USING AMPLIFICATION OF BACTERIOPHAGE LAMBDA DNA TO DETECT PCR INHIBITORS IN SKELETAL DNA

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DISCLAIMER: *This procedure is still under development and has not yet been tested using soft tissue or contemporary skeletal material. The University of Indianapolis shall not be held responsible for any tissue lost using this technique.*

Genetic Analysis is becoming increasingly important in the field of forensic anthropology. Genetic technology can compliment morphological analysis, creating a more complete profile of the individual being examined. DNA can potentially address questions involving the sex, identity, and familial relatives of the individual.

The ability to analyze the small highly degraded DNA samples associated with skeletal DNA is possible because of the polymerase chain reaction, or PCR. PCR exponentially amplifies a specified piece of the DNA strand. Several things are needed for the PCR reaction: the DNA sample, a primer (which is a synthesized piece of DNA that flanks the segment of interest), DNA polymerase (which helps to catalyze new DNA synthesis), and free nucleotides (which are used to build the new DNA strand).

PCR consists of three major steps. The temperature of the reaction is raised to break the double stranded DNA into single strands. The temperature is then lowered to allow the primer to anneal to the DNA sample on either side of the region of interest, forming a boundary around it. The DNA polymerase then catalyzes the synthesis of new DNA strands that are exact copies of the original sample.

The new strands produced from the first cycle are then templates themselves for the PCR process. Repeating this cycle 25-30 times will yield well over one million copies of the DNA for DNA profiling purposes. However, amplification of DNA samples from bone may be challenging due to the presence of contaminating molecules that can interfere with the PCR process.

PCR inhibition can arise from a variety of sources, and is classified into two major groups: non-diffusible and diffusible inhibitors. Non-diffusible inhibitors are chemical modifications of the DNA molecule, including strand damage and molecules attached to the DNA. These modifications occur as the DNA begins to degrade after the individual's death. Diffusible inhibitors are contaminating molecules not attached to the DNA that leach into bone during degradation and co-purify with the skeletal DNA.

These contaminating molecules come from a variety of sources and may vary among samples because the different taphonomic forces that effect bone degradation also have an affect on skeletal DNA. The inhibitory substances leach into the bone during diagenesis and may not be removed with conventional methods used to extract purified skeletal DNA. Iron, tannins, and humic acids are known PCR inhibitors present in organic plant material and soil. The presence of these substances in the immediate environment of the degrading bone may lead to their incorporation into the bone.

A simple technique can be used to detect diffusible PCR inhibitors in skeletal DNA. With the early detection of the inhibitors, additional purification steps may be added to remove them from the sample. This will save time, money, and most importantly, skeletal material from loss due to fruitless PCR. This process employs the use of bacteriophage lambda DNA as a detector of diffusible PCR inhibitors because it is inexpensive and abundant in most molecular laboratories. In order to determine the concentration of lambda DNA used for each sample, the full-length bacteriophage lambda DNA molecule (48,502 bp) was used.

A specified concentration of lambda DNA was mixed with a small amount of bone DNA and amplified with lambda specific primers. If diffusible inhibitors are present in the bone DNA, they will also inhibit amplification of the lambda DNA. The presence of amplified lambda DNA would indicate the absence of diffusible inhibitors.

The first step was to find a concentration of lambda DNA that was sensitive enough to detect the inhibitors in the degraded skeletal DNA sample and not saturating to the inhibitors. Preliminary amplifications determined the desired range to be 1/2000, 1/5000, and 1/10,000 dilutions of the concentrated stock lambda DNA.

- **Stock Concentration = 9,500 pg**
- **1/2000 = 4.75 pg**
- **1/5000 = 1.90 pg**
- **1/10,000 = 0.95 pg**

The DNA tested for the inhibitors was extracted from the femora of 6 different historic human skeletal specimens. Previous testing failed to amplify human DNA from these bone samples.

Three PCR reactions were constructed for each bone sample containing 2 micro-liters of one of the three lambda DNA concentrations, 2 micro-liters of the skeletal DNA, lambda DNA specific primers, and the other reagents needed for PCR amplification. As a positive control, DNA from a human skeletal specimen that previously was successfully amplified by PCR for human specific DNA fragments was also analyzed.

The amplified region of the lambda DNA was approximately 500 base pairs in length. Negative control indicated no contamination to the samples during the amplification process. The positive control contained no bone DNA. These amplifications consisted of only lambda DNA (at the three specified concentrations) and lambda specific primers, which indicates that the primers and amplification process were successful.

A second positive control containing skeletal DNA that was previously amplified using human specific primers (or non-inhibited skeletal DNA), lambda DNA (at the three specified concentrations), and lambda specific primers was also amplified. This indicates that lambda DNA is amplifiable with non-inhibited bone DNA samples.

One of the skeletal DNA samples was mixed with lambda DNA at the three specified concentrations and amplified with lambda specific primers. All three concentrations show relatively the same amount of amplified lambda DNA, indicating no diffusible inhibitors present in this bone DNA sample. The failure to previously amplify this sample is therefore not due to contaminating molecules and may be due to non-diffusible inhibitors like DNA damage or extraction procedures. A second skeletal DNA sample was mixed with lambda DNA at the three specified concentrations and

amplified with lambda specific primers. The decreased ability to amplify the lambda DNA indicates the presence of some diffusible inhibitors present in this bone DNA sample. The failure to previously amplify this sample may therefore be due to contaminating molecules and indicates the need for further purification steps.

Out of the 6 femora tested 2 displayed diffusible inhibition. Further purification steps may be added to amplify these samples. 4 of the femora displayed no indication of diffusible inhibitors. The failure to previously amplify these samples is therefore not due to contaminating molecules and may be due to non-diffusible inhibitors like DNA damage or extraction procedures. Further destruction of these 4 femora for genetic analysis purposes is not recommended.

A range of concentrations of bacteriophage lambda DNA of 4.75pg, 1.90pg, and 0.95 pg should be used during the detection of diffusible inhibitors. Two microliters of the lambda DNA should be mixed with 2 microliters of bone DNA and amplified with lambda specific primers to perform the detection process. The absence of amplified lambda DNA indicates the presence of diffusible inhibitors.

If a skeletal DNA sample fails to yield amplified human DNA after PCR, the sample should be reamplified with lambda DNA and lambda specific primers to detect the presence or absence of diffusible inhibitors. If diffusible inhibitors are present, further purification steps may be added to remove them from the sample. The absence of diffusible inhibitors indicates that the failure to previously amplify these samples is not due to contaminating molecules and may be due to non-diffusible inhibitors like DNA damage or extraction procedures. Further destruction of these bones for genetic analysis purposes is not recommended.

In conclusion, the use of bacteriophage lambda DNA as an indicator for diffusible PCR indicators can save time, money, and valuable skeletal material during the course of genetic analysis. I would like to thank the Connective Tissue for funding this research, Dr. Mary Ritke for the use of her laboratory and guidance throughout the project, Dr. Stephen Nawrocki for donating the skeletal samples, and Carlos Zambrano for technical support.

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