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Human Mitochondrial DNA Haplotyping Kit AT

Instructor's Manual

For Teaching Purposes Only

For technical assistance call 800.227.1150 x4381

Note: Upon receipt of the kit, store proteinase K, restriction enzyme *Mse*I, 10× restriction buffer, 10× BSA, mtDNA Primer/Loading Dye Mix, and pBR322/*Bst*NI markers in freezer (approximately -20°C). Other materials may be stored at room temperature (approximately 25°C).

CAROLINA

World-Class Support for Science & Math

Human Mitochondrial DNA Haplotyping AT (Advanced Technology)

In addition to the 46 chromosomes found in the nucleus of human cells, each mitochondrion in the cell cytoplasm has several copies of its own genome. The mitochondrial (mt) genome contains only 37 genes, which are involved in the process of oxidative phosphorylation—the production of energy and its storage in ATP.

There is strong evidence that mitochondria once existed as free-living bacteria, which were taken up by primitive ancestors of eukaryotic cells. The host cell provided a ready source of energy-rich nutrients, and the mitochondrion provided a means to extract energy using oxygen. This attribute was key to survival, as oxygen accumulated in the primitive atmosphere. Mitochondria are physically in the same size range as bacteria, and the mt genome retains bacteria-like features. Like bacterial chromosomes and plasmids, the mt genome is a circular molecule. Also, very few noncoding sequences, or introns interrupt mt genes. These features are contrary to those of eukaryotic chromosomes, which are linear, and eukaryotic genes, which have numerous introns.

The entire DNA sequence of the mt genome (16,569 nucleotides) was determined in 1981, well in advance of the Human Genome Project. Genes take up the majority of the mt genome. However, a noncoding region of approximately 1200 nucleotides contains signals that control replication of the chromosome and transcription of the mt genes. The DNA sequence of the “control region” is termed “hypervariable,” because it accumulates mutations at approximately 10 times the rate of nuclear DNA. This high mutation rate results in unique patterns of single nucleotide polymorphisms (SNPs), which are inherited through generations.

In the 1980s, Alan Wilson and coworkers at the University of California at Berkeley used mtDNA polymorphisms to create a “family tree” showing ancestral relationships between modern populations. Reasoning that all human populations arose from a common ancestor in the distant evolutionary past, Wilson’s group calculated how long it would take to accumulate the pattern of mutations observed in modern populations. They concluded that the ancestor of all modern humans arose in Africa about 200,000 years ago. This common ancestor was widely reported as the “mitochondrial Eve.” This confusing simplification—which appeared to leave out Adam—is due to the peculiar inheritance of mtDNA. Mitochondria are inherited exclusively from the mother, with no paternal contribution. Normally, at fertilization, only the male pronucleus (with

23 chromosomes) enters the egg cell. In addition to 23 nuclear chromosomes, the egg cell contributes all the cytoplasm and organelles to a zygote. Hundreds of maternal mitochondria are passed on to each daughter cell when the cytoplasm divides during the final stages of mitosis.

While each cell contains only two copies of a given nuclear gene (one on each of the paired chromosomes), there are hundreds to thousands of copies of a given mitochondrial gene in each cell. Thus, mtDNA analysis is important in forensic biology, especially in cases where the tissue samples are very old or in cases where the DNA is badly degraded. For example, control region polymorphisms have been used to

- Identify the remains of the Unknown Soldier killed in the Vietnam War.
- Identify the remains of the Romanov royal family killed in the Russian Revolution.
- Determine the relationship of Neandertal remains (30,000+ years old) to modern humans.

In this experiment, the polymerase chain reaction (PCR) is used to amplify a 1070-base-pair nucleotide sequence within the control region of the mt genome. This is the easiest experiment to allow a person to visualize a discrete region of his or her own genetic material. An mtDNA sequence is amplified several thousand-fold over a nuclear sequence that has only two copies. Because of the large number of mtDNA molecules per cell, cycling time can be shortened, and hand cycling is a realistic alternative to automated thermal cyclers. On the negative side, because each student is amplifying the same region, the results (with respect to the size of the DNA product) will also be the same for each. Therefore, the purpose of this amplification is to produce enough of the desired genetic region for further analysis. In this lab, you will perform a restriction enzyme analysis of the mt control region.

The source of template DNA for this procedure is a sample of several thousand squamous cells obtained from either hair sheaths or cheek cells. Either procedure is bloodless and noninvasive. Hairs are pulled from the scalp, eyebrow, or arm, and the root ends are mixed with Chelex[®]/proteinase K. With incubation at 50°C, the proteinase K digests the membrane that contains the sheath cells; vortexing then releases cells in small clumps. Alternatively, cheek cells are obtained by a saline mouthwash, collected by centrifugation, and resuspended in Chelex[®].

In either case, the samples then are boiled to lyse the squamous cells and liberate the chromosomal DNA. The Chelex[®] binds metal ions that are released from the cells and that inhibit the PCR reaction. A sample of the

clear supernatant, containing chromosomal DNA, is combined with a buffered solution of heat-stable *Taq* polymerase, oligonucleotide primers, the four deoxynucleotide (dNTP) building blocks of DNA, and the cofactor magnesium chloride ($MgCl_2$). The PCR mixture is placed in a DNA thermal cycler and taken through 35 cycles consisting of

- a 30-second incubation at 94°C, to denature the chromosomal DNA into single strands,
- a 40-second incubation at 58°C, for the primers to form hydrogen bonds with their complementary sequences on either side of the mt control region, and
- a 45-second incubation at 72°C, for the *Taq* polymerase to make complementary DNA strands that begin with each primer.

You may set up an experiment as a time course to show the accumulation of amplification products with increased number of cycles.

The primers used in the experiment bracket the mt control region and result in selective amplification, or copying, of that region of the mt chromosome. As an optional preliminary step, a fraction (5 μ L) of the student amplification products can be loaded side by side on a 2% agarose gel, along with size markers, and electrophoresed. After staining with a visible dye, each student will show the same 1070-bp band. Because of the high yield, amplification products can be readily stained with methylene blue or *Carolina*BLU™ stain.

To carry the experiment to the next level, 15 μ L of each amplified student sample may be digested with the restriction enzyme *Mse*I. In human populations, the mitochondrial genome contains a polymorphic *Mse*I site at nucleotide position 16,297. This will generate one of two possible cutting patterns: one for one mtDNA type that has the *Mse*I restriction enzyme recognition sequence and one for the mtDNA type that lacks the *Mse*I site. (**Note:** Other cutting patterns may also be seen.) The mtDNA types are termed haplotypes. After incubation at 37°C, digests are loaded side-by-side on a 2% agarose gel, along with size markers, and are electrophoresed. After staining with a visible dye, each student will determine his or her haplotype. Because of the high yield, amplification products can be readily stained with methylene blue or with *Carolina*BLU™ stain.

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Successful amplification is closely correlated to presence of a sheath. Most people find sheaths only on some hairs, and some people are unable to find any sheaths at all. Hair roots usually yield little DNA because the cell mass is not digested by proteinase K and only cells at the edge of the mass are lysed by boiling.

However, hair sheaths and roots work almost equally well in mtDNA amplifications. This is because the large amount of mtDNA in each cell compensates for the relatively low number of cells obtained from roots. Furthermore, forensic scientists agree that mitochondria are also found on the hair shaft, although their origin is not known. For these reasons, hair preparations yield 85–95% interpretable results, on par with results from cheek cells.

Each method works best with one piece of relatively inexpensive equipment. The mouthwash method requires a clinical centrifuge (for 15-mL tubes) that develops 500–1000 × g. DNA isolation from hair sheaths requires a vortexer.

3. Ready-to-Go PCR Beads™

Each PCR bead contains reagents so that when brought to a final volume of 25 µL, the reaction contains 1.5 units of *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and 200 µM of each dNTP.

4. mtDNA Primer/Loading Dye Mix

This mix includes mt control region primers (0.25 pmol/µL of each primer), 13.8% sucrose, and 0.0081% cresol red in tris-low EDTA (TLE) buffer (4 mM Tris-HCl, pH 8.0; 0.04 mM EDTA).

5. Storing Squamous Cell DNA Samples

Student DNA samples isolated in Procedure A1 or Procedure A2 are unstable and must be kept on ice prior to setting up PCR reactions. Samples may be stored at –20°C for several weeks without significant DNA degradation.

6. Setting Up PCR Reactions

The lyophilized *Taq* polymerase in the Ready-to-Go PCR Bead™ becomes active immediately upon addition of the mtDNA primer/loading mix. In the absence of thermal cycling, “nonspecific priming” allows the polymerase to begin generating erroneous products, which can show up as extra bands in gel analysis. Therefore, work quickly, and initiate thermal cycling as soon as possible after mixing PCR reagents. Be sure the thermal cycler is set and have all experimenters set up PCR reactions coordinately. Add primer/loading dye mix to all reaction tubes, then add each student DNA

10. DNA Size Markers

Plasmid pBR322 digested with the restriction endonuclease *Bst*NI produces fragments that are useful as size markers in this experiment: 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp (this last band may be faint or not visible, especially if *Carolina*BLU™ is used). Use 20 µL of the DNA marker per gel.

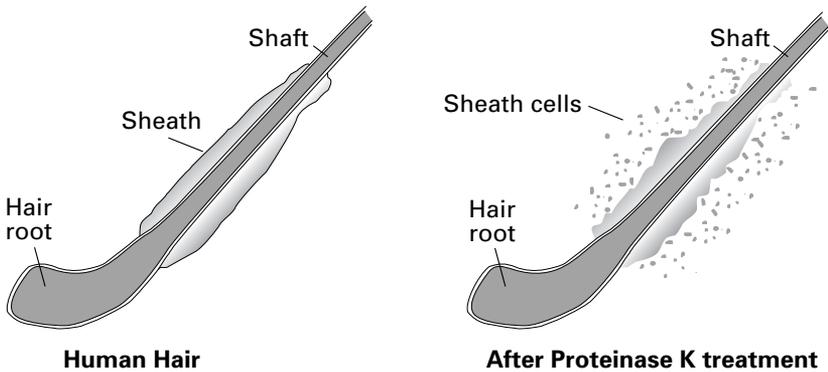
11. Viewing and Photographing Gels

View and photograph gels as soon as possible after appropriate staining and destaining. Over time (especially when ethidium bromide is used), PCR products disappear as stained bands, because they slowly diffuse through the gel.

Laboratory Procedures

Note: The following are the laboratory procedure steps in the **Student Guide**, along with *italicized responses* to some of the **Results and Discussion** questions and directions.

Note: You will do either Procedure A1 or Procedure A2.



Procedure A1: Isolate Hair Sheath DNA

1. Pull out several hairs and inspect for presence of a sheath. The sheath is a barrel-shaped structure surrounding the base of the hair, and can be readily observed with a hand lens or dissecting microscope. The glistening sheath can be observed with the naked eyes by holding the hair up to a light source. (Sheaths are most easily observed on dark hair.)
2. Select several hairs with good sheaths. Alternatively, select hairs with the largest roots. Eyebrow hair is a very good source for such hair roots.

3. Use a fresh razor blade or scalpel to cut off hair shafts just above the sheath.
4. Use forceps to transfer hairs to a 1.5-mL tube containing 100 μL of proteinase K/Chelex[®] mixture. Make sure the sheaths are submerged in the solution and not stuck on the test tube wall.
5. Incubate the sample tube in a 50°C water bath for 10 minutes.
6. Remove sample tube to room temperature. Vortex by machine or vigorously with your finger for 15 seconds to dislodge cells from hair shafts.
7. Place your sample in a floating tube rack in the boiling water bath for 8 min. Do not submerge or drop the tube into the water. Use forceps to remove your tube from the boiling water bath and allow samples to cool for 2 min. The tube may be placed on ice for faster cooling.
8. Vortex by machine or vigorously with finger for 15 seconds.
9. Put your assigned number on your sample tube and place it, along with the others, in a balanced configuration in the microcentrifuge and spin at full speed for 30 sec (1 min in nanofuge). Alternatively, let the tube sit for 5 min to allow the debris to settle.
10. Use a fresh tip to transfer 50 μL of the clear supernatant to a clean 1.5-mL tube. Be careful not to remove or disturb the Chelex[®]/cell debris at the bottom of the tube.
11. Store your sample on ice or in the freezer until you are ready to begin Procedure B.

Procedure A2: Isolate Cheek Cell DNA

1. Use a permanent marker to place your assigned number on two clean 1.5-mL tubes and on the 15-mL tube containing 10 mL saline (0.9% NaCl) solution.
2. Pour the saline solution into your mouth and vigorously rinse your mouth for a full 10 sec. Save the 15-mL tube for later use.
3. Expel the saline solution into the paper cup.
4. Carefully pour the saline solution from the paper cup back into the original tube and close the cap tightly. Save the paper cup for later use.
5. Place your sample tube, together with other student samples, in a balanced configuration in a clinical centrifuge and spin it for 10 minutes at 500–1000 $\times g$ (1500–2000 rpm in most clinical centrifuges).

If you do not have a clinical centrifuge, allow the cells to settle for 10 minutes. Remove all but 1.5 mL of the supernatant and transfer the

remaining cell pellet and supernatant to a 1.5-mL microcentrifuge tube. **Make sure** that you transfer the cell pellet. Repellet the cells by spinning for 30 seconds at full speed in a microcentrifuge.

- Carefully pour off supernatant into the paper cup. Be careful not to disturb the cell pellet at the bottom of the tube.
- Set the micropipet to 500 μL . Draw the 10% Chelex[®] suspension in and out of the pipet tip several times to suspend the resin beads. Before the resin settles, rapidly transfer 500 μL of Chelex[®] suspension to the tube containing your cell pellet.
- Resuspend the cells by pipetting in and out several times. Examine the resuspended cells in the tube against the light to confirm that no visible clumps of cells remain.
- Pipet several times to resuspend the cells and resin, then transfer 500 μL of your cell sample into a clean 1.5-mL tube. If your cells are already in a 1.5-mL tube, you do not need to transfer them.
- Place your sample in a floating tube rack in the boiling water bath for 10 min. Do not submerge or drop the tube into the water. Use forceps to remove your tube from the boiling water bath and allow it to cool for 2 min. The tube may be placed on ice for faster cooling.
- Place your sample tube, along with the others, in a balanced configuration in the microcentrifuge and spin for 30 sec at full speed (1 min in a nanofuge). Alternatively, let the tube sit for 5 min to allow debris to settle.
- Use a fresh tip to transfer 200 μL of the clear supernatant into a clean 1.5-mL tube. Be careful not to remove or disturb the Chelex[®]/cell debris at the bottom of the tube.
- Store your sample on ice or in the freezer until you are ready to begin Procedure B.
- Pour supernatant from Step 6 into the sink and rinse down with water.

Procedure B: Set Up PCR Reactions

- Use a micropipet with a fresh tip to add 22.5 μL of mtDNA primer/loading dye buffer mix to a PCR tube containing a Ready-to-Go PCR Bead[™]. Tap the tube with a finger to dissolve the bead.
- Use a fresh tip to add 2.5 μL of student DNA to the reaction tube, and tap it to mix. Pool reagents by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.

3. Label the cap of your tube with a number, as assigned by your teacher. In this way, your results will be anonymous.
4. Add one drop of mineral oil on top of reactants in the PCR tube. Be careful not to touch the dropper tip to the tube or reactants, or subsequent reactions will be contaminated with DNA from your preparation.

Note: Thermal cyclers with heated lids do not require use of mineral oil.

5. Store all samples on ice or in the freezer until you are ready to amplify according to the following profile.

Instructor: Program and run the thermal cycler with the following step file:

94°C – 30 sec

58°C – 40 sec

72°C – 45 sec

(35 cycles) link to a 4°C soak file (if your machine has this option)

Note: The 35th cycle can be followed by a 10-minute extension at 72°C. This extension can increase the amount of DNA present in the sample.

Procedure C: Load and Electrophorese PCR Products (Optional)

Note: In order to perform this procedure, you will need to order additional materials. See the “Fine Points of Lab Procedure” section. This optional step allows you to determine if your PCR reaction worked. At this stage, every student sample will look the same on the gel. Every student should see a 1070-bp PCR product on the gel. The cresol red and sucrose in the primer mix function as loading dye so that amplified samples can be loaded directly into gels.

1. Use a micropipet with a fresh tip to load 5 μ L of your PCR sample/loading dye mixture into your assigned well of a 2% agarose gel. Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well. Be sure not to get any mineral oil in your tip.
2. Load 20 μ L of the pBR322/*Bst*NI size markers into one lane of the gel.
3. Electrophorese at 130 volts for 20–30 min. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
4. Gels may be stained with *Carolina*BLU™ for 20 min and destained with distilled or deionized water for 30–45 min (optimum visualization with *Carolina*BLU™ is achieved by destaining overnight in a small volume of distilled or deionized water); alternatively, gels may be stained with 1 μ g/mL ethidium bromide for 10–20 min.

Procedure D: Restriction Digest PCR Products

1. Use a micropipet with a fresh tip to add 15 μL of your mtDNA PCR product to a fresh 1.5-mL reaction tube.
2. Use a fresh tip to add 2.25 μL of 10 \times restriction buffer to the reaction tube.
3. Use a fresh tip to add 2.25 μL of 10 \times BSA buffer to the reaction tube.
4. Use a fresh tip to add 9.5 μL of dH₂O to the reaction tube.
5. Use a fresh tip to add 1 μL of *Mse*I to the reaction tube.
6. Mix gently and pool the reagents by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.
7. Label the cap of your tube with a number, as assigned by your teacher. In this way, your results will be anonymous.
8. Incubate mtDNA PCR digest at 37°C for 60 minutes. Alternatively, this digest can be left to incubate overnight.

Procedure E: Load and Electrophorese mtDNA PCR Digests

1. Use a micropipet with a fresh tip to add 3 μL of 10 \times loading dye to your mtDNA PCR digest tube.
2. Use a micropipet with a fresh tip to add entire mtDNA PCR digest sample/loading dye mixture (33 μL) into your assigned well of a 2% agarose gel. Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well.
3. Load 20 μL of the pBR322/*Bst*NI size markers into one lane of the gel.
4. Electrophorese at 130 volts for 20–30 min. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
5. Gels may be stained with *Carolina*BLU™ for 20 min and destained with distilled or deionized water for 30–45 min (optimal visualization with *Carolina*BLU™ occurs after destaining the gel overnight in a small amount of distilled or deionized water); alternatively, gels may be stained with 1 $\mu\text{g}/\text{mL}$ ethidium bromide for 10–20 min.

Results and Discussion

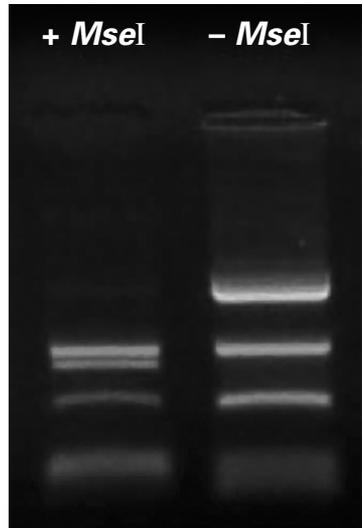
1. Observe the photograph of the stained gel containing your PCR digest and those from other students. Orient the photograph with the sample wells at the top. Interpret the band(s) in each lane of the gel:

- a. Scan across the photograph to get an impression of what you see in each lane. You should notice that student lanes contain one of two possible restriction patterns.

Note: Occasionally, you may see other patterns as well.

- b. Now locate the lane containing the pBR322/*Bst*NI markers. Working from the well, locate the bands

corresponding to each restriction fragment: 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp (the last band may be faint or not visible if you stain with *Carolina*BLU™). Working from the well, locate the bands corresponding to each restriction fragment: (the last band(s) may be faint or not visible). The expected DNA fragments, in base pair, for the +*Mse*I haplotype are: 298, 288 (not separable from 298 on an agarose gel), 253, 161, 41, and 38 (not separable from 41 on an agarose gel). The expected DNA fragments, in base pair, for the -*Mse*I haplotype are: 541, 298, 161, 41, and 38 (not separable from 41 on an agarose gel).



2. How would you use the size marker to interpret your restriction pattern fragment sizes?

Use the size marker as a guide to locate the expected restriction fragments.

3. Do you have the *Mse*I restriction site in your mitochondrial genome at nucleotide position 16,297? How can you tell?

*Students that are negative for the *Mse*I site will observe a 541-bp band on their gel. Students that are positive for the *Mse*I site will not observe this band. Instead, they will observe a 253-bp band on their gel. This band results from the cleavage of the 541-bp fragment into 253 bp and 288 bp. The 288-bp band will not be visible because it co-migrates with the 298-bp fragment on the gel.*

4. The mt control region mutates at approximately 10 times the rate of nuclear DNA. Propose a biological reason for the high mutation rate of mtDNA.

The mitochondrial genome is housed within the cell's energy producing factory, where it is exposed to reactive by-products of oxidative phosphorylation. Notably, oxygen free radicals are potent mutagens. The number of reactive by-products, in turn, increases as enzymes involved in energy production accumulate mutations that make them function less efficiently. It is hypothesized that this decline in mitochondrial efficiency is a major contributor to aging.

5. The high mutability of the mt control region genome means that it evolves more quickly than the nuclear genome. This makes the mt control region a laboratory for the study of DNA evolution. However, can you think of any drawbacks to this high mutation rate with respect to studying evolution?

The mutation rate is so high that some nucleotides have mutated several times over evolutionary history. This makes it difficult to determine the actual mutation rate and to ascertain the ancestral (original) state of a DNA sequence. This makes it difficult to accurately calibrate the "mutation clock."

6. What is the frequency of each haplotype in your class?

To calculate the frequency, first determine the number of students that were positive for the MseI site. Divide this number by the total number of students in the class. Do the same thing with the number of students who were negative for the MseI site.

7. What does this tell you about how different people in the class may be related? Are you surprised by what you find?

Although all students share a common maternal ancestor, students who are positive for the MseI site share a closer maternal relationship than those students who do not. Students are often confused that maternal genetic relationships do not always agree with racial classifications of man.

8. How are mitochondrial restriction haplotypes limited in investigating genetic relationships and human evolution?

Restriction haplotypes are limited in that restriction enzymes only analyze a small region of the mitochondrial genome. This can only provide general relationship data. For a more detailed analysis, scientists sequence the DNA of the entire mitochondrial genome.

Further Reading

- Bloom, M. 1994. Polymerase Chain Reaction. *Carolina Tips* 57: 13–18.
- Bloom, M., G. Freyer, and D. Micklos. 1995. *Laboratory DNA Science*. Benjamin/Cummings, Menlo Park, CA.
- Cann, R.L., W. M. Brown, and A. C. Wilson. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics* 106(3): 479–499.
- Kreuzer, H., and A. Massey. 2001. *Recombinant DNA and Biotechnology: A Guide for Teachers*. American Society for Microbiology Press, Washington, DC.
- Micklos, D., and G. Freyer. 2003 (2nd Edition). *DNA Science, a First Course*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Mullis, K. 1990. The unusual origin of the Polymerase Chain Reaction. *Scientific American* 262(4): 56–65.
- National Research Council. 1996. *The Evaluation of Forensic DNA Evidence*. National Academy Press, Washington, DC.
- Zischler, H., H. Geisert, A. von Haeseler, and S. Paabo. 1995. A nuclear 'fossil' of the mitochondrial D-loop and the origin of modern humans. *Nature*, vol. 378(6556):489–92.

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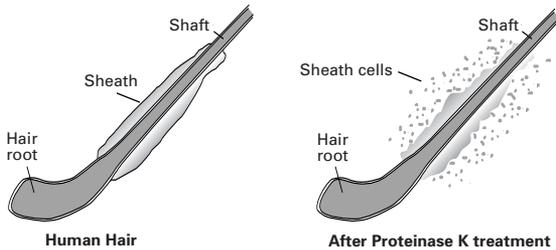
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Note: You will do either Procedure A1 or Procedure A2.



Procedure A1: Isolate Hair Sheath DNA

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2. Select several hairs with good sheaths. Alternatively, select hairs with the largest roots. Eyebrow hair is a very good source for such hair roots.
3. Use a fresh razor blade or scalpel to cut off hair shafts just above the sheath.
4. Use forceps to transfer hairs to a 1.5-mL tube containing 100 μ L of proteinase K/ Chelex[®] mixture. Make sure the sheaths are submerged in the solution and not stuck on the test tube wall.
5. Incubate the sample tube in a 50°C water bath for 10 minutes.
6. Remove sample tube to room temperature. Vortex by machine or vigorously with your finger for 15 seconds to dislodge cells from hair shafts.
7. Place your sample in a floating tube rack in the boiling water bath for 8 min. Do not submerge or drop the tube into the water. Use forceps to remove your tube from the boiling water bath and allow samples to cool for 2 min. The tube may be placed on ice for faster cooling.
8. Vortex by machine or vigorously with your finger for 15 seconds.
9. Put your assigned number on your sample tube and place it, along with the others, in a balanced configuration in the microcentrifuge and spin at full speed for 30 sec (1 min in nanofuge). Alternatively, let the tube sit for 5 min to allow the debris to settle.
10. Use a fresh tip to transfer 50 μ L of the clear supernatant to a clean 1.5-mL tube. Be careful not to remove or disturb the Chelex[®]/cell debris at the bottom of the tube.
11. Store your sample on ice or in the freezer until you are ready to begin Procedure B.

Procedure A2: Isolate Cheek Cell DNA

1. Use a permanent marker to place your assigned number on two clean 1.5-mL tubes and on the 15-mL tube containing 10 mL saline (0.9% NaCl) solution.
2. Pour the saline solution into your mouth and vigorously rinse your mouth for a full 10 sec. Save the 15-mL tube for later use.
3. Expel the saline solution into the paper cup.
4. Carefully pour the saline solution from the paper cup back into the original tube and close the cap tightly. Save the paper cup for later use.
5. Place your sample tube, together with other student samples, in a balanced configuration in a clinical centrifuge and spin it for 10 minutes at $500\text{--}1000 \times g$ (1500–2000 rpm in most clinical centrifuges). If you do not have a clinical centrifuge, allow the cells to settle for 10 minutes. Remove all but 1.5 mL of the supernatant and transfer the remaining cell pellet and supernatant to a 1.5-mL microcentrifuge tube. **Make sure** that you transfer the cell pellet. Repellet the cells by spinning for 30 seconds at full speed in a microcentrifuge.
6. Carefully pour off supernatant into the paper cup. Be careful not to disturb the cell pellet at the bottom of the tube.
7. Set the micropipet to 500 μL . Draw the 10% Chelex[®] suspension in and out of the pipet tip several times to suspend the resin beads. Before the resin settles, rapidly transfer 500 μL of Chelex[®] suspension to the tube containing your cell pellet.
8. Resuspend the cells by pipetting in and out several times. Examine the cell suspension against the light to confirm that no visible clumps of cells remain.
9. Pipet several times to resuspend the cells and resin, then transfer 500 μL of your cell sample into a clean 1.5-mL tube. If your cells are already in a 1.5-mL tube, you do not need to transfer them.
10. Place your sample in a floating tube rack in the boiling water bath for 10 min. Do not submerge or drop the tube into the water. Use forceps to remove your tube from the boiling water bath and allow it to cool for 2 min. The tube may be placed on ice for faster cooling.
11. Place your sample tube, along with the others, in a balanced configuration in the microcentrifuge and spin for 30 sec at full speed (1 min in a nanofuge). Alternatively, let the tube sit for 5 min to allow the debris to settle.
12. Use a fresh tip to transfer 200 μL of the clear supernatant into a clean 1.5-mL tube. Be careful not to remove or disturb the Chelex[®]/cell debris at the bottom of the tube.
13. Store your sample on ice or in the freezer until you are ready to begin Procedure B.
14. Pour supernatant from Step 6 into the sink and rinse down with water.

Procedure B: Set Up PCR Reactions

1. Use a micropipet with a fresh tip to add 22.5 μL of mtDNA primer/loading dye buffer mix to a PCR tube containing a Ready-to-Go PCR Bead[™]. Tap the tube with a finger to dissolve the bead.
2. Use a fresh tip to add 2.5 μL of student DNA to the reaction tube, and tap it to mix. Pool reagents by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.
3. Label the cap of your tube with a number, as assigned by your teacher. In this way, your results will be anonymous.

4. Add one drop of mineral oil on top of reactants in the PCR tube. Be careful not to touch the dropper tip to the tube or reactants, or subsequent reactions will be contaminated with DNA from your preparation.

Note: Thermal cyclers with heated lids do not require use of mineral oil.

5. Store all samples on ice or in the freezer until ready to amplify according to the following profile.

Your instructor will program and run the thermal cycler with the following step file:

94°C – 30 sec

58°C – 40 sec

72°C – 45 sec

(35 cycles) link to a 4°C soak file (if your machine has this option)

Note: The 35th cycle can be followed by a 10-minute extension at 72°C. This extension can increase the amount of DNA present in the sample.

Procedure C: Load and Electrophorese PCR Products (Optional)

This optional step allows you to determine if your PCR reaction worked. At this stage, every student sample will look the same on the gel. Every student should see a 1070-bp PCR product on the gel. **If time is limited, move on to step D.** The cresol red and sucrose in the primer mix function as loading dye so that amplified samples can be loaded directly into gels.

1. Use a micropipet with a fresh tip to load 5 μ L of your PCR sample/loading dye mixture into your assigned well of a 2% agarose gel. Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well. Be sure not to get any mineral oil in your tip.
2. Load 20 μ L of the pBR322/*Bst*NI size markers into one lane of the gel.
3. Electrophorese at 130 volts for 20–30 min. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
4. Gels may be stained with *Carolina*BLU™ for 20 min and destained with distilled or deionized water for 30–45 min (optimum visualization with *Carolina*BLU™ is achieved by destaining overnight in a small volume of distilled or deionized water); alternatively, gels may be stained with 1 μ g/mL ethidium bromide for 10–20 min.

Procedure D: Restriction Digest PCR Products

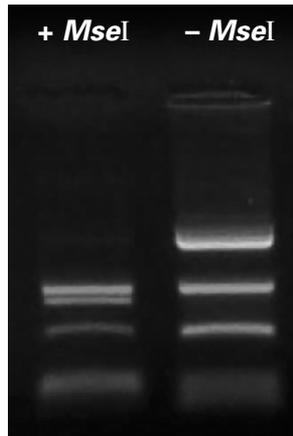
1. Use a micropipet with a fresh tip to add 15 μ L of your mtDNA PCR product to a fresh 1.5-mL reaction tube.
2. Use a fresh tip to add 2.25 μ L of 10 \times restriction buffer to the reaction tube.
3. Use a fresh tip to add 2.25 μ L of 10 \times BSA buffer to the reaction tube.
4. Use a fresh tip to add 9.5 μ L of dH₂O to the reaction tube.
5. Use a fresh tip to add 1 μ L of *Mse*I to the reaction tube.
6. Pool the reagents by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.
7. Label the cap of your tube with a number, as assigned by your teacher. In this way, your results will be anonymous.
8. Incubate the mtDNA PCR digest at 37°C for 60 minutes. Alternatively, this digest can be left to incubate overnight.

Procedure E: Load and Electrophorese mtDNA PCR Digests

1. Use a micropipet with a fresh tip to add 3 μ L of 10 \times loading dye to your mtDNA PCR digest tube.
2. Use a micropipet with a fresh tip to add the entire mtDNA PCR digest sample/loading dye mixture (33 μ L) into your assigned well of a 2% agarose gel. Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well.
3. Load 20 μ L of the pBR322/*Bst*NI size markers into one lane of the gel.
4. Electrophorese at 130 volts for 20–30 min. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
5. Gels may be stained with *Carolina*BLU™ for 20 min and destained with distilled or deionized water for 30–45 min (optimal visualization with *Carolina*BLU™ occurs after destaining the gel overnight in a small amount of distilled or deionized water); alternatively, gels may be stained with 1 μ g/mL ethidium bromide for 10–20 min.

Results and Discussion

1. Observe the photograph of the stained gel containing your PCR digest and those from other students. Orient the photograph with the sample wells at the top. Interpret the band(s) in each lane of the gel:



- a. Scan across the photograph to get an impression of what you see in each lane. You should notice that student lanes contain one of two possible restriction patterns.
Note: Occasionally, you may see other patterns as well.
- b. Now locate the lane containing the pBR322/*Bst*NI markers. Working from the well, locate the bands corresponding to each restriction fragment in the marker: 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp (the last band may be faint or not visible). The expected DNA fragments, in base pair, for the +*Mse*I haplotype are: 298, 288 (not separable from 298 on an agarose gel), 253, 161, 41, and 38 (not separable from 41 on an agarose gel). The expected DNA fragments, in base pair, for the -*Mse*I haplotype are: 541, 298, 161, 41, and 38 (not separable from 41 on an agarose gel).
Note: The 41 and 38 bp bands may not be visible on your gel.

7. What does this tell you about how different people in the class may be related? Are you surprised by what you find?

8. How are mitochondrial restriction haplotypes limited in investigating genetic relationships and human evolution?

Further Reading

- Bloom, M. 1994. Polymerase Chain Reaction. *Carolina Tips* 57: 13–18.
- Bloom, M., G. Freyer, and D. Micklos. 1995. *Laboratory DNA Science*. Benjamin/Cummings, Menlo Park, CA.
- Cann, R.L., W. M. Brown, and A. C. Wilson. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics* 106(3): 479–499.
- Kreuzer, H., and A. Massey. 2001. *Recombinant DNA and Biotechnology: A Guide for Teachers*. American Society for Microbiology Press, Washington, DC.
- Micklos, D., and G. Freyer. 2003 (2nd Edition). *DNA Science, a First Course*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Mullis, K. 1990. The unusual origin of the Polymerase Chain Reaction. *Scientific American* 262(4): 56–65.
- National Research Council. 1996. *The Evaluation of Forensic DNA Evidence*. National Academy Press, Washington, DC.
- Zischler, H., H. Geisert, A. von Haeseler, and S. Paabo. 1995. A nuclear ‘fossil’ of the mitochondrial D-loop and the origin of modern humans. *Nature*, vol. 378(6556):489–92.

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