Kennewick Man Ancient DNA Analysis: Final Report Submitted to the Department of the Interior, National Park Service

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On May 18, 2000, two bone samples from the Kennewick collection – a right eighth rib [Catalog 97.I.12d(13)] and a left third metacarpal (MC3) [Catalog 97.L.16(Mca)] – were transferred to the laboratory of D. Andrew Merriwether for ancient DNA analysis. Three people with extensive ancient DNA research experience were involved in the subsequent analysis of these samples: the principal investigator, D. Andrew Merriwether, a doctoral student, Graciela Cabana, and a laboratory technician, John McDonough. Ancient DNA analysis of the Kennewick samples included five separate extractions using four different extraction procedures and multiple DNA amplification attempts using Polymerase Chain Reaction (PCR) on these extractions. We were unable to successfully amplify any ancient mitochondrial DNA (mtDNA) using standard methods. We conclude that if any DNA remains in these particular samples, it is inaccessible via current standard methods, but may be accessible and analyzable in the future using novel methods.

Because we did not obtain positive results in this analysis, this report consists primarily of a detailed description of the methods used and explanation of the results obtained. We attach as appendices (1) a log of electrophoresis gel images documenting the results of PCRs on these samples (Appendix 1), (2) a photographic log of procedures described in the text of this report (Appendix 2), and electropherograms documenting the contaminating sequences (Appendix 3).

DNA ANALYSIS: METHODS

Below is a general description of the methods used for the ancient DNA analysis of these Kennewick bone samples.

Contamination Controls

Extraordinary care has been taken to control for exogenous human DNA contamination. Contamination in ancient DNA work, however, is inevitable and controls must be included at every step of the process to monitor its effects. We followed these preventative steps:

1. All ancient DNA work is conducted in a physically separate ancient DNA room. This room is always cleaned with a 10 % bleach solution before any procedure. The room is equipped with Ultra Violet (UV) lights to cross-link any contaminating DNA on working surfaces.

2. Ancient DNA investigators change into a clean lab coat, and exchange their street shoes for shoe covers in an ante-room. Gloves and face masks are put on immediately inside the ancient DNA laboratory. Investigators do not handle any samples or reagents for ancient DNA use without this precautionary dress.
3. All reagents are either bought as molecular grade, DNase/RNase free directly from a manufacturer, or made under stringent conditions in the ancient DNA lab. We use UV/UF filtered H_2O taken from a physically separate laboratory that does not work with human DNA. All reagents made in the ancient DNA laboratory as well as the UV/UF H_2O are tested for exogenous DNA contamination regularly.
4. All reagents and samples are mixed or stored in DNase-free Sarstedt brand conical tubes, or in glass media bottles that have been decontaminated with HCl and subsequently left under UV light for at least half an hour.
5. Controls of all extraction reagents are run with the samples during PCR.

Decontamination of Bone Surfaces & Bone Extraction Preparation

The outside of Kennewick bone samples were treated to remove any potential exogenous human DNA contamination by first scrubbing the bone with a 10 % bleach solution using a sterilized brush. All bone surfaces were then subjected to UV light for half an hour to cross-link any superficial DNA.

Bone extraction preparation varied according the type of bone sample. The MC3 was sampled by drilling a fine bone powder using a Dremel tool. The Dremel bit, parts and tool were cleaned with a 10 % bleach solution and UV-irradiated prior to use. The rib was either scraped or ground because there was little actual bone accessible with a drilling tool, and the inside of the rib was filled with a tough soil matrix. For the first extraction of the rib (Extraction #3, below), the rib sample was soaked in UV/UF H_2O for an hour in an attempt to loosen the soil matrix from the bone. The soil matrix did not dissolve, so the cortex of the rib was then shaved off with a sterile razor blade and only these shavings were used in the subsequent extraction. This method had the advantage of not including potential PCR inhibitors in the soil matrix, but also discarded any internal rib bone pieces from the extraction. For the second and third rib extraction (Extraction #4 and #5, below), a piece of rib with its soil matrix was simply placed under a sterilized piece of bench paper and ground with a sterilized hammer. The entire piece, matrix and all, was used in the subsequent extraction to maximize the amount of ancient human bone in the sample.

Extraction Procedures

We used four different extraction procedures on five separate occasions. Twice we attempted DNA extraction using a phenol-chloroform method (*e.g.*, Kaestle 1998), once with a phenol-chloroform-silica method (modified from Baron *et al.* 1996), once with the QiaQuick PCR Purification Kit (Qiagen) (Yang *et al.* 1998), and once with a guanidine isothiocyanate - silica method (modified from Hoss & Pääbo 1993). The first and second phenol-chloroform-based methods are known to produce the highest DNA yields, but have the disadvantage of co-extracting humic acids and possibly other elements typically present in soils that react with reagents used during PCR. These may subsequently inhibit PCR reactions and yield false

negative results. The other two methods produce cleaner results, meaning that inhibitory elements tend to be removed. We used a variety of methods and attempted one method twice because results do vary from one extraction to another, even with the same protocol.
The extraction methods used for the Kennewick samples can be summarized as follows:

Phenol-Chloroform (*e.g.*, Kaestle 1998)

1. A sample of 0.10 to 0.25 g of bone powder is transferred to a 15 ml conical tube with 0.5 M EDTA pH 8.0 (molecular grade). Samples rotate for 72-96 h at room temperature (~ 30 °C).
2. Change EDTA every 24 h until EDTA is clear, but no more than 3 times.
3. Wash three times with UV/UF H_2O to thoroughly remove EDTA.
4. Decalcify sample with 2 ml of Proteinase K buffer and incubate overnight at 55 °C, with slight rotation.

Proteinase K Buffer:
50 mM (.05 M) Tris pH 8.0
1 mM $CaCl_2$
1 mM DTT
0.5 % Tween 20
1 mg/ml Proteinase K

5. Centrifuge samples at 2500 RPM for 10 min.
6. Extract DNA using phenol, followed by phenol-chloroform (1:1), and then chloroform : isoamyl alcohol 24:1. Use a volume for each equal to the amount left after the Proteinase K digest.
7. Concentrate samples with Centricon 100 filtration units. Wash twice with molecular grade Tris-EDTA (TE) pH 8.0.
9. Flip the Centricon column. Add 50 µl – 100 µl TE pH 8.0 into the final (UV-irradiated) tube provided by the manufacturer.
10. Transfer extract into new irradiated final 1.5 ml tubes.

Phenol-Chloroform-Silica (modified from Baron *et al.* 1996)

1. A sample of 0.10 to 0.25 g of bone powder is transferred to a 15 ml conical tube with 1.5 ml 0.5 M EDTA pH 8.0 (molecular grade). Samples rotate for 96 h at room temperature (~ 30 °C).
2. Add 500 µl of 20 mg/ml Proteinase K (molecular grade) plus 1.5 ml of UV/UF H_2O. Incubate at 60 °C for 90 min, rotating gently.
3. Centrifuge samples for 5 min at 3000 RPM.
4. Extract DNA twice using 25:24:1 phenol : chloroform : isoamyl alcohol, followed by chloroform. Use a volume for each equal to the amount left after the Proteinase K digest.
5. To precipitate DNA, add ~ 80 µl of 2M Sodium Acetate (pH 4.5) and 3.3 ml 100 % isopropanol. Mix for 10 min.
6. Add 5 µl glassmilk, a brand of silica. Mix for 10 min at room temperature.
7. Centrifuge for 5 min at 3000 RPM.

8. Wash twice with ice cold 80 % ethanol.
9. Let samples dry for about 1 h in an incubator.
10. Elute DNA with 50 μl molecular grade TE and transfer to a smaller, 1.5 ml tube.
11. Retain glassmilk (silica) in extract in subsequent PCRs.

Silica-Based Extraction (Yang *et al.* 1998)

1. Bone sample is dissolved in 8 ml extraction buffer:
   0.5 M EDTA pH 8.0
   0.5 % sodium dodecyl sulfate (SDS)
   100 μg/ml Proteinase K
2. Sample is incubated at 55 °C overnight.
3. Sample is then incubated at 37 °C for 24 h.
4. Sample is centrifuged at 2000 x g for 5 min.
5. Sample is run through Centricon filters to reduce volume to ~ 30 μl. Final collection tube is UV-irradiated.
6. Five volumes of Qiagen PB Buffer is mixed with the sample in the Centricon collecting tube.
7. 750 μl of this mixture is loaded at a time onto the Qiagen column and the column is centrifuged at 12,800 x g for 1 min. Column is reloaded with extract until all extract has passed through the columns.
8. Sample is washed with 750 μl of Qiagen PE buffer and column is centrifuged for 1 min.
9. Qiagen columns placed into final irradiated tubes. DNA is eluted by loading 100 μl of Qiagen's EB buffer.

Guanidine Isothiocyanate - Silica (modified from Höss & Pääbo 1993)

1. The following buffers are prepared and stored ahead of time before possible exposure to the bone samples:
   • Lysis Extraction Buffer
   4.7 M GuScn
   47 mM Tris-HCl pH 7.4
   20 mM EDTA pH 8.0
   5 % N-laurel sarcosine

   • Extraction Buffer
   4.7 M GuScn
   47 mM Tris-HCl pH 7.4

2. Prepared bone sample is transferred to a 15 ml conical tubes with 10 ml of 0.5 M EDTA pH 8.0 and rotated at room temperature for about 7 h.
3. Sample is centrifuged at 3400 RPM for 10 min and supernatant discarded.
4. A 4 ml volume Lysis Extraction Buffer is added to the original 15 ml tube, vortexed and rotated for about 13 h at 56 °C.
5. Sample is centrifuged at 3400 RPM for 10 min.
6. Sample supernatant is transferred to new, UV-irradiated 15 ml tubes.

7. 50 μl of silica is added to each tube.
8. Sample is incubated for 10 to 20 min at room temperature with slight agitation.
9. Silica is pelleted by centrifuging at 1500 RPM for 2 min and supernatant discarded.
10. Silica is washed with 1.5 ml Extraction Buffer. Product is transferred to a 1.5 ml tube.
11. Silica is washed with 70 % ethanol, vortexed and centrifuged.
12. Silica is washed with acetone, centrifuged at 1300 RPM for 4 min and supernatant discarded.
13. Sample is left to dry at room temperature for 15 min.
14. Silica is resuspended with 130 μl TE (irradiated), incubated at 56 °C for 10 min, then centrifuged at 13,000 RPM for 5 min to pellet the silica.
15. Supernatant is pipetted out, leaving silica behind, and transferred into UV-irradiated 1.5 ml final tubes.

Post-Extraction "Clean-Up" Procedure using Proteinase K

To further remove inhibitors from extracted samples, a Proteinase K clean-up method was used on Extractions #1 and #2. The method follows:

1. Make a Proteinase K buffer
   - 50 mM KCL
   - 15 mM Tris-HCl pH 7.5
   - 2.5mM $MgCl_2$
   - 5 % Tween 20
   - 1 mg/ml Proteinase K
2. Place 10 μl of extract into PCR tube; add 40 μl of Proteinase K buffer.
3. Incubate for 1 h at 55 °C.
4. Incubate at 95 °C for 10 min to inactivate the Proteinase K.
5. Use 1 μl of this Proteinase K/Extract solution per 1 μl PCR reaction size.

Polymerase Chain Reaction (PCR) Amplification Procedures

1. All reagents used in the PCR cocktails were bought directly from the manufacturer as molecular grade, DNase/RNase free specifically for this work. The only exception to this was the UV/UF H_2O, which, as described above, was taken from another laboratory and is regularly tested for contaminants in all PCR reactions.
2. Individually capped PCR tubes in strips of eight are always used so that each sample tube is covered when not in use.
3. PCR cocktails were made in volumes of 50 μl per sample, and included the following:
   - 1x manufacturer's (molecular grade) buffer.
   - 0.6 mM primers (Gibco-BRL) rehydrated in molecular grade 1x TE and diluted in UV/UF H_2O (as above).
   - 1.5 mM manufacturer's (molecular grade) $MgCl_2$.
   - *Taq* polymerase from the manufacturer (molecular grade). We use either Amplitaq Gold (Perkin-Elmer) or Platinum-Taq (Gibco-BRL). These *Taq*s imitate "hot-start" PCR, which reduces non-specific priming and amplification.

• 200 mM dNTPs (Gibco-BRL) rehydrated in UV/UF H_2O (as above).
• 1 mg/ml Bovine Serum Albumin (BSA), molecular grade (Roche).
• UV/UF H_2O to bring the cocktail up to the total volume (*e.g.*, 50 µl per sample).

4. The PCR cocktail minus the *Taq* was prepared and UV-irradiated in a Stratagene for 15 min to eliminate any exogenous DNA contamination. The *Taq* polymerase was then added to the mixture, and the entire cocktail was then aliquoted into the separate PCR tubes.

We performed PCR amplifications under varying conditions depending on the *Taq* manufacturer's specifications. In addition, we used two cycling protocols, a standard PCR and a touch-down PCR.

The standard PCR cycling conditions began with an initial 4-10 min denaturation step at 94 °C, followed by 40 to 45 cycles in which the DNA samples are denatured at 94 °C for 40 sec, annealed at 52 °C for 40 sec, and extended at 72 °C for 40 sec. This cycling procedure is followed by a final extension step at 72 °C for 2 min.

The touchdown PCR involved an initial 2 min denaturation step at 94 °C, followed by 50 cycles in which the DNA samples are denatured at 94 °C for 14 seconds, annealed in the first of the 50 cycles at 58 °C for 30 seconds with a decrease in 0.1 °C in each successive cycle, and extended at 72 °C for 15 seconds.

Most PCRs included a positive control of modern DNA that is always added outside the ancient DNA room. This is to check that the PCR reaction itself functioned, even in the case of no amplification of the ancient samples.

DNA Target

Our strategy was to begin by targeting DNA from the maternally inherited mitochondrion, or mtDNA, for two reasons. First, mtDNA is valuable for population studies, in that it allows for the reconstruction of maternal lineages that often are correlated with geographical groups. In the case of the New World, it is widely accepted that most modern Native Americans fall into one of five mtDNA-based maternal lineages, or haplogroups, labeled A, B, C, D and X (*e.g.*, Smith *et al.* 1999; Merriwether *et al.* 1995; Schurr *et al.* 1990). Studies of ancient samples of ancient Native Americans confirm the presence of these maternal lineages before European contact (*e.g.*, O'Rourke *et al.* 1999; Stone and Stoneking 1998). If DNA amplification had been successful in the case of the Kennewick sample, such a haplogroup assignment might have been possible.

Second, because mtDNA exists in higher copy numbers per cell relative to nuclear DNA, it is best-suited for ancient DNA analyses. Positive amplification of mtDNA is a strong indicator of the general presence of DNA in ancient samples. If no mtDNA amplifies, it is unlikely that nuclear DNA remains in an ancient sample.

Moreover, in our experience, DNA from the displacement loop, or D-loop, of the mitochondrion tends to amplify more readily than DNA outside the mtDNA D-loop. Thus, our initial work targeted portions of the D-loop and we designed our primer pairs used in PCR amplifications

accordingly. If amplification of the D-loop had occurred, then tentative population-specific haplogroup assignments could have been made, since certain D-loop mutations are known to be closely associated with population-specific mtDNA haplogroups. At that point, the appropriate mutations outside the D-loop would have been targeted to confirm the haplogroup assignment based on D-loop sequences.

DNA Visualization

After PCR amplification, 10 μl of the PCR product along with approximately 1 μl of sucrose loading dye was loaded onto a 3 % NuSieve/1 % agarose gel stained with ethidium bromide. The gel images were "photographed" on thermal paper using a UV transilluminator attached to a digital imaging system. These gel images were subsequently scanned and appear in Appendix 1.

DNA Sequencing

When we successfully amplified DNA from PCRs that yielded negative PCR controls as well as extraction controls (see PCRs 16 and 17), we sequenced the resulting PCR products using the following method:

1. The PCR product is filtered to remove unincorporated primers and salts using a silica-based spin column and manufacturer reagents from the Qiagen PCR Purification kit.
2. The resulting purified PCR product is sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit v1.0 (Applied Biosystems).
3. The sequenced product is loaded and visualized through an ABI 377XL automatic sequencer.

Summary of Extractions and Results

DAM = D. Andrew Merriwether
GSC = Graciela S. Cabana
JM = John McDonough

Extraction Log

Extraction #1: performed by DAM from 5/22/00 to 5/25/00. A bone powder sample of the MC3 was taken, and the DNA extracted using the Qiagen method, as above.

Extraction #2: performed by DAM from 5/29/00 to 5/31/00. A bone powder sample of the MC3 was taken, and the DNA extracted using the Phenol-Chloroform method, as above.

Extraction #3: performed by GSC from 6/14/00 to 6/16/00. Bone scrapings were taken from the rib bone, and the DNA extracted using the modified Guanidine Isothiocyanate - Silica method, as above.

Extraction #4: performed by GSC from 7/4/00 to 7/6/00. A ground bone sample was taken from the rib bone, and the DNA extracted using the Phenol-Chloroform method, as above.

Extraction #5: performed by GSC from 9/3/00 to 9/6/00. The remaining rib bone was ground, and the DNA extracted using the Phenol-Chloroform-silica method, as above.

PCR Log

This log complements the gel images shown in Appendix 1.

Most PCR gels have standard size 1 kilobase ladders flanking the samples in both the left-most (lane 0) and right-most lanes.

• PCR 1 (GSC)
Primers 16192 to 16322 (mtDNA D-loop)
PCR of Extraction #1 (Qiagen method)

Lane	Sample	Amplification status
1-17	Other ancient samples	
18	Kennewick sample	Negative
19	EDTA + Extraction Buffer (1st wash)	Positive
20	EDTA + Extraction Buffer (2nd wash)	Positive
21	Proteinase K Buffer	Negative
22	PCR Negative Control	Negative
23	PCR Positive Control	Negative
24	PCR Negative Control	Negative

Result: Although the PCR negative controls did not show amplification, the Kennewick extract controls did show contamination. This does not mean, however, that the reagents used in the PCR did not contain exogenous human contamination. This is because if very low levels of exogenous DNA are in the PCR reagents, and there are more "samples" than PCR negative controls, chances are the contamination from the PCR reagents will show up in the samples. Thus, we typically wait to see the results of several PCRs before determining that an extraction product is itself contaminated. Indeed, subsequent PCRs on this extract suggested to us that the this extract was not contaminated.

• PCR 2 (GSC)
Primers 16268 to 16375 (mtDNA D-loop)
PCR of Extraction #1 (Qiagen method) and Extraction #2 (Phenol-Chloroform method)

Lane	Sample	Amplification status
1	Kennewick sample (Ext. #1)	Negative
2	EDTA + Extraction Buffer (1st wash)	Positive
3	EDTA + Extraction Buffer (2nd wash)	Positive
4	Proteinase K Buffer	Negative
5	Kennewick MC3 (Ext. #2)	Negative
6	Extract Control for MC3	Negative
7	Kennewick MC3	Negative
8	Kennewick MC3	Negative

9	Extract Control for MC3	Negative
10	Proteinase K buffer Control	Negative
11	Extract Control for MC3	Negative
12	PCR Negative Control (+ H_2O)	Negative
13	PCR Negative Control	Negative
14	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 3 (GSC)
Primers 16112 to 16237 (mtDNA D-loop)
PCR of Extraction #1 (Qiagen method) and Extraction #2 (Phenol-Chloroform method)

Lane	Sample	Amplification status
1	Kennewick sample (Ext. #1)	Negative
2	EDTA + Extraction Buffer (1st wash)	Negative
3	EDTA + Extraction Buffer (2nd wash)	Positive
4	Proteinase K Buffer	Positive
5	Kennewick MC3 (Ext. #2)	Positive
6	Extract Control for MC3	Positive
7	Kennewick MC3	Positive
8	Kennewick MC3	Positive
9	Extract Control for MC3	Positive
10	Proteinase K Buffer Control	Negative
11	Extract Control for MC3	Negative
12	PCR Negative Control (+ H_2O)	Positive
13	PCR Negative Control	Positive
14	PCR Positive Control	Positive

Result: PCR contaminated by an unknown source. The PCR reagents were discarded and samples were re-run with new reagents (below).

• PCR 4 (GSC)
Primers 16112 to 16237 (mtDNA D-loop)
PCR of 1:20 dilutions of Extraction #1 (Qiagen method) and Extraction #2 (Phenol-Chloroform method)

Lane	Sample	Amplification status
1	Kennewick sample (Ext. #1)	Negative
2	EDTA + Extraction Buffer (1st wash)	Negative
3	EDTA + Extraction Buffer (2nd wash)	Negative
4	Proteinase K Buffer	Negative
5	Kennewick MC3 (Ext. #2)	Negative

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6	Extract Control for MC3	Negative
7	Kennewick MC3	Negative
8	Kennewick MC3	Negative
9	Extract Control for MC3	Negative
10	Proteinase K buffer Control	Negative
11	Extract Control for MC3	Negative
12	PCR Negative Control (+ H_2O)	Negative
13	PCR Negative Control	Negative
14	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 5(GSC)
Primers 16268 to 16375 (mtDNA D-loop)
PCR of 1:100 dilutions of Extraction #1 (Qiagen method) and Extraction #2 (Phenol-Chloroform method)

Lane	Sample	Amplification status
1	Kennewick sample (Ext. #1)	Negative
2	EDTA + Extraction Buffer (1st wash)	Negative
3	EDTA + Extraction Buffer (2nd wash)	Negative
4	Proteinase K Buffer	Negative
5	Kennewick MC3 (Ext. #2)	Negative
6	Extract Control for MC3	Negative
7	Kennewick MC3	Negative
8	Kennewick MC3	Negative
9	Extract Control for MC3	Negative
10	Proteinase K buffer Control	Negative
11	Extract Control for MC3	Negative
12	PCR Negative Control (+ H_2O)	Negative
13	PCR Negative Control	Negative
14	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 6 (GSC)
Primers 16268 to 16375 (mtDNA D-loop)
PCR of Extraction #3 (modified Guanidine Isothiocyanate - Silica method)

Lane	Sample	Amplification status
1-14	Other ancient samples	
14	Kennewick (Ext. #3)	Negative
15	Lysis Extraction Buffer	Negative

16	Extraction Buffer	Negative
17	Extract Control	Negative
18	PCR Negative Control (+ H_2O)	Negative
19	PCR Negative Control	Negative
20	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 7 (DAM)
Primers 8195 to 8317 (Haplogroup B)
PCR of Extraction #1 (Qiagen method), Extraction #2 (Phenol-Chloroform method) and Extraction #3 (modified Guanidine Isothiocyanate - Silica method)

Lane	Sample	Amplification status
1	Kennewick sample (Ext. #2)	Negative
2	Kennewick sample (Ext. #2)	Positive
3	Kennewick sample (Ext. #2)	Positive
4	Kennewick sample (Ext. #1)	Positive
5	Kennewick sample (Ext. #3)	Positive
6	Extract Control for MC3 (Ext. #2)	Positive
7	Proteinase K Buffer (Ext. #2)	Positive
8	Extract Control for MC3 (Ext. #2)	Negative
9	Proteinase K buffer Control (Ext. #2)	Positive
10	Extract Control for MC3 (Ext. #2)	Positive
11	Lysis Extraction Buffer (Ext. #3)	Positive
12	Extraction Buffer (Ext. #3)	Negative
13	Extract Control (Ext. #3)	Negative
14	PCR Negative Control (+ H_2O)	Positive
15	PCR Negative Control	Negative
16	PCR Negative Control	Positive
17	PCR Positive Control	Positive
18	PCR Positive Control	Positive

Result: PCR contaminated by an unknown source. The PCR reagents were discarded and samples were re-run with new reagents (below).

• PCR 8 (GSC)
Primers 8244 to 8313 (Haplogroup B)
PCR of a Post-Extraction Clean-up Procedure (Proteinase K) of Extraction #1 (Qiagen method), Extraction #2 (Phenol-Chloroform method) and Extraction #3 (modified Guanidine Isothiocyanate - Silica method)
Note: this gel was made by melting down a previously-used gel, hence the stray band in the gel image.

Lane	Sample	Amplification status
1	Proteinase K of Kennewick sample (Ext. #2)	Negative
2	Proteinase K of Kennewick sample (Ext. #2)	Negative
3	Proteinase K of Kennewick sample (Ext. #2)	Negative
4	Proteinase K of Kennewick sample (Ext. #1)	Negative
5	Proteinase K of Kennewick sample (Ext. #3)	Negative
6	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
7	Proteinase K of Proteinase K Buffer (Ext. #2)	Negative
8	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
9	Proteinase K of Proteinase K buffer Control (Ext. #2)	Negative
10	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
11	Proteinase K of Lysis Extraction Buffer (Ext. #3)	Negative
12	Proteinase K of Extraction Buffer (Ext. #3)	Negative
13	Proteinase K of Extract Control (Ext. #3)	Negative
14	Negative Control of Proteinase K buffer (+ H_2O)	Negative
15	Negative Control of Proteinase K buffer	Negative
16	PCR Negative Control (+ H_2O)	Negative
17	PCR Negative Control	Negative
18	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 9 (DAM)
Primers 16192 to 16375 (mtDNA D-loop)
PCR of a Post-Extraction Clean-up Procedure (Proteinase K) of Extraction #1 (Qiagen method), Extraction #2 (Phenol-Chloroform method) and Extraction #3 (modified Guanidine Isothiocyanate - Silica method)

Lane	Sample	Amplification status
1	Proteinase K of Kennewick sample (Ext. #2)	Positive
2	Proteinase K of Kennewick sample (Ext. #2)	Positive
3	Proteinase K of Kennewick sample (Ext. #2)	Positive
4	Proteinase K of Kennewick sample (Ext. #1)	Positive
5	Proteinase K of Kennewick sample (Ext. #3)	Positive
6	Proteinase K of Extract Control for MC3 (Ext. #2)	Positive
7	Proteinase K of Proteinase K Buffer (Ext. #2)	Positive
8	Proteinase K of Extract Control for MC3 (Ext. #2)	Positive
9	Proteinase K of Proteinase K buffer Control (Ext. #2)	Negative
10	Proteinase K of Extract Control for MC3 (Ext. #2)	Positive
11	Proteinase K of Lysis Extraction Buffer (Ext. #3)	Positive
12	Proteinase K of Extraction Buffer (Ext. #3)	Positive
13	Proteinase K of Extract Control (Ext. #3)	Positive
14	Negative Control of Proteinase K buffer (+ H_2O)	Positive

15	Negative Control of Proteinase K buffer	Positive
16	PCR Negative Control (+ H_2O)	Positive
17	PCR Negative Control	Positive
18	PCR Positive Control	Positive
19	PCR Positive Control	Positive
20	PCR Negative Control	Positive

Result: PCR contaminated.

• PCR 10 (JM)
Primers 16112 to 16237 (mtDNA D-loop)
PCR of a Post-Extraction Clean-up Procedure (Proteinase K) of Extraction #1 (Qiagen method), Extraction #2 (Phenol-Chloroform method) and Extraction #3 (modified Guanidine Isothiocyanate - Silica method)

Lane	Sample	Amplification status
1	Proteinase K of Kennewick sample (Ext. #2)	Negative
2	Proteinase K of Kennewick sample (Ext. #2)	Negative
3	Proteinase K of Kennewick sample (Ext. #2)	Negative
4	Proteinase K of Kennewick sample (Ext. #1)	Negative
5	Proteinase K of Kennewick sample (Ext. #3)	Negative
6	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
7	Proteinase K of Proteinase K Buffer (Ext. #2)	Negative
8	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
9	Proteinase K of Proteinase K buffer Control (Ext. #2)	Negative
10	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
11	Proteinase K of Lysis Extraction Buffer (Ext. #3)	Negative
12	Proteinase K of Extraction Buffer (Ext. #3)	Negative
13	Proteinase K of Extract Control (Ext. #3)	Negative
14	Negative Control of Proteinase K buffer (+ H_2O)	Negative
15	Negative Control of Proteinase K buffer	Negative
14	PCR Negative Control (+ TE)	Negative
15	PCR Negative Control (+ H_2O)	Negative
16	PCR Negative Control	Negative
17	PCR Negative Control	Negative
18	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 11 (JM)
Primers 16268 to 16375 (mtDNA D-loop)
PCR of a Post-Extraction Clean-up Procedure (Proteinase K) of Extraction #1 (Qiagen method), Extraction #2 (Phenol-Chloroform method) and Extraction #3 (modified Guanidine Isothiocyanate - Silica method)

Lane	Sample	Amplification status
1	Proteinase K of Kennewick sample (Ext. #2)	Negative
2	Proteinase K of Kennewick sample (Ext. #2)	Negative
3	Proteinase K of Kennewick sample (Ext. #2)	Negative
4	Proteinase K of Kennewick sample (Ext. #1)	Negative
5	Proteinase K of Kennewick sample (Ext. #3)	Negative
6	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
7	Proteinase K of Proteinase K Buffer (Ext. #2)	Negative
8	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
9	Proteinase K of Proteinase K buffer Control (Ext. #2)	Negative
10	Proteinase K of Extract Control for MC3 (Ext. #2)	Negative
11	Proteinase K of Lysis Extraction Buffer (Ext. #3)	Negative
12	Proteinase K of Extraction Buffer (Ext. #3)	Negative
13	Proteinase K of Extract Control (Ext. #3)	Negative
14	Negative Control of Proteinase K buffer (+ H_2O)	Negative
15	Negative Control of Proteinase K buffer	Negative
16	PCR Negative Control (+ H_2O)	Negative
17	PCR Negative Control	Negative
18	PCR Negative Control	Negative
19	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 12 (JM)
Primers 16192 to 16322 (mtDNA D-loop)
PCR of a serial dilution of Extraction #1.

Lane	Sample	Amplification status
1	1:5 dilution of Kennewick sample (Ext. #1)	Negative
2	1:10 dilution of Kennewick sample (Ext. #1)	Negative
3	1:20 dilution of Kennewick sample (Ext. #1)	Negative
4	1:50 dilution of Kennewick sample (Ext. #1)	Negative
5	1:10 dilution of Extract Control #2 (Ext. #1)	Negative
6	1:10 dilution of Extract Control #3 (Ext. #1)	Negative
7	1:10 dilution of Extract Control #4 (Ext. #1)	Negative
8	PCR Negative Control (+ H_2O)	Negative
9	PCR Negative Control (+ H_2O)	Negative
10	PCR Positive Control	Positive

Result: No amplification except for positive control.

• PCR 13 (GSC)

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Primers 642 to 708 (Haplogroup A)
PCR of a small fragment (66bp) of Extraction # 3 and #4.

Lane	Sample	Amplification status
1-4	Other ancient samples	
5	Kennewick sample (Ext. #3)	Positive
6	Extract Control for Ext. #3	Positive
7	Kennewick sample (Ext. #4)	Negative
5	Extract Control for Ext. #4	Positive
8	PCR Negative Control (+ H_2O)	Negative
9	PCR Negative Control	Negative
10	PCR Positive Control	Positive

Result: One Kennewick DNA sample and 2 extraction controls amplified. PCR product was subsequently digested with the *Hae* III enzyme (below).

• Digest 1 (GSC)
Product of PCR 13 digested the *Hae* III enzyme to detect the mutation defining Haplogroup A.

Lane	Sample	Digestion status
1	No sample	
2-4	Other ancient samples	
5	Kennewick sample (Ext. #3)	No digest
6	Extract Control for Ext. #3	No digest
5	Extract Control for Ext. #4	No digest

Result: No Kennewick sample or extract control digested.

• PCR 14 and 15 (GSC)
Primers 16112 to 16237 (PCR 14) and Primers 16192-16322 (PCR 15) (mtDNA D-loop).
PCRs of Extraction # 5 (modified Phenol-Chloroform-silica method).

Lane	Sample	Amplification status
PCR 14		
1	Kennewick sample (Ext. #5)	Negative (with smear)
2	Extract Control for Ext. #5	Negative
3	PCR Negative Control (+ H_2O)	Negative
4	PCR Negative Control	Negative
PCR 15		
5	Kennewick sample (Ext. #5)	Negative (with smear)
6	Extract Control for Ext. #5	Negative
7	PCR Negative Control (+ H_2O)	Negative

8	PCR Negative Control	Negative

Result: The Kennewick samples did not yield specific amplification products in that no distinct bands at the appropriate levels on the gel appeared. However, a smear of non-specific DNA appeared in the Kennewick sample lanes that is typical when bacterial and fungal DNA are also present. This is not unlikely given that the rib sample included a soil matrix. The co-extraction of bacterial and fungal DNA also suggests that humic acids and other potential inhibitors present in soils might be affecting our ability to detect any human DNA. One way to lessen their effect is to dilute the extract with the hopes of diluting inhibitory elements. PCRs 16 and 17 (below) represent PCRs based on these dilutions.

• PCR 16 (GSC)
Primers 16112 to 16237 (mtDNA D-loop).
PCRs of dilutions of Extraction # 5 (modified Phenol-Chloroform-silica method).

Lane	Sample	Amplification status
1	1:5 dilution of Extract Control for Ext. #5	Negative
2	1:5 dilution of Kennewick sample (Ext. #5)	Negative
3	PCR Negative Control (+ H_2O)	Negative
4	PCR Negative Control	Negative
5	1:50 dilution of Extract Control for Ext. #5	Negative
6	1:50 dilution of Kennewick sample (Ext. #5)	Positive
7	PCR Negative Control	Negative

Result: Positive amplification of Kennewick sample (Lane #6), while PCR and extraction negatives showed no DNA amplification. The resulting PCR product was sequenced, and found to match the sequence of JM.

• PCR 17 (GSC)
Primers 16192 to 16322 (mtDNA D-loop).
PCRs of dilutions of Extraction # 5 (modified Phenol-Chloroform-silica method).

Lane	Sample	Amplification status
1	1:5 dilution of Extract Control for Ext. #5	Negative
2	1:5 dilution of Kennewick sample (Ext. #5)	Positive
3	PCR Negative Control (+ H_2O)	Negative
4	PCR Negative Control	Negative
5	1:50 dilution of Extract Control for Ext. #5	Negative
6	1:50 dilution of Kennewick sample (Ext. #5)	Positive
7	PCR Negative Control	Negative

Result: Positive amplification of Kennewick sample (Lanes #2 and 6), while PCR and extraction negatives showed no DNA amplification. The resulting PCR product was sequenced, and found

to match the sequence of JM (see appendix three which contains electropherograms of the sequences from PCRs 16 and 17, with the sites differing from the published reference sequence circled). Note that for the region sequenced, all three mutations match the sequence of JM and there are no positions that do not match JM. All are consistent with a European origin of the sequence. The observed sequence is not found in genebank or HVRbase, only in JM from the Merriwether lab. The 16145 mutation has not been observed before. Thus the best explanation for this sequence is contamination of the extract by unknown means by the DNA from JM.

CONCLUSION

We were unable to obtain reliable ancient DNA amplification results from the Kennewick samples. This means that either (1) no original DNA was preserved in the bone samples transferred to our laboratory, or (2) original DNA was preserved in the bone samples but we were unable to extract it. The latter could occur either because the extraction methods used in this analysis were inadequate, or because we were unable to overcome PCR inhibitors once the DNA was extracted. However, we used a variety of methods to overcome PCR inhibition, including an additional Proteinase K clean-up step to further break down proteins in the samples, dilutions of the extractions reduce the proportion of inhibitors relative to DNA, and the use of BSA in the PCR reaction (BSA may bind to inhibitors, thus inhibiting the inhibitors).

Moreover, any DNA amplification we obtained seems to have been a product of exogenous human contamination. This is immediately clear in two cases: (1) when the PCR negative controls are contaminated, and (2) when the PCR product is sequenced and matched to an investigator (*e.g.*, an excavator or other analyst). The first case implies that at the very least the reagents used in the PCR are contaminated, and reagents become easily contaminated through multiple uses. In this case, we threw out the old PCR reagents and performed a new PCR with fresh reagents. The second case demonstrates that even under the most stringent conditions, modern contaminating DNA is problematic in ancient DNA analysis.

Finally, it is possible that an amplified DNA product is the result of exogenous human contamination despite the fact that negative controls yield no amplification. If very low levels of exogenous DNA exist in the PCR reagents, and there are more "samples" than PCR negative controls, chances are the contamination from the PCR reagents will show up in the samples, rather than in the PCR controls. For similar sampling reasons, low levels of exogenous DNA in DNA extracts and disposable pipette tips, tubes, etc., also will amplify sporadically, from one PCR to another. Thus, in addition to the use of controls, we monitor the results of positive amplifications by sequencing the amplification products. For example, PCRs 14, 15, 16, and 17 showed no positive amplification of PCR or extract controls, yet the sequences (shown in Appendix 3) were an exact match of our laboratory technician.

To conclude, we attempted a variety of techniques to both extract DNA from the sample and reduce the effects of potential PCR inhibitors. When DNA amplified, it was always from an exogenous source. This, combined with the fact that even very low levels of contaminating DNA will preferentially amplify when the sample itself contains little or no DNA of its own, leads us

to conclude that little or no DNA remained in the Kennewick samples transferred to our laboratory. However, our conclusion should not preclude further DNA testing using future novel methods on other, perhaps more DNA-rich, bone samples from the Kennewick remains. We are still of the belief that teeth are the best source for clean DNA free of exogenous contamination and would still argue for testing of the teeth (even with the caveat that Xrays can damage DNA and all but two of the teeth were apparently X-rayed).

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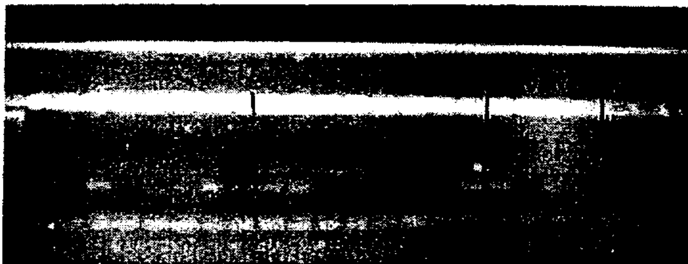
DOI 09733

Appendix 1
PCR and Digest Gel Images

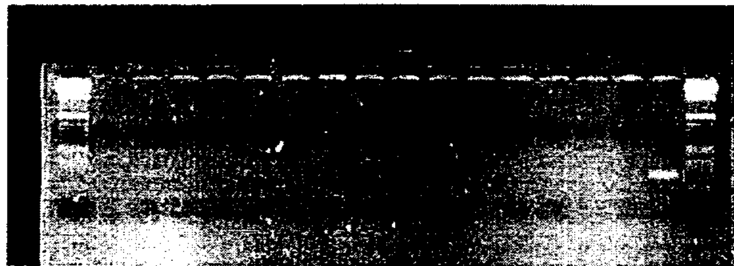
PCR 1

[illegible]



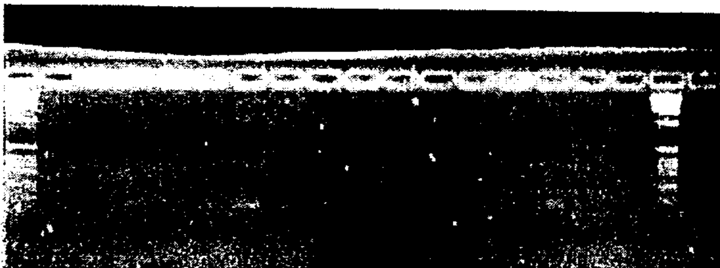
PCR 2

[illegible]



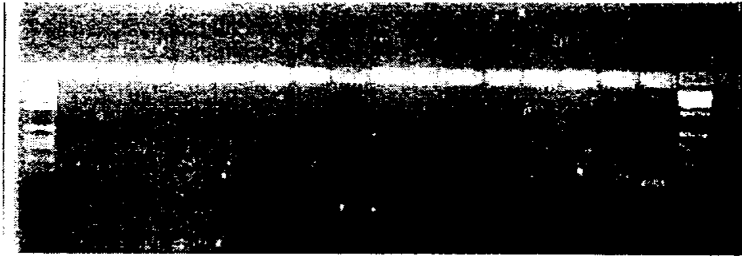
PCR 3

[illegible]



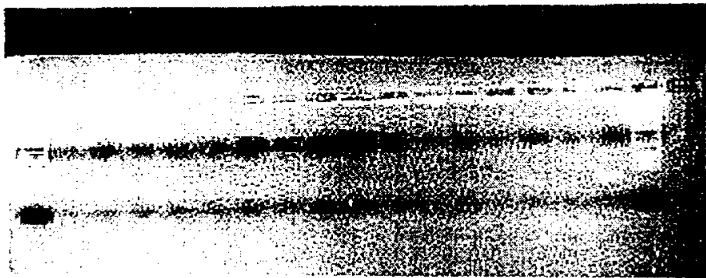
PCR 4

STRATAGENE EAGLEEYE II [illegible]
[illegible]
IMAGE SIZE [illegible]
INT PERIOD = [illegible] COUNTS.
IMAGE CREATED ON [illegible] JUN [illegible] 2000.



PCR 5

STRATAGENE EAGLEEYE II 06/16/00 05:58:27
[illegible]
IMAGE SIZE (640 - 480 - 8).
INT PERIOD = 1.49 SEC OR 45 COUNTS.
IMAGE CREATED ON FRI JUN 16 05:58:24 2000.

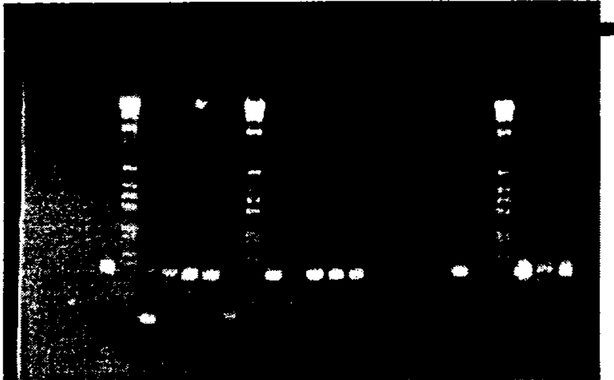


PCR 6

STRATAGENE EAGLEEYE II [illegible]
[illegible]
IMAGE SIZE [illegible]
INT PERIOD = [illegible] COUNTS.
IMAGE CREATED ON [illegible] JUN [illegible] 2000.

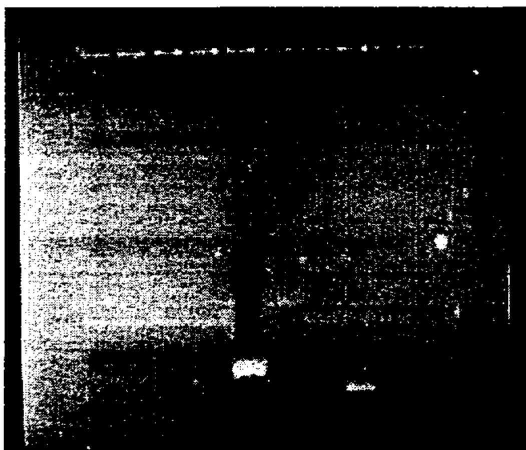
PCR 7

STRATAGENE EAGLE EYE II 05/28/00 07:[illegible]
[illegible] PCR [illegible] 8195-8237 [illegible] contaminated
IMAGE SIZE (640 X 480 X 8).
INT PERIOD = 0.46 SEC OR 20 COUNTS.
IMAGE CREATED ON SUN JUN 25 07:[illegible] 2000.



PCR 8

STRATAGENE EAGLE EYE II 07 02 00 [illegible]
[illegible]
IMAGE SIZE (640 X 480 X 8).
INT PERIOD = 1.33 SEC OR 40 COUNTS.
IMAGE CREATED ON MON JUL 03 [illegible] 2000.



PCR 9

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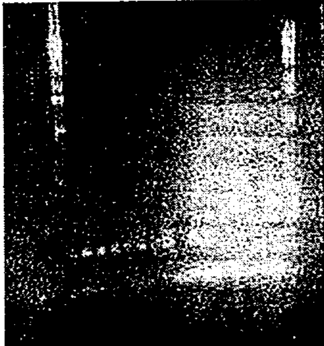
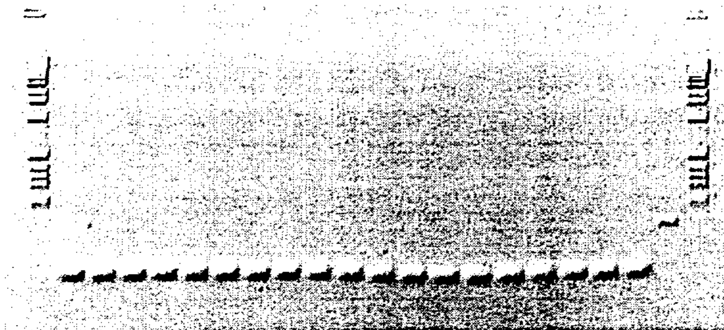
[illegible]
IMAGE SIZE (640 × 480 × 8).
[illegible] COUNTS.
IMAGE CREATED ON [illegible] 2000.

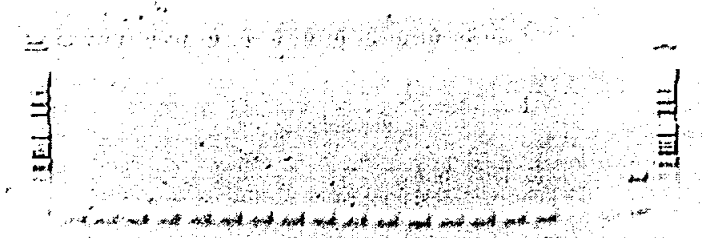


PCR 10

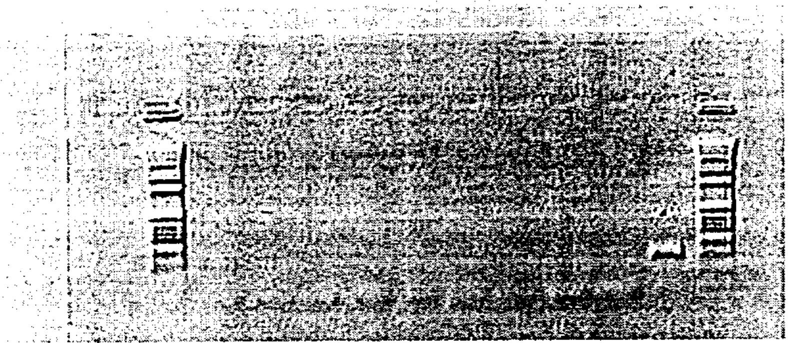
[illegible]
[illegible]
[illegible]
[illegible] COUNTS.
IMAGE CREATED ON SAT JUL [illegible] 2000.



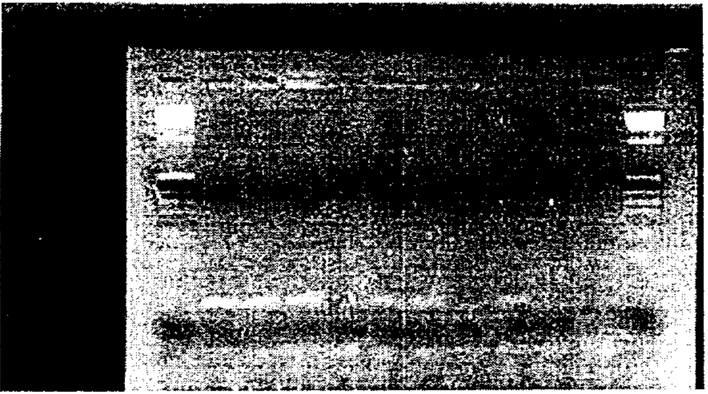
PCR 11



PCR 12

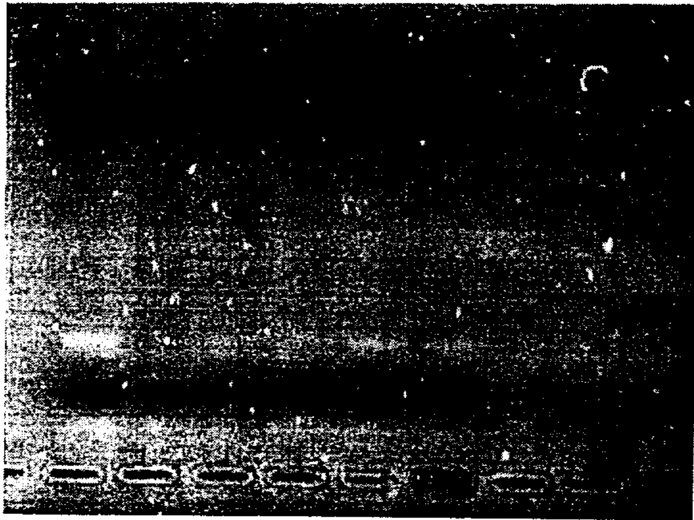


PCR 13

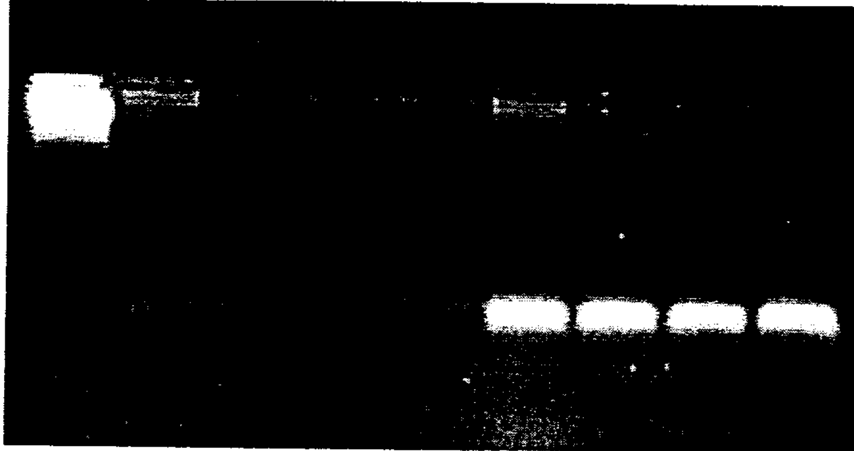


DOI 09738

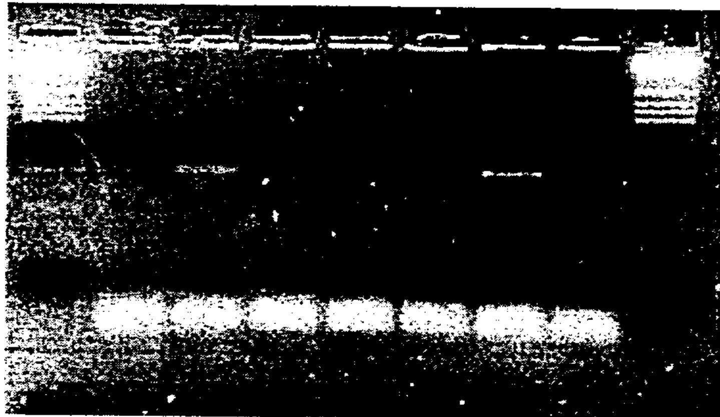
Digest 1



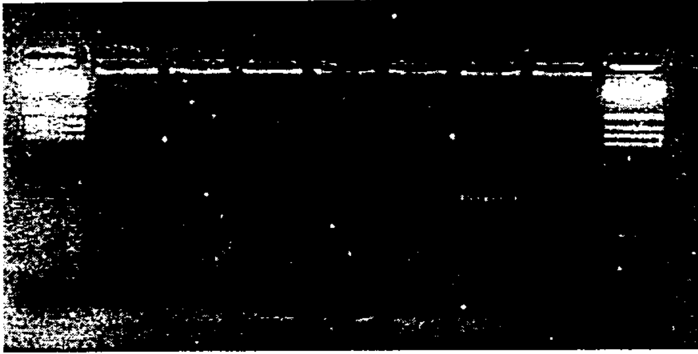
PCR 14 and 15



PCR 16



PCR 17



Appendix 2
Photographic Log

Photo 1: Sample storage

Samples are stored in DNase/RNase free Sarstedt tubes.

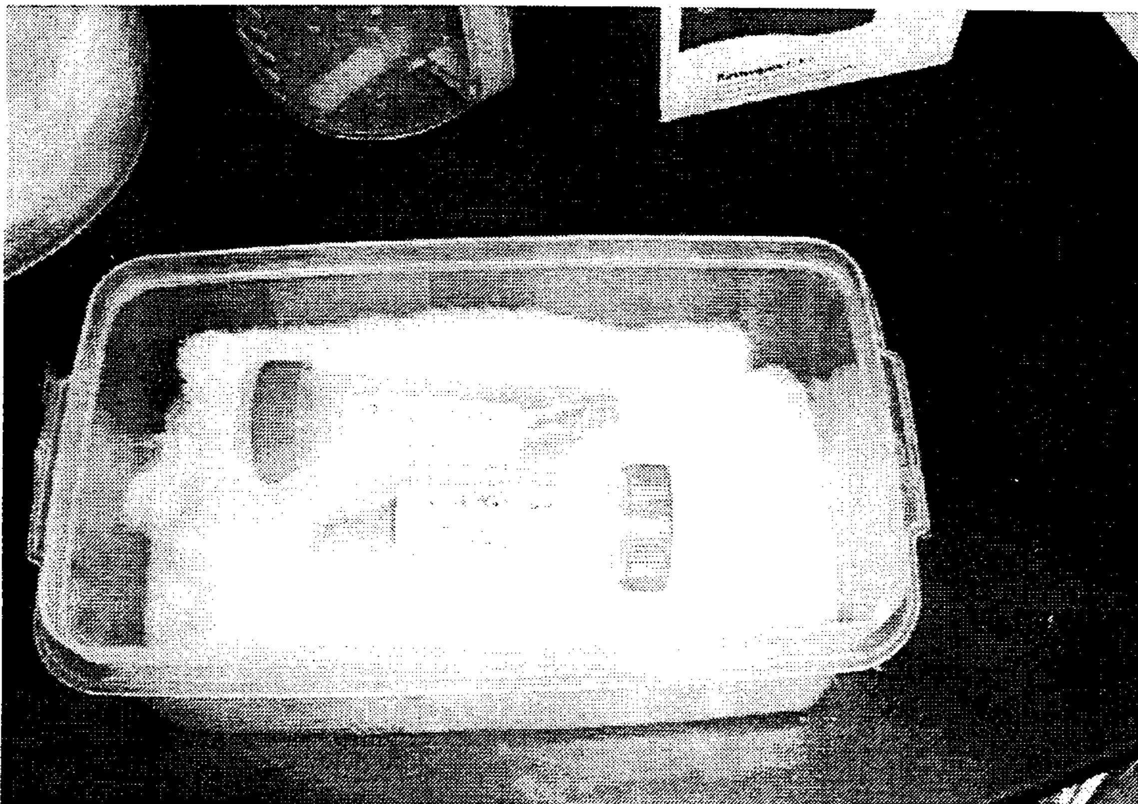


Photo 2: Post decontamination

Kennewick MC3 sample has been scrubbed with a 10% bleach solution and UV-irradiated on all sides. Sample is ready for drilling.

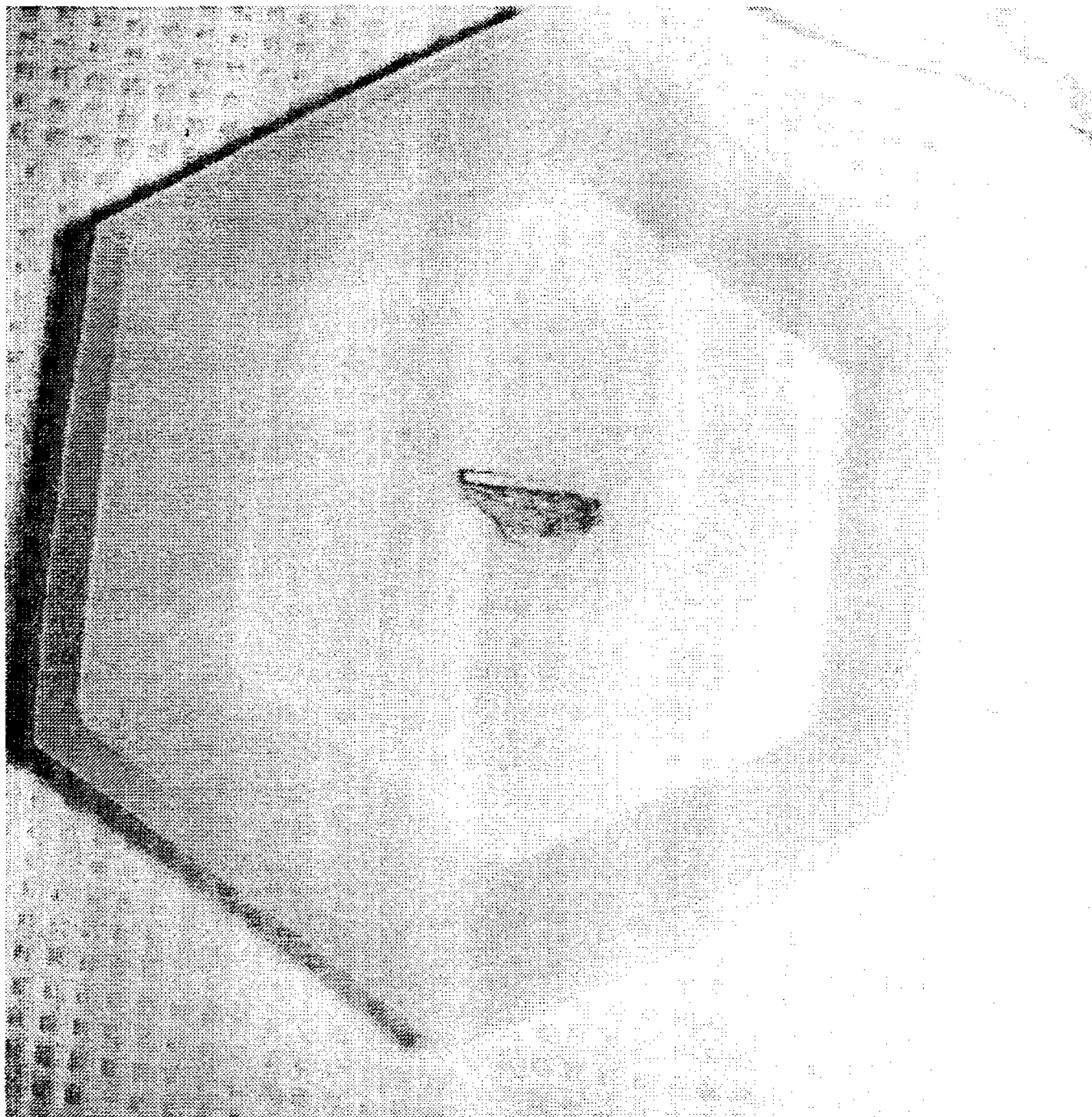


Photo 3: Drilling

MC3 sample is being drilled with a dremmel tool by DAM in the ancient DNA laboratory.

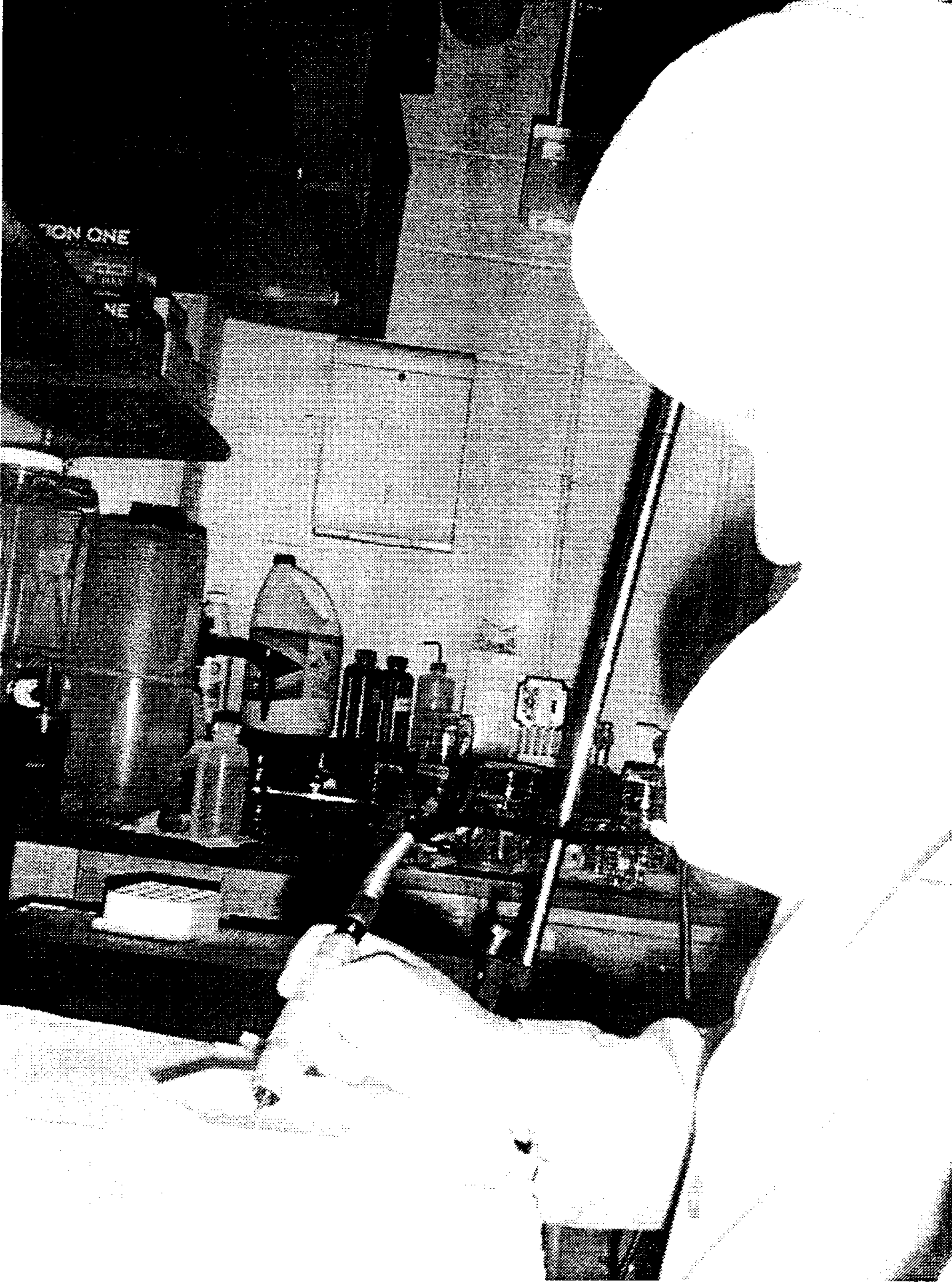


Photo 4: Detail of drilling

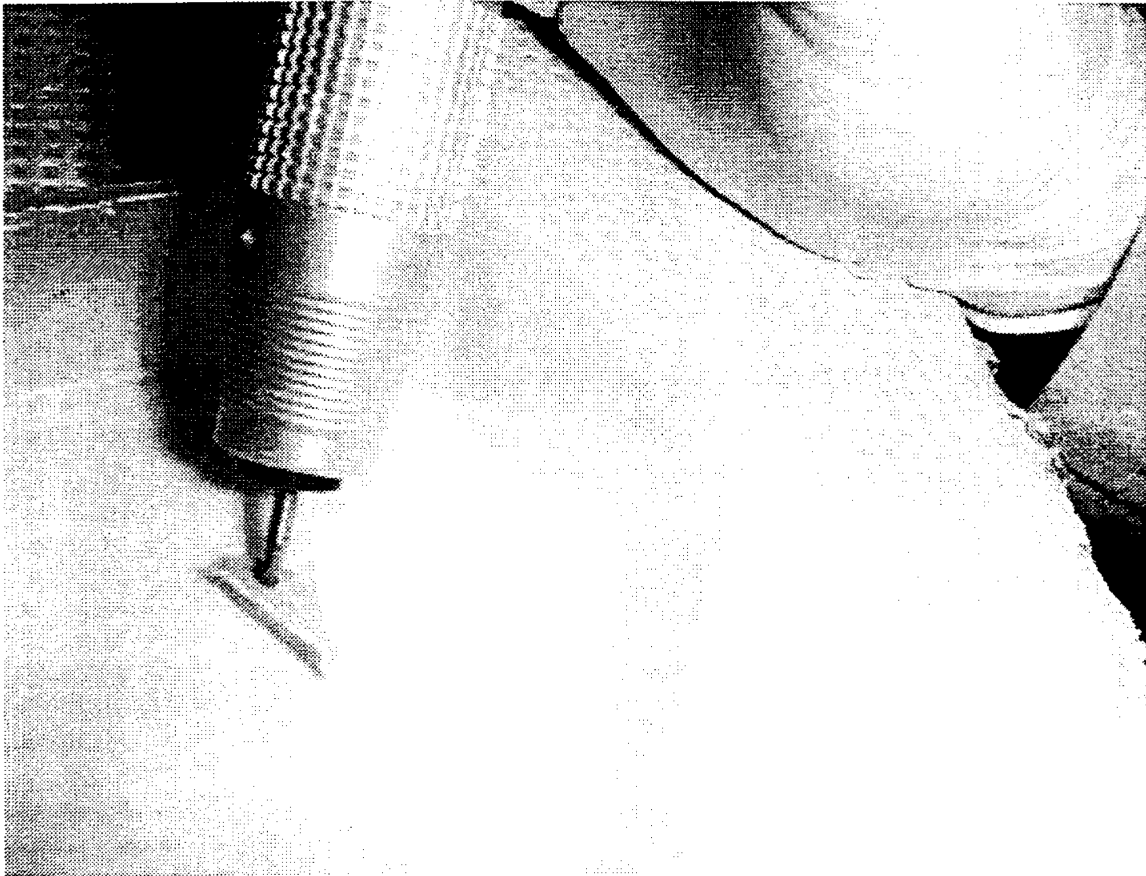


Photo 5: Bone sampling

A portion of the MC3 has been drilled into a fine powder. This powder will be used in the subsequent DNA extraction.

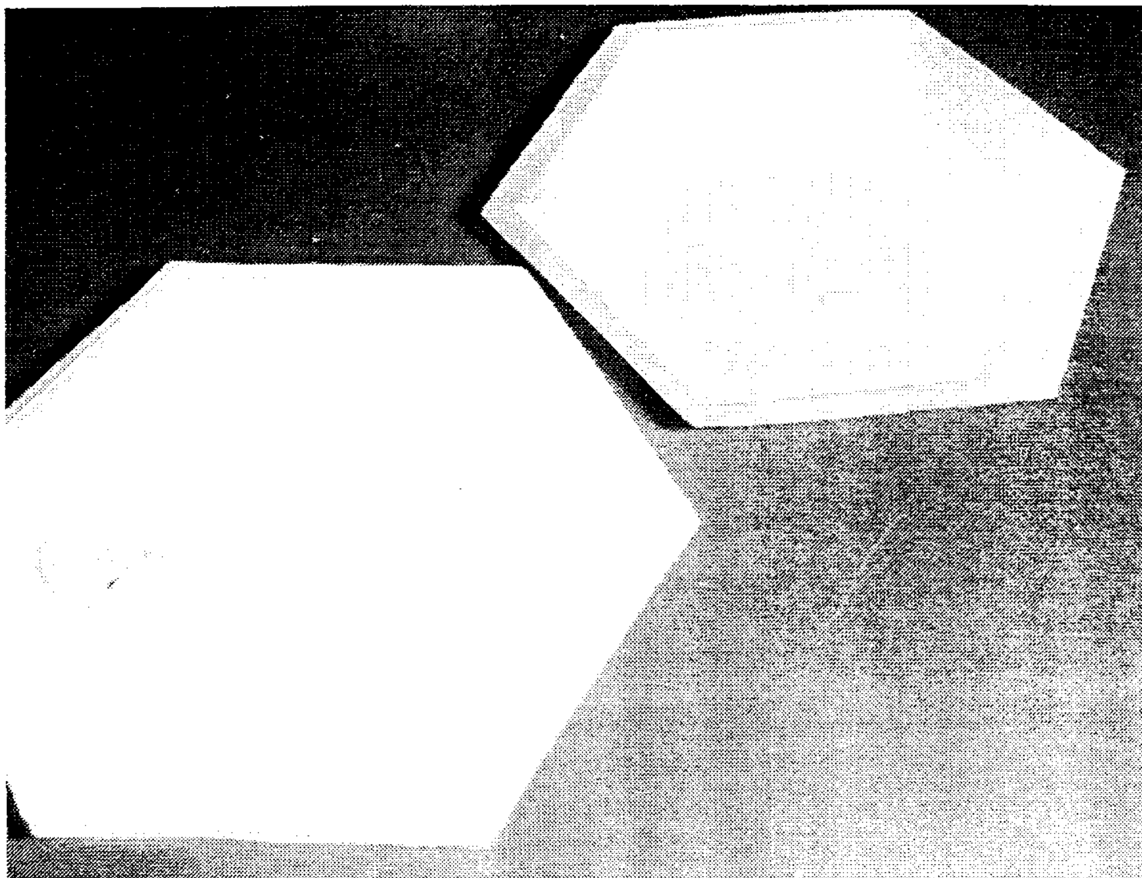


Photo 6: Samples in early phase of ancient DNA extraction

Samples shown here are in the early phases of ancient DNA extraction. Most protocols require an initial demineralization step in EDTA pH 8.0, and need to rotate slowly in 56^0C. We seal the samples in a DNase/RNase free Sarstedt tube with EDTA, place them on an automatic rotator within an incubator that maintains a steady temperature.

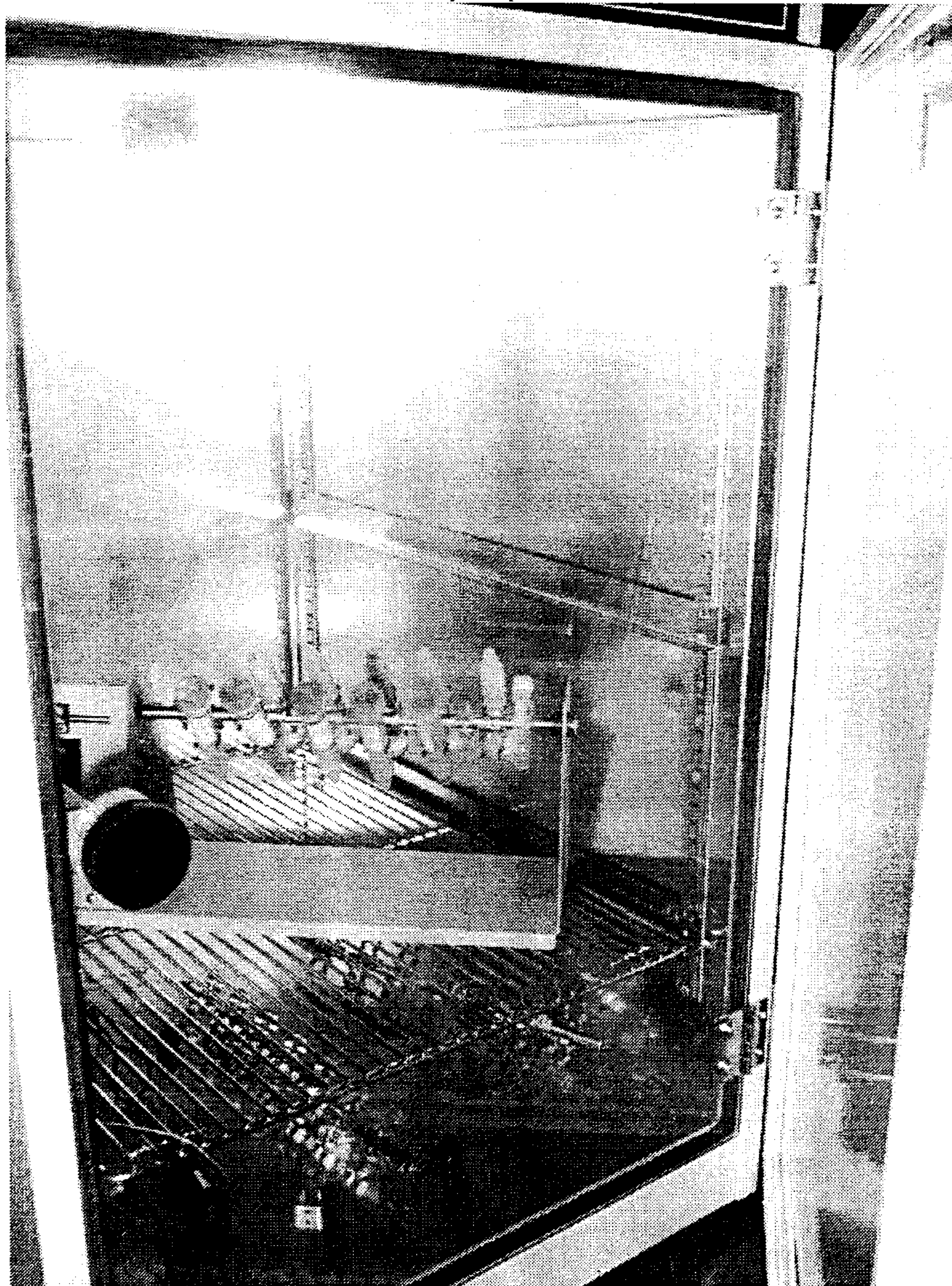
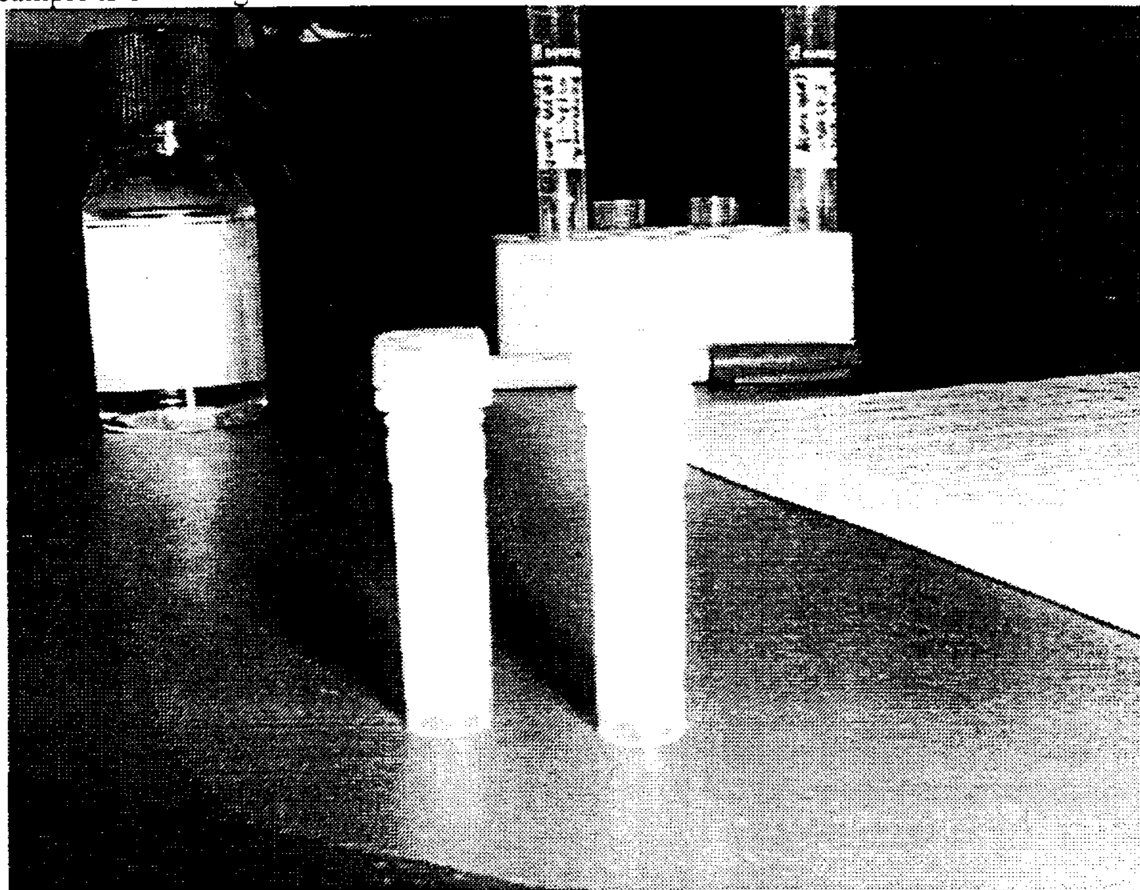


Photo 7: Samples undergoing Phenol-chloroform DNA extraction

These samples are being extracted using a phenol-chloroform method. The samples have just had chloroform added to them.

Photo 8: Extraction outcome

Shown here are the samples after having undergone a phenol-chloroform extraction. The actual sample is on the right and an extract control on the left.



Appendix 3

Annotated Sequence Electropherograms

• Electropherogram 1: Kennewick 1:50 dilution.

Primers 16112 to 16237, reverse direction.

Note: Forward direction did not sequence and therefore is not available.

ABI PRISM

Model 377
Version 3.3
LR-377
Version 3.3.1b2

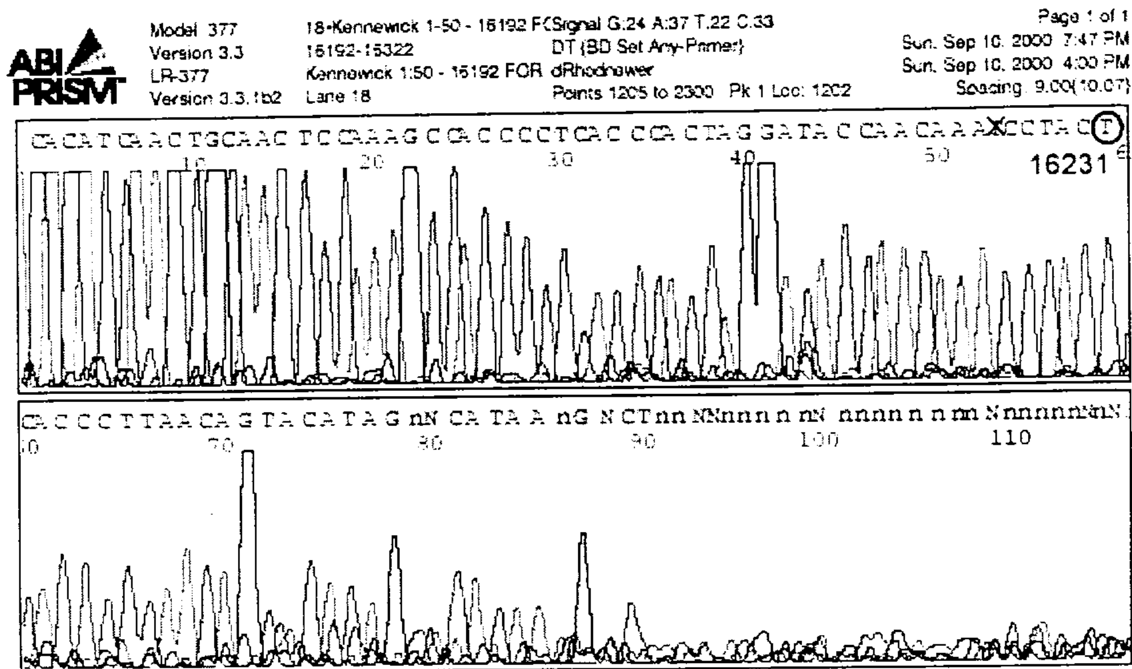
04-Kennewick 1-50 - 16237 R Signal G:375 A:120 T:94 C:245
16112 - 16237 DT {BD Set Any-Primer}
Kennewick 1:50 - 16237 REV dRhodrewer
Lane 4 Points 1050 to 2300 Pk 1 Loc: 1050

Page 1 of 1
Sun, Sep 10, 2000 7:34 PM
Sun, Sep 10, 2000 4:00 PM
Spacing: 7.00(9.08)

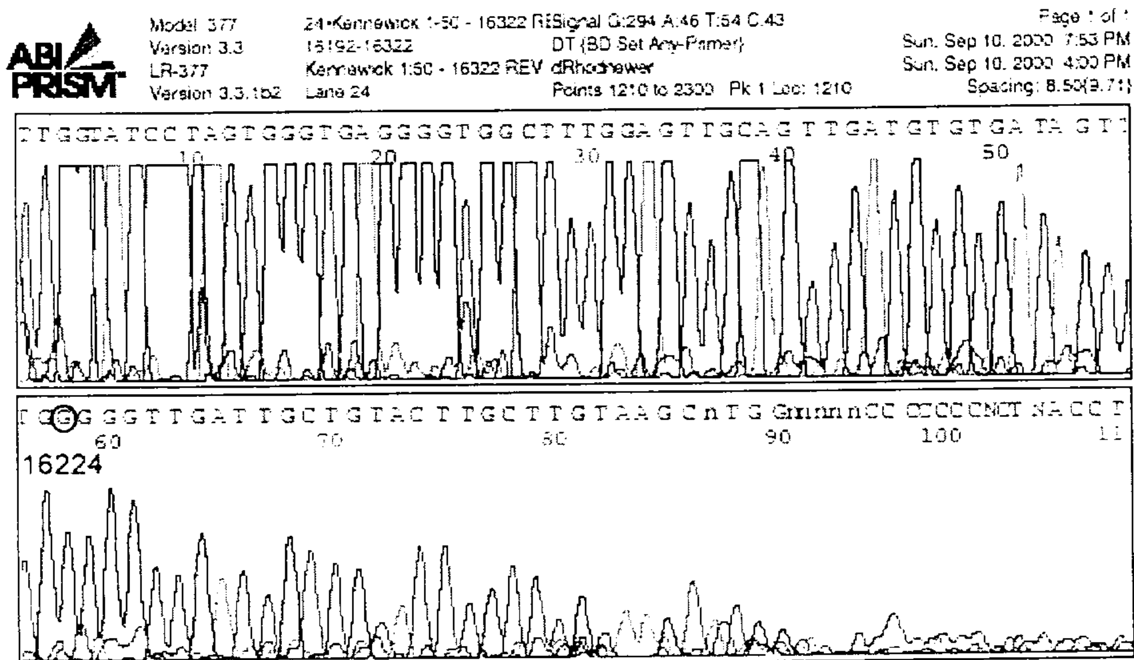
16145

DOI 09749

• Electropherogram 2: Kennewick 1:50 dilution.
Primers 16192 to 16322, forward direction.



• Electropherogram 3: Kennewick 1:50 dilution.
Primers 16192 to 16322, reverse direction.



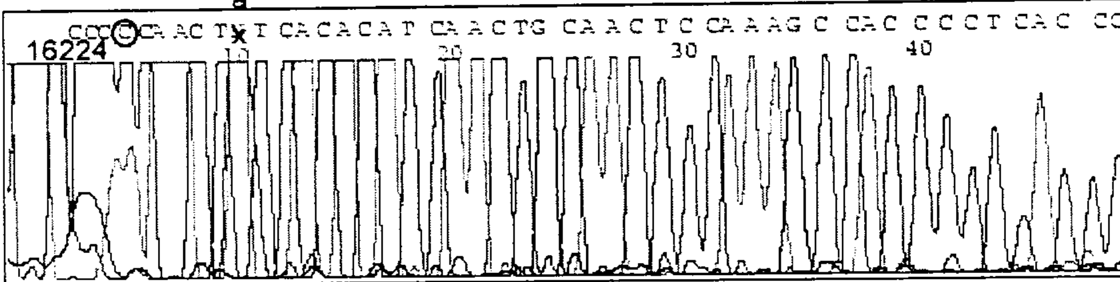
• Electropherogram 4: Kennewick 1:5 dilution
Primers 16192 to 16322, forward direction.

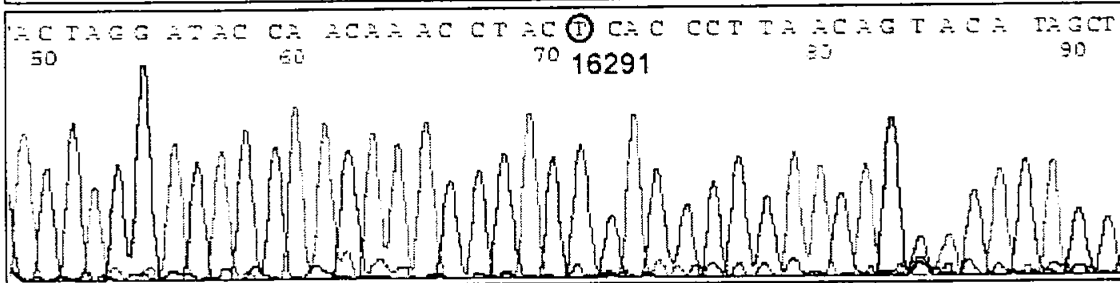
Model 377
Version 3.3
LR-377
Version 3.3 ...b2

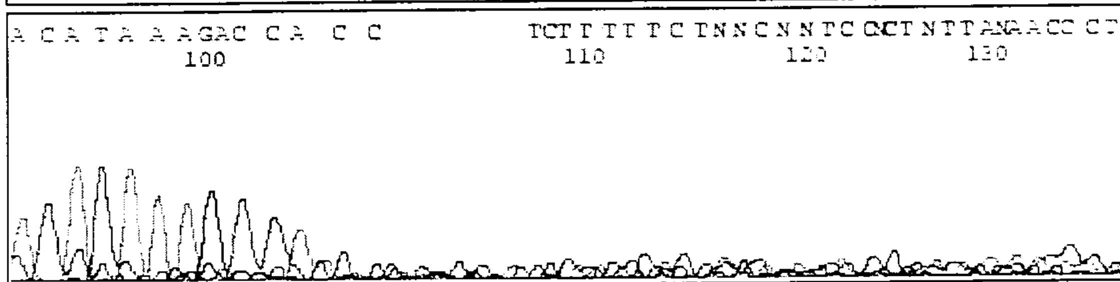
10-Kennewick 1-5 - 16192 FO
16192-16322
Kennewick 1:5 - 16192 FOR
Lane 10

Signal G:38 A:64 T:42 C:55
DT {BD Set Any-Primer}
dRhodnewer
Points 1100 to 2300 Pk 1 Loc: 1100

Page 1 of 1
Sun. Sep 10, 2000 7:31 PM
Sun. Sep 10, 2000 4:00 PM
Spacing: 7.00(9.53)







• Electropherogram 5: Kennewick 1:5 dilution.
Primers 16192 to 16322, reverse direction.

ABI PRISM

Model 377
Version 3.3
LR-377
Version 3.3.1b2

12•Kennewick 1-5 - 16322 RE'Signal G:453 A:74 T:87 C:71
16192-16322
Kennewick 1:5 - 16322 REV
Lane 12

DT {BD Set Any-Primer}
dRhodnewer
Points 1210 to 2300 Pk 1 Loc: 1210

Page 1 of 1
Sun. Sep 10. 2000 7:36 PM
Sun. Sep 10. 2000 4:00 PM
Spacing: 8.00(9.39)

TGGTATC CTAGTGGGTGAGGGGTGGC TTTGGAGTTGC AGT TGATGTGTGATA

XGTTGGGGGTTGATTGCTGTACTTGCTTGTAAGCATGG ACNNNNNNNNCN

16224