African Journal of Biotechnology Vol. 8 (20), pp. 5163-5168, 19 October, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Quantification and presence of human ancient DNA in burial place remains of Turkey using real time polymerase chain reaction

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Accepted 29 June, 2009

Archaeometry and forensic laboratories are increasingly confronted with problematic samples from the scene of samples, containing only minute amounts of deoxyribonucleic acid (DNA), which may include polymerase chain reaction (PCR) inhibiting substances. Efficient DNA extraction procedures, as well as accurate DNA quantification methods, are critical steps involved in the process of successful DNA analysis of such samples. Genomic DNA was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. This method is a sensitive for the extraction of DNA from a wide variety of forensic samples, although it is known to be laborious compared with single tube extraction methods. The relatively high DNA recovery and the quality of the extracted DNA speak for itself. For reliable and sensitive DNA quantitation, the application of real time PCR is described. A published real-time PCR assay, which allows for the combined analysis of nuclear or ancient DNA and mitochondrial DNA, was modified. This approach can be used for recovering DNA from the surface of fossil bone remains in Turkey via a simple procedure that permits a direct quantitative and qualitative assessment of molecular markers. Using quantitative RT-PCR, the available sources of total aDNA was shown to consists of intact DNA that is virtually free of RNA, resulting in a more accurate representation of gene expression using RT-PCR and PCR amplification methods. In this study, the results demonstrate that RT-PCR method can be useful for the improved ancient DNA extraction in anthropology and archeology.

Key words: Ancient DNA, fossil bone, RT-PCR.

INTRODUCTION

Ancient DNA research, defined as the retrieval and analysis of DNA sequences from various degraded biological source materials, has promoted many biological and medical research fields during the last two decades. In particular, historical anthropology and paleoanthropology stand to benefit from direct access to back-dating genetic data, as has already been shown through applications ranging from individual identification, reconstruction of kinship and marriage patterns to human phylogeny. The DNA-based prerequisites and basic methodological strategies for access to the various types of information are explained, as well as the characteristics of ancient DNA that limit the different approaches. Major restrictions arise from the degradation of ancient DNA down to fragment sizes of at the most only a few hundred base pairs. This fact links ancient DNA analysis almost exclusively to the PCR technique that enables us to deduce genetic information from degraded nucleic acids (Holland et al., 1991). Futhermore, ancient DNA extracts regularly consist of only a few intact target sequences, which may additionally reveal sequence deviations due to the degradation process. Both these factors make the analysis vulnerable to the generation of non authentic results. These pitfalls of ancient DNA analysis are explained and discussed in detail with reference to the most recent relevant literature. Wherever possible and available, suggestions for strategies to overcome commonly experienced obstacles in ancient DNA analysis are highlighted and evaluated. Molecular analysis of fossil and archaeological remains has been established as a powerful tool in providing new insight in phylogenetic investigations. The overlapping set

Abbreviations: aDNA, ancient DNA; **nDNA,** nuclear DNA; **RT-PCR,** real time-polymerase chain reaction.

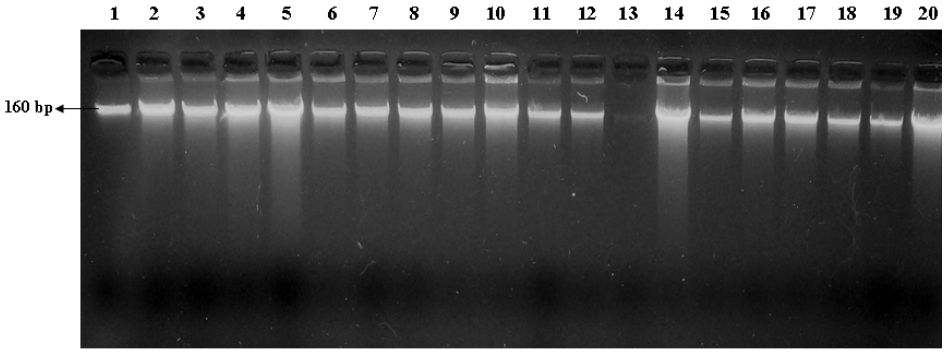

Figure 1. Genomic DNA was isolaled from fossil bone tissue remains with Bio Robot EZ1 and subjected to electrophoresis in 1% agarose gel.

of molecular modifications and degradation that forensic samples share with archaeological specimen suggests the application of similar technical approaches to the respective biological material.

Fairly recently, a new method of PCR quantification has been invented (Gerard et al., 1998). This is called “real-time PCR” because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. The scientific, medical, and diagnostic communities have been presented the most powerful tool for quantitative nucleic acids analysis: real-time PCR (Bustin, 2004). This new technique is a refinement of the original polymerase chain reaction (PCR) developed by Kary Mullis and coworkers (Saiki et al., 1985). Real time polymerase chain reaction (RT-PCR) is the molecular technique of choice for the retrieval of specimen deoxyribonucleic acid (DNA) molecules. Real-time quantitative PCR (qPCR) is a powerful tool for quantifying specific DNA target sequences. Although determination of relative quantity is widely accepted as a reliable means of measuring differences between samples, there are advantages to being able to determine the absolute copy numbers of a given target. One approach to absolute quantification relies on construction of an accurate standard curve using appropriate external standards of known concentration. Furthermore, real-time PCR allow for quantitative measurement of DNA or RNA molecules. The RT-PCR method can be useful for the improved ancient DNA extraction in anthropology and archeology. The PCR standard is a fragment of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or cDNA bearing the target sequence. A simple protocol for constructing a cDNA standard for one-step PCR can be found in Fronhoffs et al. (2002), while a DNA standard for two step real-time PCR can be synthesized by purifying a conventional PCR product or directly synthesizing the target nucleic acid. The standard used must be a pure species or human blood in this work. DNA standards have been shown to have a larger quantification range and greater sensitivity, reproducibility, and stability than RNA standards (Liss, 2002). A protocol for the automated preparation of high-molecular weight DNA from the fossil bones of burial place remains of Turkey suitable for multiple PCR analyses is described. Subsequent Light-Cycler amplification of an endogenous gene allowed the exact quantification of the amount of prepared DNA and the presence of aDNA and modern DNA was analysed in fossil and living.

MATERIALS AND METHODS

Collection of fossil bone samples

A subset of 100 bones from the total set obtained from Mugla in Turkey was analyzed in this study. Upon recovery of the skeletal remains, the bones were described in terms of sex, estimated age, and some of the skeletal weathering stages. In order to allow ratings on individual bones, a new staging system was developed at Archeometry Laboratory in Selcuk University, Arts and Science Faculty, and assigned as period or era each bone based on visual inspection for the DNA study (Table 1). The bone samples of more than 100 individuals were chosen to study the genetics of this skeletal population.

Isolation of DNA

Approximately 1 cm^3 of bone was cut from the source section using a Dremel MultiPro tool and was collected in a tube. Samples were then immersed in filter-sterilized wash buffer (1% SDS, 25 mM EDTA) and 0.1 mg/ml proteinase K, and incubated for one hour at room temperature. Following the incubation, the wash buffer was poured off and each sample was washed with 1 ml of sterile dH_2O six consecutive times. Samples were allowed to air dry. Bone powder from the dried bone samples was collected in one of two ways. Bone was either ground to powder drilled using a the Dremel tool both fitted with 1/16 microfuge tube and weighed. Four hundred microliters of digestion buffer (20 mM Tris, 100 mM EDTA, 0.1% SDS) and 0.4 mg/ml proteinase K was added to each ground bone sample and incubated overnight at 56°C. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. Extraction of fossil bone was performed with the same kit according to the manufacturer's instructions Amount and purity of extracted DNA from ancient bones were measured by spectrophotometer. In addition to spectrophotometric measurement, extracted DNA was applied to

Table 1. The ancient ruins of Koranza and Necropal area are situated in the region of modern city Mugla in Turkey. More than hundred ancient tombs has been excavated and a lots of grave gifts and skeletal remains were found in this graves.

Number of bone samples	Codes	Period	Locatıon site	Excavatıon region
2	05BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM22	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM09	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM29	HELLENİSTİK-4.YY	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM21	GEÇ HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	GEÇ GEOMETRİK-M.Ö.730-680	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM23	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM05	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM37	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM85	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM118	GEOMETRİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	GEÇ KLASİK-M.Ö.377	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM27	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM14	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM41	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM22	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM17	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM64	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM37	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM31	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM26	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM30	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM58	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM95	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM106	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM18	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM100	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM10	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM11	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM55	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM23	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM45	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA

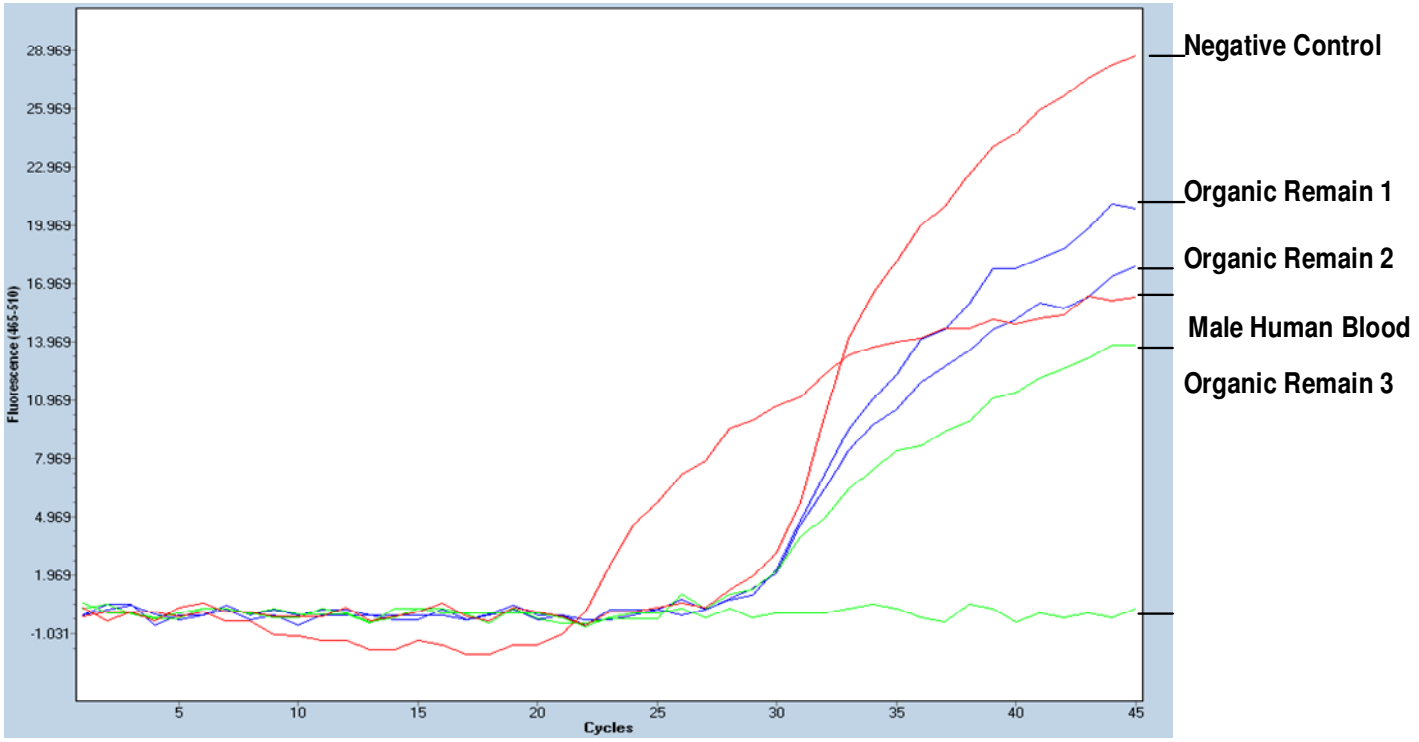

Figure 2. Quantification of nDNA or modern DNA isolated from human blood and aDNA isolated from human fossil bone in Turkey using RT-PCR.

1% agarose gel, stained and imaged under ultraviolet (UV) irradiation (Figure 1).

Ancient DNA quantity

Genomic DNAs isolated from fossil bone remains were showed by spectrophotometric analysis. DNA quality and concentrations were evaluated nearly 1.8. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. Amount and purity of extracted DNA from ancient bones were measured by spectro-photometer and then extracted DNA was applied to 1% agarose gel, stained and imaged under ultraviolet (UV) irradiation.
As a result, 50 ng pure DNA was extracted from ancient bones. Several precautions were taken to prevent contamination during the experiments.

Quantitative RT-PCR

2 µl of DNA and 3 µl primer mix was used in a final volume of 20 µl according to the manufacturer's directions. LightCycler amplification and Real-Time PCR detection with SYBR Green was done as described by the supplier using the Fast Start DNA Master SYBR Green I (Roche Applied Science) with 8 mM $MgCl_2$ in the reaction mixture. Amplification conditions were 95°C for 10 min and 45 cycles, each cycle at 95°C for 10 s, 56°C for 10 s and 72°C for 20 s. The LightCycler amplification and Real-Time PCR detection with fluorescence labeled hybridisation probes was done following the protocol provided either for the LightCycler. Positive and neagtive controls were included in all reactions (Figure 2).

RESULTS AND DISCUSSION

Until recently, the ancient DNA field has been driven by empirical approaches to DNA recovery. Researchers have typically divided samples into two categories: those that will amplify and those that will not. Little thought has been give to the reasons for success or failure. Quantitative real-time PCR is a fluorescence detection system that monitors DNA amplification during the exponential phase of the reaction. In the exponential phase, the amount of fluorescence is directly proportional to the number of starting template molecules in the reaction. RT-PCR data are important in aDNA research and forensics for several reasons; foremost being that the results of different extraction methodologies can be compared accurately. The removal of inhibitors such as humic acids and tannins is also crucial to successful amplification. Many ancient DNA PCR reactions "fail" not due to the lack of DNA but due to the presence of inhibitors which inhibit polymerase activity. RT-PCR provided a means by which to identify when PCR inhibition is occuring. RT-PCR offers another key benefit to aDNA research programs by determining the number of starting template molecules in a PCR. Reactions that start off a small number of starting templates are more susceptible to contamination from exogenous sources and more likely to yield sequence data containing post-mortem DNA damage or contamination. The develop-

ment of good quantitative assays allows systematic assessment of DNA preservation. RT-PCR approaches will not only maximise our ability to extract and amplify older and more degraded templates, but will also contribute to the prediction of other sites favourable to the preservation of aDNA. This real-time DNA quantification assay has proven to be highly sensitive, enabling quantification of single DNA copies. Although certain limitations were apparent, the system is a rapid, cost-effective, and flexible assay for analysis of forensic case work samples. At its simplest, real-time PCR can be used as a qualitative assay. PCR specificity is strongly dependent on primer design. With real-time PCR, amplification and detection of target are performed simultaneously. In case of nonspecific amplification, amplicon detection must be specific. The disadvantage of using a double stranded-DNA (dsDNA) dye for real time PCR is that the dye detects any double-stranded DNA generated during PCR (Rasmussen et al., 1998). Specific amplicons and primer dimers, for instance, are detected equally as well. It is often said that the use of fluorogenic probes eliminates this problem (Pfaffl et al., 2004). For absolute quantification, the accuracy of external standard quantification depends entirely on the accuracy of the standards. Care is thus needed for the design, synthesis, purification and calibration of DNA standards. Absorbance at 260 nm, fluorescence measurement with DNA dyes and limiting dilution assays are ways to calibrate standards. If the quantification method is relative, it is not important to know the actual copy number of the standards.

Statistical analysis

For each of the two platforms independently, a two-way analysis of variance (ANOVA) was used to test for significant differences. The two factors examined were plates and samples (results not shown). The results from both platforms showed that at the 80% level of confidence, there was a non-significant interaction ($P > 0.05$) between the samples and the three replicate plates within a platform, implying that the samples were behaving consistently between the plates.

Conclusions

In this study, tissue card and convenient DNA investigator kit was used to isolate DNA for conventional PCR and real-time RT-PCR. The on-column DNA removal step ensures sensitive RT-PCR results without amplification products generated from genomic DNA targets. Moreover, DNA investigator kit generates high purity, intact total aDNA from a range of bone tissues. The powerful amplification potential of PCR has assured its use in the detection of low abundance aDNA in fossil bone tissues. RT-PCR is currently the most sensitive technique available for mRNA and DNA detection and quantification. The method requires very little DNA and differs from standart and efficient methods because it is somewhat tolerant of degraded DNA. aDNA isolated from the fossil samples was analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain. This method of analysis is at best semi-quantitative and, in many cases, the amount of product is not related to the amount of input DNA making this type of PCR a qualitative tool for detecting the presence or absence of a particular DNA. The data here show that of the available sources of total aDNA consists of intact DNA that is virtually free of RNA, resulting in a more accurate representation of gene expression using RT-PCR and PCR amplification methods. Real-time PCR is now a common method for measuring gene expression, it is increasingly important for users to be aware of the numerous choices available in all aspects of this technology. Unlike traditional PCR, there are many complexities with real-time PCR that can affect overall results. However, with a well-designed experiment performed with the proper controls, real-time PCR can be one of the most sensitive, efficient, fast, and reproducible methods of measuring gene expression and DNA quantification.

LightCycler Real-Time PCR using SYBR Green for detection was applied to quantify the actual amount of the prepared DNA. For every sample, a primer pair amplifying a single copy region of the genome was designed amelogenin primers. The specificity of the PCR reaction was tested after every run by determining the melting point of the respective product. All reaction products showed single peaks and the product size was verified to be in the expected range by gel electrophoresis. As expected, yields were generally higher for crushed ancient DNA compared to soaked modern DNA seemingly due to the higher surface area contact with lysis buffer during homogenisation. Independent from the homogenisation method, the highest amount of DNA was prepared from blood and fresh tissue samples and clearly less yield was obtained from fossil bone. The high variation in DNA yield from the different species was quantitatively reproducible and can be explained by differences in the relation to the tissue freshness and pureness. Similar experiences with clear differences in the DNA yield have also been made by others comparing several species. Still, the amounts of DNA obtained with this method from any source or treatment were more than sufficient for multiple PCR analyses. The developed amplification systems can serve as valuable internal standards in the quantitative detection of specific sequences in DNA preparations or aDNA.

ACKNOWLEDGMENTS

The author is grateful to Dr. A. Ahmet Tırpan and his working group for the access to valuable archaeological skeletal material.

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