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Guidelines for work with ancient DNA developed at the Archaeological Research Laboratory

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Ancient DNA is highly degraded and extremely sensitive to contamination which makes it hard for the analyser to know whether he is working with authentic ancient DNA or a modern contamination. However, there are ways to avoid and detect contamination when it appears. We suggest a set of rules and controls to help the analyser to confirm the authenticity of his work.

Introduction

When we started to work with ancient DNA and PCR technology at the Archaeological Research laboratory in 1993 the method still had some of the "exotic glamour" due to the mythology that always comes with new techniques practised in multiple disciplines. Only seven years had passed since Karry Mullis et al., (Mullis et al. 1986) published the polymeras chain reaction (PCR), and maybe eight years since Mullis went on the famous car ride where the thought popped up in his mind. Today the method is widely spread and not as spectacular as it used to be. Any field where DNA is being analysed, be it medicine, molecular systematics or archaeology, benefits from PCR technology. Although many people know the method and work with it daily, it is still just as much of a trouble maker as it is a savour. The method is sensitive enough to amplify a limited number of molecules and it is efficient enough to produce large amounts of amplicons from this limited number of molecules. It is thus easy to imagine that the starting molecules in the PCR may just as well be a contamination from an external source as DNA from the specimen meant to be analysed.

Contamination

An individual who died and was buried during prehistory must have been treated by other members of the society, although the degree of treatment varied with the culture. He/she may have been buried in a multiple grave where remains from different individuals came to pile upon each other. Ages later the grave is excavated by archaeologists and the remains may be examined by a physical anthropologist before they are stored for future research. When they are being sampled for DNA analysis another physical anthropologist may help the DNA researcher to select the bones for the analysis. In between the death of the individual and the drilling of the bones, the bones may have been in contact with more than 10 other individuals or remains of individuals. Now how can a DNA researcher working with these bones be sure that he is analysing DNA belonging to the original bone and not somebody else's DNA? The answer lies in the way contamination is avoided and detected, and the knowledge of what kind of contamination that may cause problems. Many different techniques and measurements have been suggested for protection against and the detection of contamination (Handt et al. 1994a; Austin, Smith & Thomas, 1997). We argue that some of these cannot be used as routine on antiquarian material for different reasons. One is that DNA analysis is a destructive method which destroys the tissue. Working with unique antiquarian material there may, e.g. not be enough to spend on a competitive PCR and for the same reason it may not be realistic to ask another laboratory to reproduce several sex identifications. However, some precautions can and should be taken in order to avoid contamination.

Detection

All possible precautions against contamination should be considered, although we will never experience a con-

tamination free laboratory working with ancient human DNA. A general level of contamination among many laboratories that work with ancient human DNA seems to be 15%. Working with archaeological DNA you have to be your own expert on differentiating between authentic ancient DNA and contamination. Contamination appears in different shapes and can be defined into different categories; As *sample contamination*, a specific positive indication will appear in all samples, including the blanks, i.e. if all samples are treated the same way. As *the Carrying effect*, if the contamination is of a small amount it may only appear in the samples where DNA from micro-organisms and proteins forces it out into the solution while it gets lost in tube and pipette walls in the blanks (Cooper, 1992). As *sporadic contamination*, if the contamination consists of a few molecules it may express itself in some samples but not in all (Heinrich, 1991).

In order to detect contamination one should first of all use multiple negative controls. One, *the extraction blank*, follows the sample all the way from the beginning of the extraction until the analysis of the amplification, this will reveal *sample contamination*. Another [illegible] should be added that follows the sample from the beginning of the extraction the same way as the extraction blank but with DNA added to which the primers used do not anneal. This is *the carrying effect blank*. Finally a water blank should be added right before the PCR is prepared, this is the *PCR blank*. These blanks will reveal if sample contamination and the carrying effect appear, they will also provide information [illegible] where the contamination was introduced, i.e. before or during the PCR preparation. By setting up two [illegible] PCR reactions from every extraction and by [illegible] two extractions on each sample sporadic contamination may be detected. In order to exclude [illegible] contamination all four amplifications should [illegible] same result. Ancient DNA should also be subject to a size control since ancient DNA is degraded (Jeffreys et al. 1992). This could be done by [illegible] of primers that anneals well to a modern [illegible] 400 or 700 base pairs. If ancient DNA amplifies with such a primer set there are well grounded [illegible] suspect modern contamination. Finally the [illegible] frequencies should be regarded when appropriate, i.e. a horse should look like a horse on the [illegible] level and not like a human, and a group of [illegible] should look like a group of individuals and [illegible] monozygous twins if a polymorphic marker is [illegible]

[illegible] can occur during different events and [illegible] be divided into four categories; *historic* [illegible] i.e. prehistoric contamination or contamination from the archaeologists and physical anthropologists that have worked with the bones. Micro-organisms that have been living in the bones may also cause contaminations and contamination-like patterns. *Contamination in the laboratory*, i.e. cross-contamination between samples, contamination from people working with the sample, from tools, chemicals, primers etc. *Carry-over* of PCR products and primers from previous reactions to subsequent PCR amplifications. Finally, *PCR jumping*, which is not really a contamination but presents problems much like it. Here small DNA fragments from the sample (no matter what origin) will build up to unspecific products during the PCR (Desalle, Barcia & Wray, 1993).

Historic contamination may be avoided by UV irradiating the bone surface, washing it in 1M HCl and then taking the sample from the core of the bone.

Since the amount of DNA in ancient tissue always is small (Handt et al. 1994b) sample *cross-contamination* should never present a large problem. However, by keeping the extraction series small (preferably around five, definitely below ten) the risk is minimized for cross-contamination. Washing all working surfaces with sodiumhypochlorite (5%), UV irradiating all the tools and washing them with HCl (1M) all improve the working environment. Autoclaving and UV irradiating all reagents which can support these treatments (e.g. Taq polymerase and uracil N-glycocylase), will help to keep solutions clean. However, it may also be a good thing to keep primers and nucleotides away from heavy UV irradiation. The actual working area for sampling and extraction should be physically separated from the rest of the laboratory by using a sterile bench or a PCR working station with UV light. These steps should take care of contamination from the environment. Cross-contamination may be avoided by using one laboratory for extraction and setting up PCR reactions and another one for working with the PCR products. The routine should be set up in such a way that people as well as material and samples enter the PCR product working area from the extraction area and never the other way around. A method based on Uracil-N-glycocylase should be used to make sure that all PCR products that may enter into the reaction are destroyed (Heinrich, 1991). In this way PCR products are kept away from the PCR reactions, and if the samples were to get contaminated by PCR products anyway, they would not be amplified since the uracil N-glycocylase will destroy the contaminating products. *PCR jumping* may be avoided by using "hot start", if amplitaq gold™ (Perkin Elmer); alternatively if an uracil N-glycocylase system is used the tubes do not even have to be opened in the PCR machine, neither is any extra step needed by the handling of a hot start wax. Things like lab coats, latex gloves, eppendorf cap openers and filter tips should of course be used all the time the way ordinary good laboratory work demands.

Conclusion

Most of these precautions have been suggested individually or in other more lengthy protocols before, but we argue that the above combination of precautions and detection systems offers the most realistic system for ancient DNA work and will still provide a reliable answer to the authenticity question. Only one extra PCR to determine the quality (fragment length) of the extracted DNA is set up, so valuable sample material is not wasted. Further, the kind of false positives that would slip through this system would emerge due to bad knowledge of the sample and would not be detected if the experiment is repeated in a different laboratory. We argue that the control system used at the Archaeological Research Laboratory is sufficient for detecting and avoiding contamination and that a total reproduction of all results in other laboratories, i.e. as routine, would not improve the system enough to justify the increased cost in time, resources and loss of antiquarian material.

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