# Investigation into effect of sample storage time on DNA analysis from human femur

**Authors**: Khaing Yee Mon Aung[a], Prapaporn Khaiprapai[b], Chanidapa Srinongwa[b], Worawee Waiyawuth[b], Siwaporn Mejoo Smith[c], and Nathinee Panvisavas[d]*

[a]Forensic Science Program, Multidisciplinary Unit, Faculty of Science, Mahidol University

[b] Bureau of Forensic Biology, Central Institute of Forensic Science, Ministry of Justice

[c] Department of Chemistry, Faculty of Science, Mahidol University

[d] Department of Plant Science, Faculty of Science, Mahidol University

* Corresponding author/ Major Advisor

## Abstract

Prolonged storage of bone samples after the first DNA analysis is inevitable in cases of unidentified human remains because these skeletal remains may be the only piece of evidence for positive identification. Often the re-testing for results confirmation would be required. However, the long term storage could impact the chance for obtaining useful results in future analysis due to the possibility of DNA degradation in the samples. Therefore, the present study aims at investigating the effect of sample storage time on human bone DNA analysis. For the purpose of comparison, post-storage DNA analysis was carried out on 10 femur samples of unidentified human skeletal remains, exactly as pre-storage analysis. The results indicated the occurrence of DNA degradation in both pre- and post-storage bone samples. The STR typing results of post storage analysis revealed increased nuclear DNA degradation as indicated by allelic and loci drop-outs, lower peak heights, peak imbalances, and degraded profile pattern. Post-storage mtDNA HV fragment analysis of 9 samples revealed that the HVI and HVII fragments were intact; in contrast, fragmentation of large HV fragment was evidence. These suggested the possibility of successful mitotyping for HV I and HV II regions.

**Keywords**: Sample storage, DNA analysis, human femur

## Introduction

In the field of forensic DNA analysis, storage of biological samples is an important necessity for preservation of the integrity of forensic samples. For cases of unidentified human remains, sometimes, DNA may be the only item of evidence that can be recovered. As long as these human skeletal remains are still unidentified, the bone pieces or bone powder have to be kept for long-term because of the possibility of retesting these samples.

Because of profound degradation of DNA extracts at room temperature (Christophe Frippiat *et al.* , 2104), to preserve DNA, freezing of the extracts becomes the most commonly used method for storage. However, this method is costly and requires a large number of freezer-spaces and back-up generators. For these reasons, after the routine analysis, DNA evidences of unidentified human skeletal remains are usually stored in the form of the analyzed data and the original sample, such as bone pieces or bone powder. However, it is important to detect the effect of long-term sample storage time on the further replicate tests because degradation of DNA in the samples could impact obtaining useful results.

Therefore, in the present study, the effect of sample storage time on DNA analysis was investigated using 10 forensic human femur samples. Last 2 and 3 years, forensic DNA analysis was conducted for these bone samples and the remaining samples had been keeping in sterile plastic containers at room temperature, either in the form of bone pieces or bone powder. To detect degradation of DNA in each sample during storage period, quantitative and qualitative data of new extracts after 2 or 3 years of storage were compared with corresponding previous data obtained from pre-storage analysis. The steps and protocols for post-storage nuclear DNA analysis was carried out as exactly as in the pre-storage analysis. Evaluation of the extent of mtDNA degradation in post-storage extracts was conducted by PCR amplification and detection of 3 human mitochondrial HV fragments of defined sizes with 3 human HV primer sets and gel electrophoresis.

**Objectives**;

1. To investigate the effect of bone sample storage time on human DNA analysis

## Research methodology

*Materials*

A number of femurs from 10 unidentified human skeletal remains stored in Forensic DNA laboratory, Central Institute of Forensic Science (CIFS), in which 6 were bone pieces and 4 were bone powder samples, were used as starting materials. The average dimension of each bone piece was 8×2×0.8 cm (length×width×thickness). Among 10 samples, 5 samples were submitted for DNA analysis in 2011 and the other 5 samples in 2012. To date, storage time was approximately 3 and 2 years, respectively. Bone pieces were stored in sterile plastic containers, and dried bone powder samples were stored in sterile polypropylene tubes. All samples were stored in the bone cabinets at room temperature (25-30ºC).

For bone piece samples, bone surfaces were mechanically cleaned with a sterile scalpel, and then chemically with 10% commercial bleach and 95% ethanol solutions, and allowed to air-dry. Then, bone pieces were grinded into fine powder under liquid nitrogen, using the cryogenic 6750 Freezer/Mill® (SPEX SamplePrep, USA).

*DNA recovery*

An aliquot of 2 g of bone powder was decalcified in 10 ml of 0.5 M EDTA, pH 7.5, and incubated overnight in a shaking-incubator at 30ºC. An aliquot of 100 µL sample solution was taken to check the end point of decalcification by adding 100 µL of 5M ammonium oxalate solution. The presence of calcium in the sample would make the test solution become turbid. Then, the decalcified sample was centrifuged to collect the pellet, and further washed with distilled water for 3 times. Then, DNA was extracted by using DNA IQ™ kit (Promega, USA) according to the manufacturer's protocol. Samples were then quantified by using Quantifiler Human DNA Quantification Kit (Applied Biosystems, USA).

*Analysis of human DNA*

For nuclear DNA analysis, samples were DNA-typed by using the PowerPlex® 16 HS System (Promega, USA) according to the manufacturer's protocol. The PCR products were separated in ABI Prism 3130 Genetic Analyzer Instrument (Applied Biosystems, USA), and analyzed with GeneMapperID® software (Applied Biosystems, USA).The DNA profiles of the samples were compared to the laboratory staff database, to ensure that there were no exogenous contaminations (i.e. drop-in STR alleles).

For mitochondrial DNA fragment analysis, 4 human mitochondrial HV primers were designed from the revised Cambridge Reference Sequence of human mitochondrial DNA complete genome (accession number: NC_012920.1) and optimized. Details of primers are shown in table 1. Amplification was carried out in a Veriti®thermocycler (Applied Biosystems, USA) by touch-down PCR in a total reaction volume of 25 μL, containing 20-40 ng of DNA template and PCR MasterMix (5μL of 5X Go Buffer, 1.5 μL of 0.3 mM Betaine, 2.5 μL of 2.5 mM $MgCl_2$, 0.5 μL of 10 mM dNTP, 1 μL of 10 pmol of corresponding forward and reverse primers, 0.2 μL of 5 U/μL Go Taq Polymerase, sterile deionized nuclease free water to fulfill final volume). Expected mtDNA PCR product sizes were 461, 368, 1066 bp for HV I, HV II, and HV I+II fragments, respectively. Then, PCR products were separated in a 2% (w/v) ethidiumbromide-stained agarose gel/TBE, and photographed using the gel documentation system (Syngene, USA).

**Table 1** Human mitochondrial HV primers

| Primer name | 5'-Sequence-3' | Tm °C | Length | position | GC ratio |
|---|---|---|---|---|---|
| HV I F | 5'-CTCCACCATTAGCACCCAAAGCT-3' | 70 | 23bp | 15975 to 15997 | 55% |
| HV I R | 5'-TTCACGGAGGATGGTGGTCAAGG-3' | 72 | 23bp | 16394 to 16416 | 56% |
| HV II F | 5'-ACGGGAGCTCTCCATGCATTTGG-3' | 72 | 23bp | 32 to 54 | 60% |
| HV II R | 5'-AATCTGGTTAGGCTGGTGTTAGG-3' | 68 | 23bp | 370 to 392 | 45% |

## Results

### 1. Quantitative results

Comparison of Real-Time quantification results between pre- and post-storage extractions, were presented in table 2. The average DNA yield of 10 samples from pre-storage analysis was (0.861±0.771) ng and that of the same samples after 2 and 3 year storage was (0.372±0.249) ng. The quantity of E7 extracts after 2 years of storage was undetermined. It was found that total DNA yields of post-storage extracts were lower than those of the corresponding pre-storage data except E4, E6, and E10. No PCR inhibition was detected in all post-storage DNA extracts.

### 2. Qualitative results

Before making a comparison of profile quality, the electropherograms of STR typing of pre-stored samples were evaluated for the presence of DNA degradation. Degraded profile pattern was detected in pre-storage STR typing (Figure 3A).

Analysis results of 3 year-stored samples, showed 1 full STR profile, 3 partial STR profiles, and 1 sample gave no STR profile. In case of 2 year-stored samples, it was found that 4 samples gave partial STR profiles, and 1 sample gave no STR profile. The alleles present in the post-storage profiles were consistent with the corresponding reference profiles, and no allelic drop-in was identified. However, allelic dropouts were found in all post-storage samples, except E4 (table 2). In addition, peak height of each allele was lower than that of respective data obtained from pre-storage analysis. And it was also found that peak heights were inversely proportionate to the lengths of amplicons i.e. degradation pattern in the electropherogram (Figure 3).

For the human HV mtDNA fragment analysis, the two shorter fragments were detected in electrophoresis of 9 post-storage samples. The longest HV I+II fragment were detected from only two samples, which were E4 and E5 (Table 2). For E2, 3, 7 and 8 samples, amplification of the longest fragment could not be performed due to insufficient quantities of DNA extracts.

## Discussion

### 1. Evidence of DNA degradation during storage

Approximation of DNA yield per one gram of human bone with 7 days postmortem and without exposure of harsh environmental conditions, was more than 10,000 ng (Li, 2006). In the present study, DNA yields per gram of bone were much lower than this values (<2.0 ng in pre-storage analysis and <0.5 ng in post-storage analysis). These findings suggested the occurrence of DNA degradation in samples before and during storage. However, Real-Time quantitative results were not considered as a criterion to assess DNA degradation during sample storage because it cannot accurately predict the extent of DNA degradation and the amplification success of STR loci (Cupples C. M. *et al*., 2009). Degradation pattern of STR profile, peak height, loci dropouts, and analysis of mitochondrial DNA fragmentation were used as the criteria for detection of DNA degradation (William Goodwin *et al*., 2011). Therefore, these were applied as criteria for investigation in this study.

Comparison of STR typing results showed that degraded STR profile patterns were clearly showed in post-storage analysis (Figure 3 B). According to post-storage typing results (Figure 2), it was found that allelic dropout number between 2 to 4, 5 to 6, and 6 to 9 occurred in STR loci size range of 100-200, 200-300, and over 300 bases, respectively. Occurrence of dropout was lowest in D3S1358 (one of the shortest STR loci), and highest in FGA locus (one of the longest loci). This indicated the increase in number of allelic dropouts of longer STR fragments in the progressing time period. These were demonstrating the effect of storage time on degradation of longer nuclear DNA fragments.

Peak height ratios (PHR) of pre- and post-storage samples were then analyzed. It was found that PHR of most STR loci with sizes over the range of 200-300 bases were lower than 70% (Figure. 2). It indicated the occurrence of DNA degradation before and during storage of these 10 samples. In post-storage STR profiles, PHR of larger size alleles (>300 bases) were significantly lower (<60%), except in Penta D locus. This suggested an increase in tendency of DNA degradation of larger sized loci as the storage time progressed. Therefore, the degraded STR profile patterns together with allelic and loci drop-outs, lower peak heights, and peak imbalances in post-storage DNA profiling indicated nuclear DNA degradation during storage period.

Regarding mtDNA analysis of post-storage samples, the presence of HVI and HVII fragments but not longest HVI+II fragment was an indication of mtDNA fragmentation. Faint positive DNA bands for the longest fragment in E4 and E5 also suggested the possibility of their mtDNA degradation. These findings altogether revealed the presence of mitochondrial DNA degradation during 2 or 3 years of bone sample storage. In addition, the presence of HVI and HVII amplification products suggested the intactness of these 2 shorter mtDNA fragments, and high possibility to obtain successful mitotying results of the HV I and HV II regions.

Among 10 samples, only E4 sample gave full STR profile and amplification results of all mtDNA HV fragments. However, allelic peak heights were lower than those in the pre-storage STR profile. And the longest mtDNA HVI+II amplification product was observed as a faint DNA band in the ethidium bromide-stained agarose gel. These indicated the occurrence of DNA degradation in this sample during 3 years of storage.

**2. Comparison of DNA degradation between 2 year and 3 year storage time**

Among the five samples that were kept for 3 years, E2 sample showed severe degradation because of undetectable STR loci and mtDNA HV fragments. Among the 2 year-stored samples, although there was one sample (E7) giving no STR profile, its mitochondrial DNA HV regions were still preserved. Therefore, according to comparison of the two samples (E2 and E7), it showed that longer storage time increased the level of degraded DNA.

However, the longest mtDNA fragment was detected in two of the 3-year-stored samples (E4 and E5). This finding suggested two possibilities. Firstly, these two samples might have relatively shorter postmortem interval than other samples. It was supported by the study of (Christina Kaiser *et al*., 2008) in which fragments larger than 700 bp could be detected within first 8 years postmortem. The second possibility was the difference in environmental conditions exposed to each sample before submission to DNA laboratory. If the samples were exposed to adverse environmental conditions (high humidity, high temperature, low pH) before forensic analysis, the chance of obtaining informative results would be reduced as the storage time progresses (M. P. Solar *et al*., 2011). In this experiment, the information of all samples regarding estimated postmortem interval and environmental conditions of recovery sites were unknown due to limited accessibility of confidential data. Therefore, further analysis of large number of samples with complete information are required for establishing the relationship between sample storage time and DNA degradation.

**3. Comparison of DNA degradation between bone pieces and bone powder**

When comparing 2 samples that gave no STR profile, two shorter mtDNA HV fragments were still detected in samples stored as bone piece (E7), while no nuclear nor mtDNA was detected from sample (E2) that was stored as bone powder. Therefore, bone piece sample, E7 gave better results than bone powder sample, E2. This better result in the bone piece sample might also be contributed by other factors such as postmortem interval or environmental conditions. Further analysis of large number of samples with complete information are required for establishment of the relationship.

## Recommendation

In routine forensic DNA casework, the possibility of getting degraded skeletal remains is higher than to use good quality bone samples. This experiment pointed out the facilitation of DNA degradation at room temperature in degraded bone samples. Moreover, it was found that DNA degradation occurred in either bone piece or bone powder state. Therefore, to get useful information, DNA analysis of forensic bone samples should be done as soon as possible after recovery of the skeletal remains. Otherwise, it is required to store samples in a cool and dry place to obtain useful results for future analysis.

**Table 2** Summary of DNA quantification results, mtDNA analysis, and STR typing results obtained from pre- and post-storage DNA analysis.

| Sample No. | Storage period | State of stored material | Real-Time Quantity Total DNA yield (ng) | | mtDNA analysis | | | STR typing results Number of loci | |
|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | HV I | HV II | HV I+II | A | B |
| E1 | 3 years | powder | 2.29 | 0.2625 | √ | √ | X | 16 | 14(+2) |
| E2 | 3 years | powder | 1.985 | 0.1175 | X | X | NR | 16 | X |
| E3 | 3 years | piece | 0.7625 | 0.0425 | √ | √ | NR | 16 | 3(+1) |
| E4 | 3 years | powder | 0.355 | 0.5375 | √ | √ | √ | 16 | 16 |
| E5 | 3 years | piece | 0.5625 | 0.55 | √ | √ | √ | 16 | 9(+2) |
| E6 | 2 years | piece | 0.3925 | 0.745 | √ | √ | X | 16 | 13(+3) |
| E7 | 2 years | piece | 0.325 | UD | √ | √ | NR | 16 | X |
| E8 | 2 years | piece | 1.45 | 0.175 | √ | √ | NR | 16 | 13(+3) |
| E9 | 2 years | powder | 0.045 | 0.29 | √ | √ | X | 16 | 4(+6) |
| E10 | 2 years | piece | 0.4425 | 0.6275 | √ | √ | X | 16 | 13(+3) |

Note: UD represents 'undetermined result'; 'A' and 'B' refers to analysis 'before' and 'after' storage, '√' refers to presence of expected PCR product, 'X' refers to 'no PCR product' for mtDNA analysis and 'no STR loci', and 'NR' refers to 'no results' because of insufficient DNA extracts, and numbers in ( ) represent half-locus.

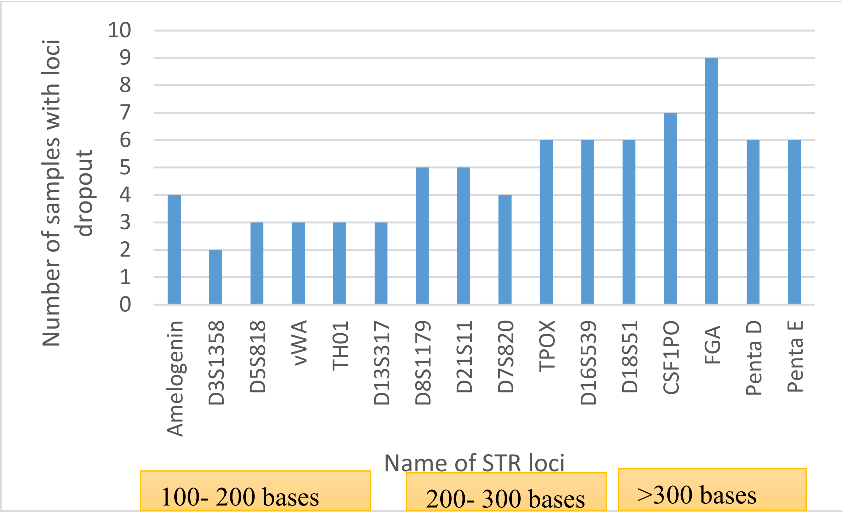


**Figure 1** Occurrence of allelic dropout observed in 10 STR profiles of the post-storage samples according to the allele size range.

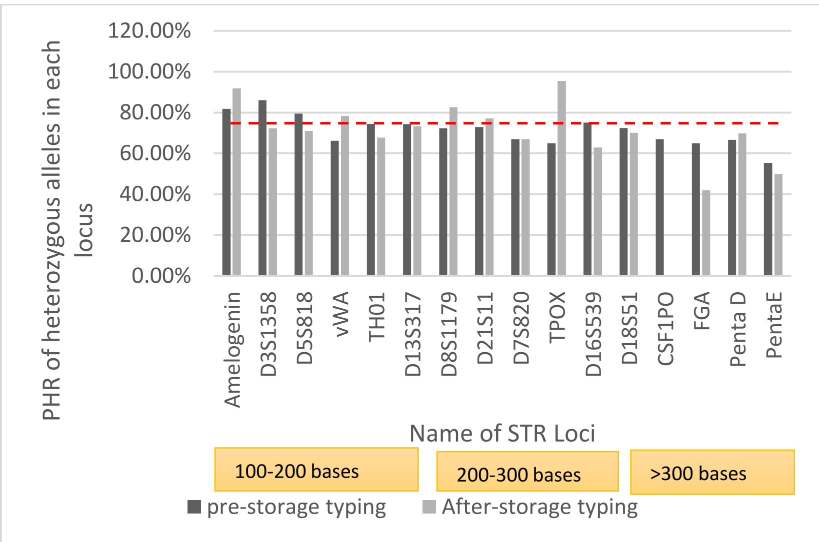


**Figure 2** Comparison of peak height ratios of heterozygous alleles in each STR loci of pre- and post-storage DNA samples (red dash line represents PHR 70%)

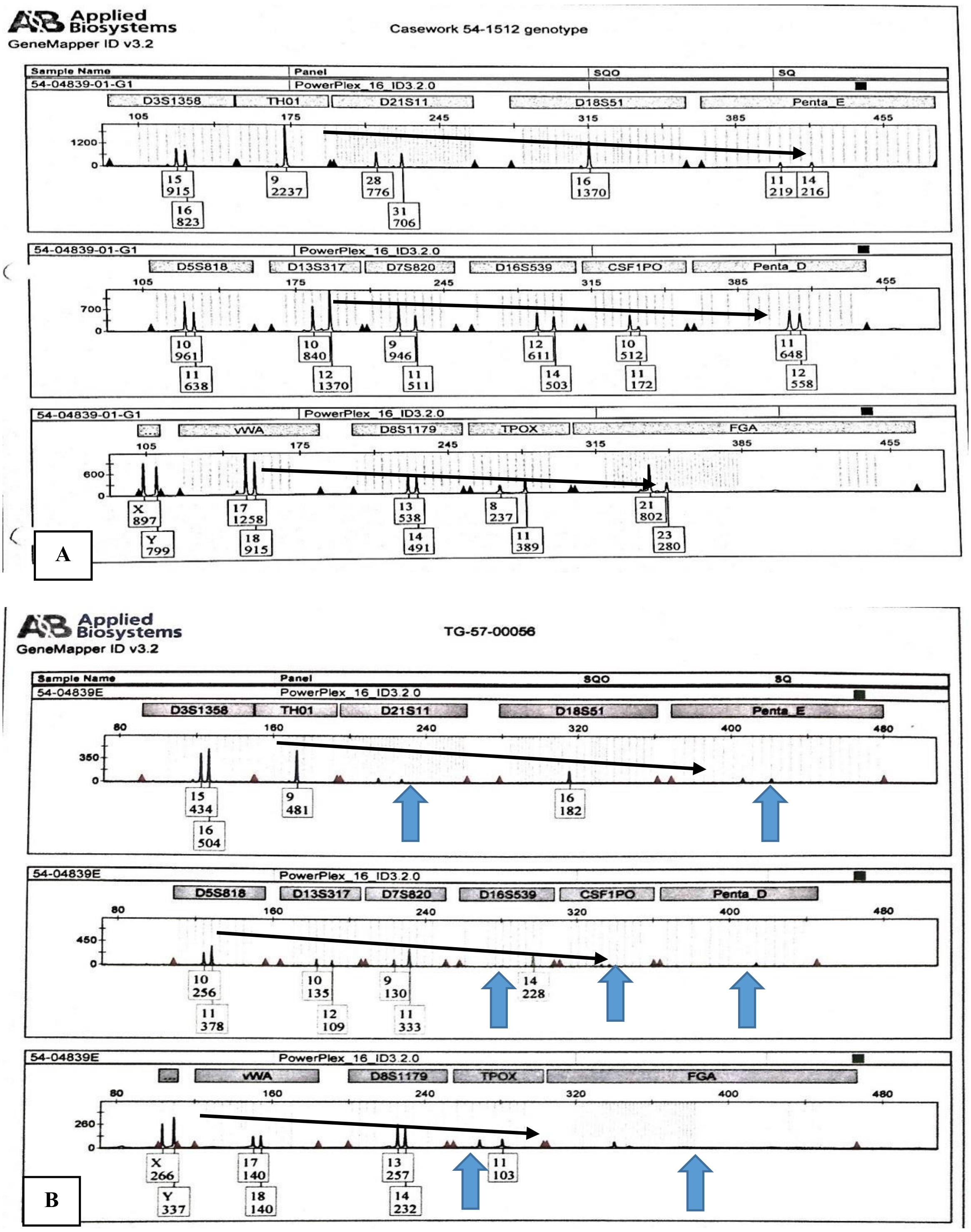


**Figure 3** An example of STR profile results of sample E5 (A) before storage, and (B) 3 years after storage. The degradation pattern was observed in both pre- and post-storage analysis (shown by black arrows) and allelic and loci drop-outs were found in post-storage typing (shown by blue arrows).

## Acknowledgement

The first author is supported by the China Medical Board (CMB) Scholarship to Myanmar. And this study is a part of the project approved by Mahidol University Institutional Review Board (MU-IRB) by an Exemption Review Process (COE. No. MU-IRB 2013/009.0409).


## References


Amy Mundorff *et al.* (2014). Examination of DNA yield rates for different skeletal elements at increasing post mortem intervals. *Forensic Science International* , 55-63.

Christina Kaiser *et al.* (2008). Molecular study of time dependent changes in DNA stability in soil. *Forensic Science International* , 32-36.

Christophe Frippiat *et al.* (2014). Efficiency of a novel forensic room-temperature DNA storage medium. *Forensic Science International* , 81-84.

Cupples C. M. *et al.* (2009). STR profiles from DNA samples with "undetected" or low quantifiler results. *Journal of Forensic Science* , 103-7.

Li, R. C. (2006). *Application of Proteinases for DNA Isolation of Bone Specimens.* Retrieved from https://www.ncjrs.gov/pdffiles1/nij/grants/227502.pdf.

M. P. Solar *et al.* (2011). Morphological and DNA analysis in human skeletal remains exposed toenvironmental conditions in Brazil. *Forensic Science International*, e339-340.

William Goodwin *et al.* (2011). *An introduction to forensic genetics (second edition).* Willey-Blackwell.