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DNA Extraction from Blood in Soil

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Degree of Bachelor Science in Forensic Science

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ABSTRACT

DNA analysis from crime scene samples involve blood that mixed with soil had currently brought difficulties to forensic scientist due to the presence of inhibitors and also environmental factors that influenced the quality of the DNA. Murder is the main crime involved in this situation. Due to difficulties, a simulation of crime scene had been setup for the study. Blood from different individuals from Blood Transfusion Centre, Universiti Sains Malaysia Hospital had been poured to the chosen soil location (A and B) and had been left for 28 days for study. The study aims for determination of DNA concentration from extracted blood on soil in both the locations; determination of concentration of DNA from soil after a period of time and also soil in different levels of depth by using Quantifiler kit (Applied Biosystems) using Real-Time PCR. Environmental factors that had been set to study towards the samples are the pH of soil, humidity of soil and weather throughout 28 days of study. The results obtained from the study are unreliable although the Real-Time PCR successfully quantitated the DNA. However, only 6 samples out of 38 samples are able to be quantitated. The remaining samples have failed to be quantitated due to the presence of natural inhibitors in the soil, and acidic pH of soil, high humidity of soil due to frequent raining during the period of study. The study should be further explored for more reliable results by demonstrated multiple extraction methods for removal of inhibitors in the soil besides optimize the quantity and the quality of the DNA extracts in the extraction process.

1.0 INTRODUCTION

1.1 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is a negatively charged polynucleotide that usually exists in the form of double-helix. The two strands linked with hydrogen bonds running in opposite directions. Each single strand DNA chain consists of a backbone made up of deoxyribose sugar molecules linked together at the 5' and 3' positions by phosphate ester groups. To accomplish the nucleotide structure a purine or pyrimidine base is attached as a C–N bond to the deoxyribose sugar molecules at the 1' position. In natural conditions DNA is a hydrated macromolecule, with 8–10 tightly bound water molecules per nucleotide residue. As a highly reactive chemical residue, DNA is the target of several physical agents and chemical reactions. DNA analysis is widely used for human identification purposes in crimes, mass disasters and terrorist attacks. In these circumstances, forensic investigation of biological items often requires the analysis of highly degraded material (Alaeddini *et al.*, 2009).

Polymerase chain reaction (PCR) is currently the method of choice for in vitro amplification of DNA molecules ahead of forensic DNA analysis. During the decomposition process, the DNA templates can become highly fragmented or chemically modified, therefore reducing the yield of intact target fragments and consequently leading to failure or poor PCR amplification or mistyping of the target loci. Awareness of DNA degradation pathways and mechanisms would assist in choosing the most appropriate target samples and in designing improved techniques for sampling, storage, purification and probable repair. Moreover consideration of the original

phenomena that drives in DNA degradation could assist in better interpretation of the results (Alaeddini *et al.*, 2009).

The ability to extract and PCR amplify DNA from biological stains is a key element in forensic DNA analysis. DNA often survives for months or years at ambient conditions in untreated dry stains. The effects of various parameters on the survival of DNA, such as UV-light, stain substrates, admixture of soil, age, humidity, and temperature have been previously assessed. However, to the knowledge; there is little information about the stability of DNA in stains when controlled conditions of humidity and temperature approach the extreme. Microbial enzymatic attack is of fundamental importance in the degradation of organic matter but depends on the availability of water. Also many of the non-enzymatic processes that degrade DNA depend on the presence of water. A priori one would therefore expect an accelerated rate of degradation of DNA in biological stains as the relative humidity approaches 100%. To determine the range of natural conditions that allow an adequate stability of DNA in stain material, blood stains without any stabilizing additives were incubated at various conditions of relative humidity (RH) and temperature. The quality and quantity of the remaining DNA was estimated by the ability of PCR to amplify long and short fragments and by quantitative assessment using real time PCR (Dissing *et al.*,2009).

1.2 DNA enzymatic degradation

Several factors affect the rate of fragmentation (and hence the detectability of the residual DNA) including the expression level of the enzymes, availability of cations and solution for the reactions, temperature and pH levels. Under favorable conditions such as low temperatures or where tissue dependent natural protective measures exist, nuclease enzymes may become inactive before they fully break down long strands of DNA into shorter, less informative fragments. The high molecular weight (HMW) fragments are produced in the presence of Mg^{2+} as the sole divalent cation, whereas oligonucleosomal fragments are produced when both Mg^{2+} and Ca^{2+} are present in the digestion buffer. Ca^{2+}/Mg^{2+} DNase have been reported as a major feature in apoptosis. DNAse activity in some tissues and conditions such as empyema might be so slight or absent that the DNA from the dead cells forms a solidified shape requiring external DNAse injections to break it down. Physiological concentrations of Zn^{2+} are also known to inhibit DNA fragmentation and Ca^{2+}/ Mg^{2+} nuclease activity (Alaeddini *et al.*, 2009).

The rupture of cell membranes during the later stages of cell death leads to the release of nutrient-rich fluids, which encourage the growth of environmental microorganisms and therefore further degradation of macromolecules. More than 70% of soil microorganisms contain nuclease enzymes. The predominating soil microorganisms are able to decompose nucleic acids and most of their degradation products. Factors such as the availability of the appropriate nutrients, pH, electron acceptors and donors, ionic concentration, and the absence of toxic metal ions will determine which microbial communities will colonize the post-mortem tissue. Few environmental situations can properly exclude the microbial activity. Incorporation of molecules

into bio-minerals might be one of those preservative conditions as it happens in extracellular bone matrix. The availability of liquid media increases the vulnerability to degradation. Such media use available during the very first stages of post-mortal period by the tissue matrix or later on through the accumulation of bacterial liquid products (Alaeddini *et al.*, 2009).

1.3 DNA admissibility

Over the past twenty years, DNA analysis has revolutionized forensic science, and has become a dominant tool in law enforcement. Today, DNA evidence is a key to the conviction or exoneration of suspects of various types of crime, from theft to rape and murder. The DNA profile of every person is considered unique (except for identical twins) , and consequently, this "DNA fingerprint" is used in police investigations to link between a crime scene and a specific individual, who is either a suspect in the case, or identified by an automatic search of the database (e.g. CODIS). In recent years, DNA evidence has become the "gold standard" of forensic testing, and is an invaluable tool for the criminal justice community (Frumkin *et al.*, 2009).

The high credibility of DNA evidence in court stems from the fact that it uses a statistical approach based on population genetics and empirical testing, in contrast to other types of forensic evidence, such as ballistics, blood-spatter analysis, and fiber analysis, which rely on expert judgment and have limited connection to established science. It is even considered to be more reliable than eyewitness evidence, which is known to suffer from a relatively high rate of errors. (Frumkin *et al.*, 2009)

Blood is one of the most common evidence of crime scenes (Turrina *et al.*, 2008). Blood stains may be found anywhere, they can be scattered on the floor, spattered on the wall and could be analyzed to reconstruction of the scene of crime. The collected material is submitted to a series of scientific techniques; aiming to compare and establish the identity of both the victim and the criminal. (Eloisa *et al.*, 2009)

Estimation of the time since death is a practical task in daily forensic case work (Henssag & Madea, 2007). In the last 60 years, numerous methods have been proposed for determination of time since death by physical evidences, environmental evidences or chemical means. None of these early methods has gained any practical value since they do not meet the demands in practice to be precise, reliable, giving an immediate result (Madea, 2005). Accurate estimation of post mortem interval remains a problem that need to be solved, for its great value to criminal investigation (Chen *et al.*, 2009). From the point of view of criminal law, a precise estimation of time of death enables to verify witness' statements, limit the number of suspects and assess their alibis (Kaliszan *et al.*, 2009).

DNA samples recovered from a crime scene are often subjected to detrimental environmental conditions, before they collected for analysis; these effects further test the scientists' ability to produce an evidentially valuable profile from a sample already compromised in terms of quality (Thacker *et al.*, 2006). This is due to doubt whether the DNA in the blood has already been decomposed or contaminated. This is because the factors that influence the decomposition of DNA in the blood are extremely high. Blood stained soils may be of great interest in forensic

incidents. Amplification of DNA from soil is often inhibited by co-purified contaminants. In this study, soil was stained with blood and samples were collected systematically after specified intervals.

In this study, we focused on obtaining DNA profiles from blood found in soil. Soil can provide important information to criminal investigations as transfer evidence because many criminal cases take place under circumstances such that soil transfers to a criminal or victim. (Thornton *et al.*,1997). Thus soil can be a good source of evidence especially in murder cases when it is stained with blood. Blood stained soil samples can be obtained after some incident. The availability of the samples from such cases and places is directly affected by soil pH, soils color, nutrients, humus and microorganism present in the soil. (Shahzad *et al.*, 2009)

The objective of this study is to determine whether the DNA profile can be generated or not according to the determined factors; i.e. moisture, soil pH, soil depth and duration of blood on the ground. With the acquisition of data from this study, the effort to get DNA profile of blood on the ground in a crime incident can be simplified. This is because after a complete review, the time period to get DNA profile of blood can be optimized.

2.0 LITERITURE REVIEW

According to research article about effect of blood stained soils and time period on DNA and allele drop out using Promega 16 Powerplex kit by Shahzad, soil can provide important information to criminal investigations as transfer evidence. Rapid, inexpensive, large-scale DNA extraction method involving minimal purification was developed. DNA was quantitated using Spectrophotometer and Fluorometer and was confirmed by agarose gel electrophoresis. DNA extracted from different soils in different periods showed a remarkable decrease in yield as well as degradation in every extraction. PCR amplification was performed using various DNA targets present in Promega 16 Powerplex System kit. Amplification could not be carried out in all loci especially in degraded samples taken after 20 days. Allele locus drop out was noticed which shows that DNA was degraded. For some loci more than 2 alleles were also noticed showing contamination while working with the blood stained soils.

Meanwhile, a previous European DNA Profiling Group (EDNAP) study about analysis of artificially degraded DNA using short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) using DNA degraded by sonication and DNAse I, and other studies using degraded body fluid stains and telogen hair roots, has demonstrated that the efficacy of low molecular weight amplicons can be used to analyze degraded DNA. The experiment described by Dixon *et al.* (2005) followed a different design to those previously described, as it simulated a time-course series of degraded stains in their 'natural state'. This was achieved by incubating material spotted with saliva and blood in 100% humidity at 37 °C. Under these conditions,

degradation was greatly accelerated compared to the dried state process and total degradation was achieved within a short time period of 12–16 weeks.

McNally *et al.*, 1989 study was designed to analyze the effect of common environmental insults on the ability to obtain DNA restriction fragment-length polymorphisms (RFLP) patterns from laboratory prepared specimens. The environmental conditions that had been studied include the exposure of dried bloodstains to varying amounts of relative humidity (0, 33, 67, and 98%), heat (37°C) and ultraviolet light for periods of up to five days. In addition, the effect of drying over a four-day period in whole blood collected with and without ethylenediaminetetraacetate (EDTA) was examined. The results of the study showed that, under the conditions studied, the integrity of DNA is not altered such that false RFLP patterns are obtained. The only effect observed was that the overall RFLP pattern becomes weaker, but individual RFLP fragments are neither created nor destroyed.

The study of DNA damage caused by pesticide-contaminated soil by Krishnamurthi *et al.*, 2006 found that the DNA strand breaks can be detected with a great sensitivity by utilizing the rate of unwinding of the DNA strands in alkali which is related to the covalent length of the strands. It is due to the low levels of base damages; as little as one break per chromosome can give a detectable increase in the rate of unwinding which are difficult to be measured by physical or chemical means.

Morgan and Pulleyblank, 1974 have reported that the ethidium bromide could bind selectively to double stranded DNA in the presence of single stranded DNA when short duplex regions in

single stranded DNA are destabilized by alkali. Fluorimetric analysis of DNA unwinding (FADU) is one of the sensitive techniques for measuring DNA damage induced by chemicals *in vivo* as well as *in vitro*. Krishnamurthi *et al.* (2006) used a methodology to assess DNA damaging potential of contaminated soil from a dumpsite near a carbamate pesticide manufacturing industry located in Central India. The procedure adopted by him was very rapid and sensitive; DNA can be analyzed directly without culturing or radio labeling. The above technique was used in the present study to evaluate the genotoxic potential of the pesticide-contaminated soil collected from a dumpsite near a carbamate pesticide manufacturing industry (Krishnamurthi *et al.*, 2006).

Forensic biology samples such blood may contain highly fragmented DNA molecules caused by various damaging agent. The current protocols in forensic DNA analysis of human remains are based on size or sequence analysis of PCR products. PCR based protocols although highly effective may encounter complication through the low copy number of the template or the modifications imposed on the template during the decay process. A diploid human cell contains ~ 6.6 pg of genomic DNA. A template DNA concentration <100 pg genomic DNA (about 15 - 17 diploid copies of nuclear DNA markers such as autosomal STRs) is considered as low copy number (Alaeddini *et al.*, 2009)

Few studies have been undertaken on the underlying causes of artefacts encountered during amplification of degraded forensic specimens. In one study performed on selected aged forensic samples the frequency of artefacts including base substitutions and ambiguities was calculated as being at least 30-fold higher than the controls, with DNA oxidation products among the most

abundant base modifications in aged samples. The three main complications observed following PCR of degraded DNA samples are the failure of amplification, preferential amplification and miscoding lesions (Alaeddini *et al.*, 2009)

In degraded samples there is a higher chance that shorter amplicons will be amplified compared to longer amplicons. This is due to the higher likelihood that longer products will be degraded during the decay process. Multiplexing the PCR reaction reduces the amount of sample material necessary for analysis and minimizes the experimental time and costs. Extra doses of Thermus aquaticus (Taq) polymerase and extra PCR cycles have been suggested to overcome the low profile amplification of low copy number templates. Therefore in multiplex genotyping profiles with a wide range of amplicon sizes, the peak positions produce a curve in which the peak heights are inversely proportional to the amplicon lengths (Alaeddini *et al.*, 2009).

As amplification and profiling of shorter fragments is more successful in degraded templates, the newer versions of primer set re-position the primers as close as possible to the repeat motif to reduce the overall amplicon length. These reduced sized targets are referred to as Mini-STRs. The smaller products would also serve as better templates for analysis by alternative technologies such as time-of-flight mass spectrometry and rapid micro channel electrophoretic separations. This strategy reportedly reduced the required amount of DNA for amplification of genomic templates up to 100 pg/25 ml. In recent years forensic scientists have focused increased attention on single nucleotide polymorphisms (SNP) analysis. A relatively small array of 50 SNP loci gives likelihood ratios equivalent to approximately 12 STRs. Short SNP fragments can be

designed to analyze the degraded samples. Nevertheless there is a greater chance of base alterations in aged post-mortem samples (Alaeddini *et al.*, 2009).

Beside the potential errors in sampling, DNA purification, DNA quantitation, PCR and automated sequencings, a major concern in analysis of forensic specimens arises from the coexistence of PCR inhibitors in the extracted material. Through a variety of mechanisms, endo- or exogenous inhibitors would hamper the progression of PCR amplifications. The inhibited and low copy number profiles may look similar at some points; however a detailed discussion on the composition or the effects of those materials on forensic genetic analysis is beyond the context of the current paper (Alaeddini *et al.*, 2009)

However, according Romanowski *et al.*, 1992; Trevors and van Elsas, 1989 to working with DNA recovered from soils is often problematic. Many soils contain organic compounds such as humic substances that inhibit restriction endonucleases and *Taq* DNA polymerase, which is the key enzyme of PCR (Porteous and Armstrong, 1991; Tsai and Olson, 1992). Humic substances are a major component of soil organic matter. Though the chemical composition is highly complex, these compounds readily co-purify with DNA and are difficult to remove without additional, laborious and time intensive treatments to obtain DNA suitable for PCR (Braid *et al.*, 2002).

3.0 OBJECTIVES OF THE STUDY

This study was conducted in order to determine the amount of DNA from blood that is available in soil after exposure to different factors such as moisture, soil pH, soil depth and duration of blood on the soil. With the acquisition of data from this study, the effort of getting DNA profile of blood in soil at a crime scene can be performed within the optimized condition. The specific objectives of this study are:

1. To determine the DNA concentration extracted from blood on soil in shaded and open area after exposure over a period of time.
2. To determine the DNA concentration extracted from blood on soil in open and shaded area at different depth.

4.0 MATERIALS AND METHODS

4.1 Materials

4.1.1 Preparation samples

a) Bags of blood

b) Two soil samplers

c) A hammer

d) Two wire cages

e) Gloves

4.1.2 Collection samples

a) 50 ml Falcon tubes

b) Spatulas

c) Weights

d) 1.5 ml Eppendorf tubes

e) Gloves

4.1.3 Samples extraction

a) 1.5ml microfuge tubes and tube rack

b) A microcentrifuge

c) Vortex

d) Water bath

e) Gloves

f) Reagents:

1X SSC buffer, 0.2M Sodium Acetate, 10% Sodium Dodecyl Sulfate (SDS), Proteinase K, Phenol/chloroform/isoamyl alcohol, Cold ethanol, TE buffer and 70% ethanol

4.1.4 Quantifier® Human DNA Quantitation kit

a) Reagents:

i. Quantifier ™ PCR Reaction Mix: it is a pre-formulated mixture containing Amplitaq Gold DNA polymerase and passive reference ROX ™ dye.

ii. Quantifier ™ Primer Mix: it is pre-formulated mixture containing primer, probe and IPC template.

iii. Quantifier ™ DNA standard: Contains genomic DNA standard.

b) Adjustable volume micropipettes: 10µL; 20µL, 100µL; 1000µL

c) Disposable sterile pipette tips: 10µL; 200µL; 1000µL

d) 1.5mL tubes and tube rack

e) MicroAmp ™ Optical 96-well reaction plate

f) Optical adhesive covers

g) Adhesive film applicator

h) Vortex and centrifuge

i) Gloves

4.2 Methods

4.2.1 Sterilization

All glassware, pipette tips, tubes, deionised water, and buffer that were used in this project were in sterile conditioned by autoclaving at 20 psi for 15 minutes before used.

4.2.2 Sample Preparation

The simulation of the crime scene had been done by pouring two bags of blood (250 ml) from different individuals onto two different chosen ground location. The blood was obtained from the Transfusion Centre of Universiti Sains Malaysia Hospital. Blood had been poured directly onto the ground in shaded (A) and open area (B). The sites were covered by wire cages for security purposes. Date and time has recorded.

4.2.3 Controls

DNA from blood was extracted prior to pouring the blood into soil which acts as reference. The soil was also collected before the blood was poured. PH and moisture content of the soil was determined. The control soil samples were collected on 1^{st}, 7^{th}, 21^{st} and 28^{th} day. The weather condition and temperature during experiment was obtained from the Malaysian Meteorology Department.

4.2.4 Sample Collection

Soils samples were collected for 6 times for DNA quantification process from 1st day, 3rd day, 7th day, 14th day, 21st day and 28th day. Soil was collected using soil sampler, a 9 inches long of hollow steel rod. The soil sampler was hammered through the soil by using hammer to collect the soil. The collected soil was divided into three parts; 1to 3 inches (part 1), 3 to 6 inches (part 2) and 6 to 9 inches (part 3). The collected soil was kept in 50 ml Falcon tubes according to its depth and labeled.

4.2.5 Separate Working Areas

Real-time PCR reaction has prepared in a flow hood to avoid extraneous contamination. The flow hood was cleaned by 70% alcohol prior and after completion of analysis. Post PCR products were handled and stored in a separate area to prevent contamination of the amplified DNA in laboratory. Set of pipettes, pipette tips and other consumables were designed to each working area to prevent cross contamination.

4.2.6 Soil Samples

Samples collected from the simulated crime scene were stored at -20 °C until further use. Approximately 100 mg of soil was used for DNA extraction process. The soil was transferred into 1.5 ml microfuge tube and weighed.

4.2.7 DNA Extraction

A total of 0.8 ml of 1X SSC buffer was added to 100 mg of soil and mixed. The samples were centrifuged for 1 minute at 12,000 rpm. The supernatant was removed and discarded into waste container. One ml of 1X SSC buffer was added again; the samples were vortexed and centrifuged for 1 minute, and all supernatant was removed. 375 µl of 0.2M sodium acetate was added into each tube and vortexed briefly for homogeneity. A total of 25 µl of 10% SDS was added into the tubes and continued with addition of 20 µl of Proteinase K. Tubes were vortexed briefly and incubated overnight at 55°C in waterbath.

On the next day, 120 µl phenol chloroform isoamyl alcohol was added into the tubes and vortexed for 30 seconds. The samples were centrifuged for 2 minutes at 12,000 rpm. The aqueous layer was carefully removed to a new 1.5 ml sterile microcentrifuge tube. One volume of phenol chloroform isoamyl was added and the tubes were mixed vigorously. The tubes were centrifuged at 12,000 rpm for 2 minutes and supernatant formed was transferred into a new 1.5 ml microfuge tube. An amount of 20 µl of 2 M sodium acetate was added into the tubes. A total of 500 µl of cold ethanol was added, mixed, and centrifuged for 1 minute at 12,000 rpm. The supernatant was decanted and the precipitate was rinsed with 1 ml of 70% ethanol and centrifuged again for 1 minute at 12,000 rpm. The supernatant was again decanted and the tubes were left for complete drying overnight at room temperature. DNA was resuspended by adding 30 µl of TE

buffer and briefly vortexed to dissolve DNA. Finally, the tubes were stored at -20°C until further use.

4.2.9 DNA Quantitation using Quantifier kit on ABI 7500 Real-Time PCR

4.2.9.1 Creating a plate document:

Running a reaction plate on the 7500 requires creation and setting up plate document using the SDS software. A plate document is a representation of the arrangement of samples (standard and unknown). The SDS software uses the plate document to coordinate the instrument operation, such as thermal cycling and data collection. Organizing and storing the data gathered during the run and analyzed the data from the run.

After the SDS software was launched, a new file was created. The document wizard was filled in with sample name, type of detector and color for each detector (Human: FAM, IPC: VIC).

The human detector and IPC detector were selected for all samples, including standards. The quantity for each standard was entered in the respective column as listed in Table 1. After all the samples information was entered, the sample plate was saved and is now ready to use.

4.2.9.2 Preparation of DNA standards

Before use, all reagents had been brought to room temperature. The Quantifier ™ DNA standard was vortexed and centrifuged before use while preparing dilutions. The quality of tips and pipette in preparation of standards are important as they affect accuracy and duplicates of standards were run for each assay.

Microfuge tubes (1.5 ml) was obtained and labeled 1 to 8. Fifteen µl of TE buffer was dispensed in tube 1 and 10µL of TE buffer in tubes no.2 to 8. Ten µL of DNA standard was dispensed to tube 1 and the tube was vortexed and centrifuged. A total of 5µL was withdrawn from tube 1 into tube 2. Tube 2 was then vortexed and centrifuged. This process was repeated subsequently until tube no. 8.

4.2.9.3 Preparation of Quantifier reactions

Number of samples, standards and controls were determined before the master mix was prepared. The primer mix was vortexed and centrifuged while the reaction mix was gently swirled to mix its content. The appropriate amount of primer mix and reaction mix (as determined by the total reaction required) was combined into microfuge tube. The master mix was vortexed and centrifuged. Additional sample number (+5) was

added to the total samples in order to compensate pipetting error. A total of 18.4μL of the master mix was aliquoted into each well. The standards prepared earlier and the unknown samples were vortexed, spin down and 1.6 μl was dispensed into each respective well as recorded on the plate. Next, the 96 wells plate was sealed using adhesive cover and placed into the Real-time PCR machine with the notched edge in the upper right hand corner. Finally, the start tab was clicked and the amplification process was started.

Table 1: Series of standard DNA dilutions.

STANDARD	CONCENTRATION	PREPARATION
A1 and A2	50.000 ng/µl	15 ng/µl TE buffer + 5 ng/µl DNA standard
B1 and B2	16.700 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. A]
C1 and C2	5.560 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. B]
D1 and D2	1.850 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. C]
E1 and E2	0.620 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. D]
F1 and F2	0.210 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. E]
G1 and G2	0.068 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. F]
H1 and H2	0.023 ng/µl	10 ng/µl TE buffer + 5 ng/µl [Std. G]

Table 2: Master mix preparation volume per sample.

Component	Number of samples (n)	Volume per sample (µl)	(n+5) x volume per sample = total volume
Primer mix	48	8.4	445.2 µl
Reaction mix	48	10	530.0 µl

5.0 RESULT AND DISCUSSION

5.1 DNA Extraction

In this study phenol chloroform extraction method was used to extract DNA from blood in the soil samples. This method was chosen since it is cost effective and easy to perform. In this method, several chemicals were involved, which have their own function in the analysis. SSC buffer (1X) was added to the samples in order to break the cell wall. The addition of Proteinase K was to digest protein and release DNA in solution. In order to make sure that the protein digestion was complete, the samples were incubated overnight. Phenol was added to precipitate protein and separate it from the supernatant containing DNA. Chloroform-isoamyl alcohol was added in order to wash away any remaining protein and phenol. This step is important since phenol can act as inhibitor in the Real-Time PCR process.

The chilled ethanol was used to precipitate the DNA. Ethanol causes every component used to stay in solution except DNA. Sodium acetate (2M) was used to enable DNA to precipitate out of alcohol solution because it shields the negative phosphate end of DNA, causing the DNA strands to come closer together. DNA that was precipitated at the bottom of the microfuge tube was rinsed using 70% ethanol. Salt residues were removed by 30% water during the wash. The tubes were left open on bench overnight at room temperature to make sure the samples were free from any ethanol residues. Finally, TE buffer was added into the tubes to resuspend DNA. TE Buffer was used since it can protect DNA from degradation.

5.2 DNA Quantitation using Quantifier kit on ABI 7500 Real-Time PCR

Real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA or sequence specific probes. Real time PCR facilitates the monitoring of the reaction as it progresses.

One can start with minimal amount of nucleic acid and quantify the end product accurately when using Real-Time PCR. Moreover, there is no need for the post PCR processing which saves the resources and the time. The advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantitation of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation.