbits are used as experimental models [29, 36, 44]. Designing a multiplex that can also be used with human DNA does offer some advantages to making it species-specific. When using it in the field experiment described, an advantage was that commercial human DNA standards could be obtained—these are routinely supplied as components of commercial quantification kits and PCR amplification kits. This allowed accurate quantification standards to be used in real-time PCR and also gives more confidence when assessing the sensitivity of the assay than using only in-house standards.

The utility of the multiplex has been tested in a series of taphonomic experiments to assess the persistence of DNA in muscle tissues. The trends seen in the results are as expected, with warmer temperatures leading to higher rates of DNA degradation. What is somewhat surprising is the time that DNA is present in the muscle tissues—even in summer; with daytime temperatures frequently exceeding 20°C, DNA over 384 bp long could be detected up to 24 days. In May a factor limiting the retrieval of DNA was the availability of soft muscle tissue, which was not present after 27 days. In the February series, the muscle tissue maintained its mechanical integrity and did not change much in appearance; in the May and September experiments, the muscle tissue again maintained its physical properties until the very last stages of the experiment (typically the penultimate or final sample) where the mechanical integrity of the muscle broke down with the muscle becoming soft and sticky—once the muscle reached this state it did not persist for more than 3–4 days. In all studies we did not see significant changes in muscle color with only slight fluctuations between pink and red.

The persistence of DNA in muscle tissue could influence practices in sampling of cadavers for the purpose of human identification, at least in temperate climates, with DNA successfully extracted from muscle recovered from what would be considered by most to be highly decomposed bodies. The results presented here can be compared with other studies, but direct comparison is difficult. A similar experiment carried out in Australia presented unusual results in that DNA could not be recovered and amplified from samples on day 2, and in several cases on day 0 [3], which suggest that either the PCR assay or extractions were not robust. Other experiments used excised tissues rather than whole animals [5], and in some cases these were not exposed to the environment [8, 14], which will have affected the DNA persistence. It is necessary to carry out more detailed studies in warmer climates before making any generalized statements about how long DNA might be available in muscle tissue. When applying the conclusions of these experiments to forensic casework care also has to be taken not to overinterpret the results as these are based on an experimental animal model. However, pigs have been used in many taphonomic studies as they have several features, such as size, anatomy and metabolic rate similar to humans and have been used extensively as a model in taphonomic research in Europe [29, 44–46].

Another utility of the multiplex is that it can be used for studying factors that affect persistence in other types of forensic evidence. Several studies have been described that have used commercial PCR kits to assess the level of degradation

[12–14]. This multiplex offers advantages over the commercial kits in that it is much less expensive and it is also amplifying homozygous nonvariable loci, removing the complication of samples from different donors having different sized alleles and combinations of homozygous and heterozygous loci. The addition of the IACs enhances the utility of this system over commercial systems in that the effect of inhibitors over the range of the multiplex can be assessed.

There is also the potential to use a multiplex such as this one as a screening tool for forensic analysis of material that is believed to be degraded, because such multiplex can potentially provide valuable information and minimize the waste of expensive reagents. Looking at the results in Table 2, it is possible to see samples where there is only amplification of the 70 and 194 bp amplicons, which would indicate that the use of a kit that is optimized to amplify small amplicons would be most beneficial. In other cases, the 70 bp amplicon had persisted after the other amplicons failed to be amplified—this would highlight the benefit of analyzing the samples with an alternative markers system that could utilize smaller amplicons, such as the SNPs and indels, where amplicons can be as small as 70 bp. The addition of internal amplified controls [30, 31] allows detection of PCR inhibitors and differentiation between DNA degradation and PCR inhibition, thereby enhancing the multiplex's ability to be used as a tool to assess degradation in different types of forensic evidence and to be used as a screening tool for complex forensic samples. However, if the system is used for screening complex samples to assess for inhibitors, it should be considered that different commercial PCR kits have different sensitivities, for example the Minifiler kit is more robust to inhibitors than the Identifiler kit [19] and in general later generations of commercial kits have higher resistance to inhibitors. Therefore, the relative impact of inhibition on this multiplex and the commercial kit of choice would have to be assessed to avoid overinterpretation. It should be noted that this same limitation exists with real-time PCR detection of inhibition in commercial kits, where the sensitivity of the detection varies between the real-time systems, for example Quantifiler and PCR multiplexes [30]. The best approach would be to incorporate the IACs into the commercial multiplex kit, however, this is a complex process [30, 31] and may not be practical in a commercial environment.

We would like to express our thanks to the staff and students working at TRACES, University of Central Lancashire, UK. In particular, we would like to thank Tal Simmons and Peter Cross for providing access to the facilities and logistical support throughout the project.

The authors have declared no conflict of interest.

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ARTICLE IN PRESS

Forensic Science International: Genetics Supplement Series xxx (2013) e1–e2

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

journal homepage: www.elsevier.com/locate/FSIGSS

Evaluation of five DNA extraction systems for recovery of DNA from bone

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ARTICLE INFO

Article history:
Received 4 September 2013
Accepted 2 October 2013

Keywords:
DNA extraction
Bone
Silica-based extraction
Forensic

ABSTRACT

Five DNA extraction systems were assessed for their DNA extraction efficiency on samples of fresh pig bone. Four commercially available silica-based extraction kits (ChargeSwitch® gDNA Plant Kit (Life Technologies), DNA IQ™ System Kit (Promega), DNeasy® Blood & Tissue Kit (Qiagen) and PrepFiler® BTA Forensic DNA Extraction Kit (Life Technologies)) and a conventional phenol-chloroform method were tested in this study. Extracted DNA samples were quantitated with GoTaq® qPCR Master Mix (Promega) using an Applied Biosystems® 7500 Real-Time PCR System and the extracts were amplified using an in-house multiplex system. The phenol-chloroform extraction produced higher yields of DNA than the silica-based extraction methods. Among the silica-based extractions ChargeSwitch® gDNA Plant Kit recovered the highest amounts of DNA. However, all methods produced DNA that could be amplified and none of the extracts contained any detectable inhibition.

1. Introduction

Isolation of DNA from skeletal human remains can be problematic. In addition to DNA degradation, which is enhanced by high temperature and humidity, there are often potent PCR inhibitors present within the samples. It is therefore important to extract the maximum amount of available DNA whilst removing any PCR inhibitors that may be present [1]. Phenol-chloroform-based extraction methods have been used for extracting DNA from bone for many years [2]. However, the use of phenol-chloroform method has reduced because of the toxicity of the chemicals used; in contrast silica-based methods have gained popularity due to their relative ease of use, their ability to remove many PCR inhibitors and also the potential to automate some steps in the extraction process [3,4]. However, all extraction methods are still in use in forensic laboratories.

2. Materials and methods

2.1. Sample collection and preparation

Fresh pig bone samples (femur and rib) were purchased from a butcher. Samples were stored at −20 °C and prior to extraction any soft tissue on the bones was removed. The bones were then soaked in bleach for 15 min, rinsed with water and then dried. Portions of each bone were prepared weighing between 1 and 2 g; after cutting any bone marrow was removed before pulverization under liquid nitrogen.

2.2. DNA extraction

DNA extraction was carried out using ChargeSwitch® gDNA Plant Kit, DNA IQ™ System Kit, DNeasy® Blood & Tissue Kit and PrepFiler® BTA Forensic DNA Extraction Kit and a conventional phenol-chloroform method, according to the manufacturer's instructions and published methods. Approximately 50 mg of pulverized bone samples were used in each extraction. Extractions were carried out in triplicate for each method and the final elution volume was set at 100 μl in each extraction.

2.3. DNA quantification and amplification

The extracted DNA samples were quantified with GoTaq® qPCR Master Mix using an Applied Biosystems® 7500 Real-Time PCR System. A 70 bp amplicon was targeted in reactions comprising 6.25 μl 2× GoTaq qPCR Master Mix, 4.75 μl H_2O, 0.5 μl primers and 1.00 μl template DNA. The thermal cycler conditions were: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The DNA concentration for each sample was estimated in ng/μl. All DNA extracts were amplified using an in-house multiplex [5] and inhibition was tested for using internal amplification controls [6].

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http://dx.doi.org/10.1016/j.fsigss.2013.10.090

Please cite this article in press as: S. Iyavoo, et al., Evaluation of five DNA extraction systems for recovery of DNA from bone, Forensic Sci. Int. Gene. Suppl. (2013), http://dx.doi.org/10.1016/j.fsigss.2013.10.090

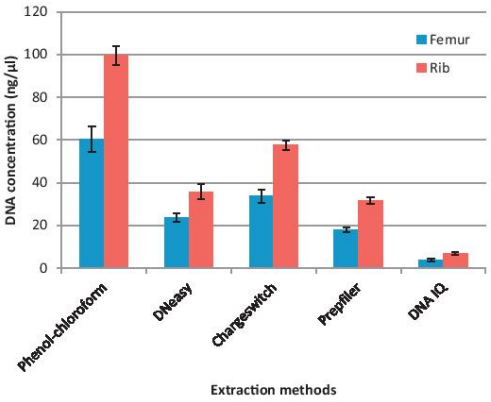

Fig. 1. Comparison of DNA yield from fresh pig bone samples (femur and rib) using different extraction methods: phenol-chloroform, DNeasy® Blood and Tissue kit, Chargeswitch® gDNA Plant kit, PrepFiler® BTA Forensic DNA Extraction Kit and DNA IQ™ System Kit. The final volume of all extraction methods was set at 100 µl.

3. Results

DNA was successfully extracted from bone samples using all tested extraction methods. The comparison study shows that the phenol-chloroform extraction method yields more DNA than the silica-based extraction methods (Fig. 1).

Although phenol-chloroform method produced the highest DNA yields, all samples could be successfully amplified using an in-house multiplex that targets four amplicons that range in size between 70 bp and 384 bp [5]. No inhibition was detected in any of the samples when amplifying two internal amplification controls [6].

4. Discussion

The main aim of this research was to assess the capability of five DNA extraction systems on bone samples. The ultimate aim of the extraction process is to obtain the maximum amount of DNA from the samples submitted. At the same time, it is also important to get a pure DNA extraction by eliminating inhibitors which can reduce the efficiency of the amplification process, thus selection of extraction techniques is very important for samples containing PCR inhibitors.

All of the tested methods have proven to be suitable in that amplifiable DNA has been extracted. However, the samples tested so far are fresh bones which contain abundant DNA. When dealing with more challenging samples the increased recovery of DNA seen with the phenol-chloroform method may become more important. Although phenol-chloroform method produces high DNA yield in this study, previous studies show that silica-based extraction methods can be more efficient at removing inhibitors from bone samples [7]. Also, when using with environmentally challenged samples the ability to remove PCR inhibitors will become more relevant [1].

Further studies will be undertaken using degraded bone samples and also bone samples that have been shown to contain high levels of PCR inhibitors. This will allow the most appropriate method for extracting DNA from samples that have been exposed to environmental insult and are likely to be problematic.

Role of funding

This study was funded by a grant from Public Service Department of Malaysia.

Conflict of interest

None.

Acknowledgements

We thank the Director General, Section Head and the staff at the Forensic DNA Section, Forensic Division, Department of Chemistry (Kimia), Ministry of Science, Technology and Innovation in Malaysia.

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Please cite this article in press as: S. Iyavoo, et al., Evaluation of five DNA extraction systems for recovery of DNA from bone, Forensic Sci. Int. Gene. Suppl. (2013), http://dx.doi.org/10.1016/j.fsigss.2013.10.090