

Human Mitochondrial DNA Kit AT

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 of 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 may be 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 mt DNA. 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 440-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 their 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.

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 37°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 (MgCl₂). The PCR mixture is placed in a DNA thermal cycler and taken through 30 cycles consisting of

- a 30-second incubation at 94°C, to denature the chromosomal DNA into single strands,
- a 30-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 30-second incubation at 72°C, for the *Taq* polymerase to make complementary DNA strands that begin with each primer.

Your teacher may have you set up the 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. Student amplification products are loaded side by side on a 1.5% agarose gel, along with size markers and are electrophoresed. After staining with a visible dye, each student will show the same 440-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, amplified student samples may be submitted to the Sequencing Service of the Dolan DNA Learning Center (DNALC) which will generate student mtDNA sequences and post the results via Internet for no charge. For information on how to have your mitochondrial DNA sequenced:

1. Go to www.geneticorigins.org/mito/mitoframeset.htm on the DNALC Web site (www.dnalc.org).
2. Look for and click on the Sequencing Service link at the top of the page.
3. Follow the instructions shown for submitting samples to the DNA Learning Center for sequencing. The sequencing service is free.

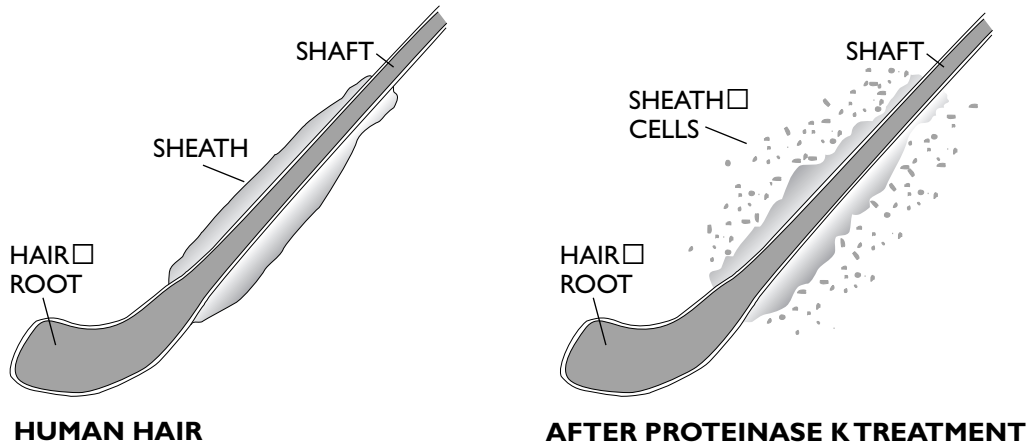
Visit the DNALC at <http://dnalc.org> to view or download animations on PCR, DNA sequencing, and DNA fingerprinting (click on **Resources** and then **Biology Animation Library**). Explore online genome resources, test theories of human evolution, and solve forensic DNA cases drawn from the current research literature. All these facilities can be found on the Dolan DNALC website. Many of these resources can be linked through the *Genetic Origins* icon on their home page.

Laboratory Procedure

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 one to several hairs with good sheaths. Alternatively, select hairs with the largest 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 300 μ L of Chelex[®]/proteinase K. Make sure sheath is submerged in the solution and not stuck on the test tube wall.
5. Incubate sample tube in 37°C water bath for 10 min.
6. Remove sample tube to room temperature. Vortex by machine or vigorously with finger for 15 sec to dislodge cells from hair shaft.
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 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 sec.
9. Put your assigned number on your sample tube and place it, along with 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 debris to settle.
10. Use a fresh tip to transfer 200 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 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 xg (1500–2000 rpm in most clinical centrifuges). Alternatively, 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 microtube. **Make sure** that you transfer the cell pellet. Re-pellet 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 micropipet to 500 μ L. Draw 10% Chelex suspension in and out of the pipet tip several times to suspend the resin beads. Before the resin settles, rapidly transfer 500 μ L of Chelex suspension to the tube containing your cell pellet.
8. Re-suspend the cells by pipetting in and out several times. Examine against light to confirm that no visible clumps of cells remain.
9. Pipet several times to re-suspend the cells and resin, then transfer 500 μ L 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, with 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.
12. Use a fresh tip to transfer 200 μ L 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 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 μ L 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 μ L 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.

Instructor: Program and start thermal cycler with a step file:

94°C – 30 sec

58°C – 30 sec

72°C – 30 sec

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

Note: The 30th 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

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 add the entire PCR sample/loading dye mixture (25 μ L) into your assigned well of a 1.5% 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. (**Note:** If you intend to use your sample as a DNA source to generate mtDNA sequence, load only 15 μL and store the remaining 10 μL at 4°C or in the freezer.) Be sure not to get any mineral oil in your tip.

2. Load 20 μL of the pBR322/*Bst*NI size markers into one lane of the gel.
3. Electrophorese at 130 volts for 20–30 minutes. 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, or they 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 sample 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 virtually all student lanes contain one or two prominent bands.
 - 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).
 - c. The amplification product of 440 bp should roughly align with the 383-bp marker.
 - d. It is common to see a second band lower on the gel. This diffuse (fuzzy) band is “primer dimer,” an artifact of the PCR reaction that results from the primers overlapping one another and amplifying themselves. Primer dimer is approximately 50 bp, and should be in a position ahead of the 121-bp marker.
 - e. Additional faint bands, at other positions on the gel, occur when the primers bind to chromosomal loci other than mt control region and give rise to “nonspecific” amplification products.
2. How would you interpret a lane in which you observe “primer dimer,” but no 440-bp band?
3. 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.
4. 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?
5. There are numerous insertions of mtDNA into nuclear chromosomes. Notably, scientists recently discovered a 540-bp fragment of the mt control region that inserted into chromosome 11 approximately 350,000 years ago (see the Zischler reference in the *Further Reading* section). Would you expect any difference in the mutation rates of the control region sequence in the mt genome versus the chromosome 11 insertion? What implication does this have in the study of human evolution?
6. Since their initial discovery in the Neander Valley of Germany in 1856, the bones of the heavy-set Neandertal have fascinated scientists and the general public. Neandertal was an archaic member of the genus *Homo* who lived in Europe beginning about 300,000 years ago and became extinct about 30,000 years ago. Clearly, during part of its span on earth, Neandertal shared its European habitat with modern humans (*Homo sapiens*). There has long been controversy about whether or not Neandertal was the direct ancestor of modern humans. Alternatively, if Neandertal and *Homo sapiens* were separate, was there any significant exchange of genes between the two populations?

According to the multiregional model, modern humans developed concurrently from several different archaic populations living in different parts of the world. Under this model, Neandertal was the ancestor of modern Europeans, while Java man (*Homo erectus*) was the ancestor of modern Asians.

According to the displacement model, better known as “Out of Africa,” *Homo sapiens* arose from a single founding population that emerged from Africa in the last 100,000–200,000 years. This group migrated successively to Europe and Asia, displacing archaic hominids.

In 1997, an international research team headed by Svante Paabo, extracted DNA from the humerus of the original Neandertal specimen, amplified the sample by PCR, and cloned the resulting products in *E. coli*. The cloned fragments were then used to reconstruct a 379-bp stretch of the mt control region. Now, you will use the DNA Sequence Server at the DNA Learning Center website (<http://dnalc.org> under *Bioservers*) to recreate this study and answer the questions posed above. You will obtain mt control region sequences from several sources and then move them onto the analysis workspace of the DNA Sequence Server Page. The Sequence Server can also be reached by clicking on *Genetic Origins* on the DNA Learning Center Homepage, then on the picture labeled *Mitochondrial Control Region* and then on *Continue on to the Mitochondrial Control Region* at the bottom of the page. You will see the link to the Sequence Server at the top of the Mitochondrial Control Region page.

Note: Be aware that the DNA Learning Center continually updates the website, so some of the following details may be different.

- a. Determine the average number of nucleotide differences in the mt control region between Neandertal and 5 modern humans in the DNALC's own database.
 1. Click on the Sequence Server icon on the *Mitochondrial Control Region* page. Log into the Sequence Server either as a guest (click on *Enter*), or using a registered account.
 2. Click *Manage Groups* to open a new window in which you can view mt control region sequences currently on the Sequence Server database.
 3. Use the pull-down menu in the upper right hand corner to select and view a list of student classes and/or several teacher workshops. Each class or workshop contains 5–30 individual DNA sequences.
 4. Click in the check box to select classes or workshop sequences you want to make available for analysis (for example, *Department of Energy Workshop*). (If you like, click *View* to open a new window showing the sequences in the selected group.) Click *OK*. The window will close and the selected sequences will now appear in a list in the Sequence Server workspace.
 5. Go back to the pull-down menu in the upper right hand corner of the *Manage Groups* screen to select and view DNA sequences in other categories.
 6. Click in the check boxes to select *Neandertal* (under pre-historic), *Primate* (under non-human), *European, Australian and Pacific Islander, Native American, Asian, and African* (under *Modern Humans*), mitochondrial DNA sequences. You will have to select the sequences from each category (pre-historic, modern, etc.) separately.
 7. After selecting the sequence from each category, click *OK*. The window will close, and the selected sequences will now appear in a list in the Sequence Server workspace.
 8. In the Sequence Server workspace, visually gauge the quality of several student or teacher sequences in the following way:

Use the pop-down menu displaying the sample names to select a specific student or teacher sequence. Then, click the adjacent *Open* to open a new window showing the nucleotide sequences of the selected entry.

Every sequence will begin with nucleotides (A,T,C,G) interspersed with *Ns*, indicating that the nucleotides could not be determined at these positions. In "good" sequences, where experimental conditions were near optimal, the beginning *Ns* will end abruptly. The remaining sequence will have very few, if any, internal *Ns*. Then, at the end of the "read," the number of *Ns* will abruptly increase again

In non-optimal cases, a large number of *Ns* will be interspersed throughout the sequence. When possible, use good sequences (those without internal *Ns*) in your subsequent comparisons.
 9. Now, compare the Neandertal sequence with a modern sequence, using the ClustalW algorithm available in the Sequence Server workspace. Click in the check boxes to the left of the Neandertal sequence and one student or teacher sequence. Then click on *Compare* to send your checked sequence to the ClustalW tool at the European Bioinformatics Institute in Hinxton, UK (or to a local server at Cold Spring Harbor Laboratory). Your results will be returned in a new window.

10. Count the number of differences between the Neandertal sequence and the student/teacher sequence:
- A yellow box indicates positions with a nucleotide difference. A gray box indicates positions with an *N*, where a nucleotide could not be determined.
- Read into the sequence until you get past the initial stretch of interspersed *N*s. Then begin counting the number of yellow highlighted nucleotide differences. Do not count any internal *N*s.
- Also count dashes (–), which indicate a deletion, a nucleotide that is absent at that position in the sequence.
- Record the number of differences.
11. If the readable student or teacher sequence does not contain as many bases as the Neandertal sequence, use the sequence coordinate numbers to estimate the length of the comparable region. If the student or teacher “read” was shorter than the Neandertal sequence, “normalize” the differences in this way:
- Normalized Differences = (379, the length of Neandertal sequence) divided by (student/teacher sequence length) × observed differences
12. Repeat steps 9–11 to do four more comparisons of the Neandertal sequence to a different teacher or student sequence. For each pairwise comparison, record the raw number of differences and the Normalized value.
- b. Now, determine the average number of differences between any two modern humans. Repeat steps 9–11 to compare five pairs of student or teacher sequences. For each pairwise comparison, record the number differences.
- c. Next, repeat steps 9–11 to compare a modern human sequence to a primate sequence. When viewing the results of these analyses, you may find alignments that contain one or more short runs of dashes. In these instances, count the entire run of dashes as a single nucleotide difference.
- d. Next, repeat steps 9–11 to determine the average number of differences between selected pairs of Diverse Modern Humans. Because we want to test the theory that Neandertal was a direct ancestor to modern Europeans, be sure to include the European group in your comparisons.
- e. Finally, repeat steps 9–11 to compare the Neandertal sequence to the Diverse Modern Human sequences you used in step d.
7. The number of differences in mt sequence provides a measure of the genetic distance between populations—that is the amount of time that has elapsed since divergence from a common ancestor. Before one can use mt mutations as a “molecular clock,” one must set the clock by some reference. The reference for hominid evolution is the estimated divergence between humans and chimpanzees 4 million years ago.
- a. Assuming that mt mutations occur at a constant rate, use the human-chimp divergence estimate and the average number of chimp-human sequence differences to calculate the average time span between mt mutations. **Hint:** The unit of your answer will be years/mutation.
- b. Now, use this value to calculate a divergence time for all modern humans.
- c. Scientists have used mt and chromosomal DNA mutations to calculate a divergence time for modern humans of about 150,000 years. Why is this number less than your calculation?

d. Now, calculate the divergence time for Neandertal-modern humans, using the modern human divergence estimate of 150,000 to set your mt clock.

e. What does this tell you about the relationship between Neandertal and modern humans?

Further Reading

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