STUDENT LAB INSTRUCTIONS

INTRODUCTION

In addition to the 46 chromosomes found in the nucleus of each human cell, each mitochondrion in the cell cytoplasm has several copies of its own genome. The mitochondrial (mt) genome contains only 37 genes, many of 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 that were taken up by a primitive ancestor of eukaryotic cells. In this endosymbiotic relationship, the host cell provided a ready source of energy-rich nutrients, and the mitochondria provided a means to extract energy from the nutrients using oxygen. This attribute was key to survival as oxygen accumulated in the primitive atmosphere. Mitochondria are in the same size range as bacteria, and the mitochondrial genome retains several bacterial features. Like bacterial chromosomes and plasmids, the mt genome is a circular molecule, and mitochondrial genes are not interrupted by introns. These features are different from those of the nuclear genome in eukaryotes, where chromosomes are linear and genes contain numerous introns.

The entire DNA sequence of the human mt genome (16,569 nucleotides) was determined in 1981, well in advance of the Human Genome Project. Genes make up the majority of the mitochondrial genome. However, a noncoding region of approximately 1200 nucleotides contains signals that control replication of the chromosome and transcription of the mitochondrial genes. The DNA sequence of this control region is termed "hypervariable" because it accumulates point mutations at approximately 10 times the rate of nuclear DNA. This high mutation rate results in distinctive patterns of single nucleotide polymorphisms (SNPs).

The combination of SNPs inherited by each person is termed a haplotype. The mt genome provides only a half set of genes because it is inherited exclusively from the mother with no paternal contribution. The female egg is a huge cell, with on the order of 100,000 mitochondria; in contrast, the tiny sperm cell is powered by fewer than 100 mitochondria at the base of the flagellum. Any male mitochondria that may enter the egg cell at conception are identified by ubiquitin surface proteins as "foreign" and are actively destroyed by enzymes in the egg cytoplasm.

In the 1980s, Allan Wilson and coworkers at the University of California at Berkeley used mtDNA polymorphisms to create a "family tree" showing ancestral relationships among 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 mitochondrial 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."

Rarely are mtDNA sequences from ancient humans found, but several key ones aid in the study of human evolution. One sequence is from "Otzi the

Iceman," a 5300-year-old human discovered frozen in the Ötztal Alps between Austria and Italy. Although Otzi had the tools and weapons of a hunter, he carried the domesticated wheat of an agricultural society.

An archaic member of the genus *Homo*, Neandertal lived in what is now Europe and central Asia—beginning about 300,000 years ago and becoming extinct about 30,000 years ago. Modern humans and Neandertal both descend from *Homo erectus*, although their relationship to each other is a long-standing controversy. During part of its span on earth, Neandertal shared its European and Middle Eastern range with modern humans (*Homo sapiens*). According to the multiregional model, modern humans developed concurrently from different archaic populations in Africa, Europe, and Asia—and Neandertal is the direct ancestor of modern Europeans. According to the displacement model ("Out of Africa"), the ancestors of modern humans left Africa only 50,000–70,000 years ago and so share with Neandertal an ancient ancestor in Africa.

In 1997, an international research team headed by Svante Pääbo tried to settle the debate about the relationship of Neandertals to modern humans. They extracted DNA from the humerus of the original Neandertal specimen, amplified the sample by PCR, and cloned the resulting products in *Escherichia coli*. The cloned fragments were then used to reconstruct a 379-bp stretch of the mt control region. Since then, mt control region sequences have been added from throughout the Neandertal range, as well as from another ancient hominin group, the Denisovans, defined by a single bone and two teeth found in a cave in Siberia. The Neandertal and Denisovan nuclear genomes were published in 2010 and 2013, making possible detailed comparisons with modern humans.

Although each cell contains only two copies of a given nuclear DNA sequence—one on each of the paired chromosomes—there are hundreds to thousands of copies of a given mtDNA sequence in each cell. This increases the chances that enough mtDNA can be obtained for forensic analysis when tissue samples are old or badly degraded and offers a simple means to visualize a discrete region of one's own genetic material. Polymorphisms in the mitochondrial hypervariable region have been used to

- Identify remains from wars and natural disasters.
- Identify the remains of the Romanov royal family assasinated during the Russian Revolution.
- Determine the relationship of Otzi, the Tyrolean ice man, and ancient hominins to modern humans.

This experiment examines a sequence within the hypervariable region of the mitochondrial genome. A sample of human cells is obtained by saline mouthwash (alternatively, DNA may be isolated from hair sheaths). DNA is extracted by boiling with Chelex resin, which binds contaminating metal ions, and the control region sequence is amplified by PCR. The PCR products (amplicons) are then visualized on agarose gels. However, because SNPs do not change the size of the amplicon, gel electrophoresis

shows no differences among student samples. To analyze SNPs that vary from person to person, the nucleotide sequence of each student amplicon must be determined.

This laboratory includes bioinformatics exercises that complement the experimental methods. The individual nucleotide sequences from students in the class are evaluated and compared with mitochondrial sequences from other modern human populations. Tools for comparing student sequences, generating mitochondrial haplotypes, and studying human evolution are found at the DNA Learning Center's *BioServers* Internet site (www.bioservers.org). BLAST is used to determine the exact size of the fragment amplified by the primer set and to identify sequences in biological databases, and the features of a complete human mitochondrial chromosome sequence are examined. Comparisons of modern human, Neandertal, and Denisovan sequences use mt SNPs as a "molecular clock" to answer fundamental questions about hominin evolution.

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Krause J, Fu Q, Good JM, Viola B, Shunkov MV, Derevianko AP, Pääbo S. 2010. The complete mitochondrial DNA genome of anunknown hominin from southern Siberia. *Nature* **464:** 894-897.

Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M, Pääbo S. 1997. Neandertal DNA sequences and the origin of modern humans. *Cell* **90**: 19–30.

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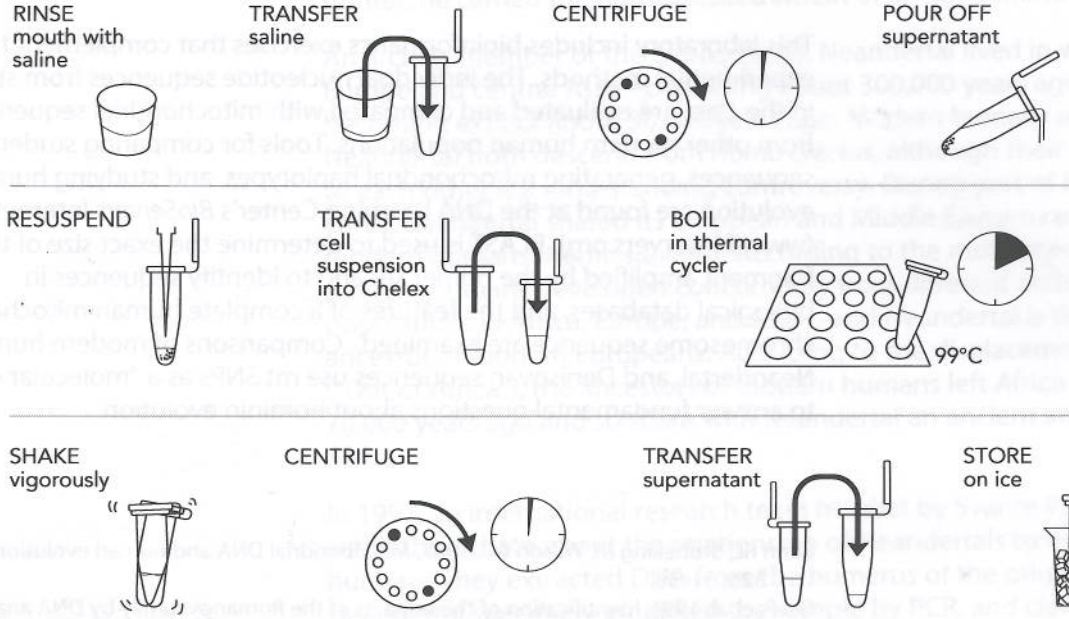
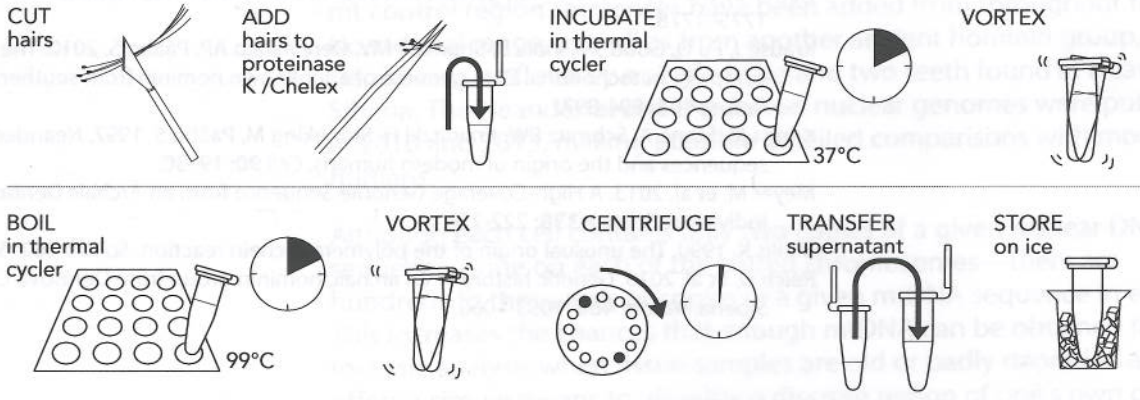
Reich D, et al. 2010. Genetic history of an archaic hominin group from Denisova Cave in Siberia. *Nature* **468**: 1053-1060.

LAB FLOW

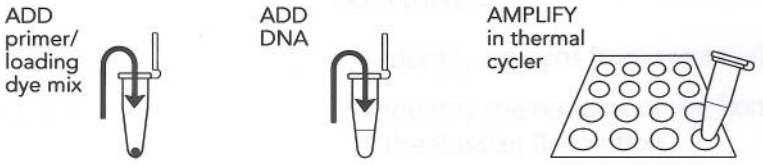
I. ISOLATE DNA FROM CHEEK CELLS



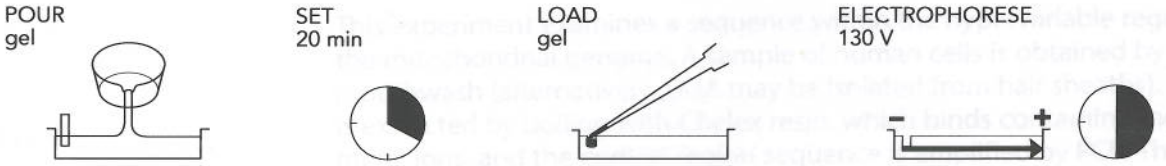
I. (ALTERNATE) ISOLATE DNA FROM HAIR SHEATHS



II. AMPLIFY DNA BY PCR



III. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS



METHODS

I. ISOLATE DNA FROM CHEEK CELLS

Reagents	Supplies and Equipment
0.9% saline solution, 10 mL in a 15-mL tube	Permanent marker
10% Chelex®, 100 µL in 0.2- or 0.5-mL PCR tube (or in a microcentrifuge tube)	Paper cup
	Micropipets and tips (10-1000 µL)
	1.5-mL microcentrifuge tubes
	Microcentrifuge tube rack
	Microcentrifuge adapters for 0.2- or 0.5-mL PCR tubes
	Microcentrifuge
	Thermal cycler (or water bath or heat block)
	Container with cracked or crushed ice
	Vortexer (optional)

1. Use a permanent marker to label a 1.5-mL tube and paper cup with your assigned number.
2. Pour all 10 mL of saline solution into your mouth and vigorously rinse your cheek pockets for 30 seconds.
3. Expel saline solution into the paper cup.
4. Swirl cup gently to mix cells that may have settled to the bottom. Use a micropipet with a fresh tip to transfer 1000 µL of the solution into your labeled 1.5-mL microcentrifuge tube.
5. Place your sample tube, along with those from other students, in a balanced configuration in a microcentrifuge. Centrifuge the tubes at full speed for 90 seconds.

If your microcentrifuge tube is graduated, the volume remaining after Step 6 will approximately reach the 0.1-mL mark.

6. After centrifuging, check to see if your pellet is firmly attached to the bottom of the tube.
   - If the pellet is firmly attached, carefully pour off the supernatant into the paper cup. Gently tap the inverted tube against the cup to remove most of the supernatant, but be careful not to disturb the cell pellet at the bottom of the tube.
   - If the pellet is loose or unconsolidated, centrifuge for 90 seconds. Then, carefully use a micropipet with a fresh tip to remove as much saline as possible.

Food particles will not resuspend. Your teacher may instruct you to examine a sample of the cell suspension under a microscope.

7. Set a micropipet to 30 µL. Resuspend the cells in the remaining saline by pipetting in and out. Work carefully to minimize bubbles.
8. Withdraw 30 µL of cell suspension and add it to a PCR tube containing 100 µL of 10% Chelex. Label the cap and side of the tube with your assigned number.

As an alternative to Steps 8 and 9, you may add the cell suspension to Chelex in a 1.5-mL tube and incubate for 10 minutes in a boiling water bath or 99°C heat block.

9. Place your PCR tube, along with those from other students, in a thermal cycler that has been programmed for one cycle of the following profile:

Pros = 99

The near-boiling temperature lyses the cell and nuclear membranes, releasing DNA and other cell contents.

Boiling step: 99°C 10 minutes

The profile may be linked to a 4°C hold program.

10. After boiling, vigorously shake your tube for 5 seconds. Use a vortexer if available.

Skip Step 11 if your sample is in a microcentrifuge tube (not in a PCR tube).

11. To prepare your PCR tube for centrifugation, "nest" it within adapter tubes as follows:
    - If your sample is in a 0.5-mL PCR tube, nest it within a capless 1.5-mL tube.
    - If your sample is in a 0.2-mL PCR tube, nest it within a capless 0.5-mL tube and then place both tubes into a capless 1.5-mL tube.
12. Place your tube, along with those from other students, in a balanced configuration in a microcentrifuge. Centrifuge the tubes at full speed for 90 seconds.
13. After centrifugation, use a micropipet with a fresh tip to transfer 30 µL of the clear supernatant into a clean 1.5-mL microcentrifuge tube. Be careful to avoid pipetting any cell debris or Chelex beads.
14. Use a permanent marker to label the cap and side of the tube with your assigned number. This tube contains your DNA and will be used for setting up the PCRs in Part II.
15. Store your sample on ice or at –20°C until you are ready to continue with Part II.

the sheath.

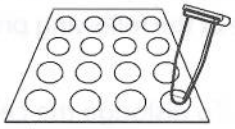
II. AMPLIFY DNA BY PCR

Reagents (at each student station)	**Supplies and Equipment**
*Cheek cell or hair sheath DNA 2.5 µL (from Part I) *mt primer/loading dye mix, 25 µL Ready-To-Go™ PCR beads (in 0.2 mL or 0.5 mL PCR tube)	Permanent marker Micropipet and tips (1-100 µL) Microcentrifuge tube rack Thermal cycler Container with cracked or crushed ice
Shared Reagent	
Mineral oil, 5 mL (depending on thermal cycler)	
*Store on ice	

1. Obtain a PCR tube containing a Ready-To-Go PCR™ Bead. Label the tube with your assigned number.
2. Use a micropipet with a fresh tip to add 22.5 µL of mt primer/loading dye mix to the tube. Allow the bead to dissolve for approximately 1 minute.

The primer/loading dye mix will turn magenta as the PCR bead dissolves.

If the reagents become splattered on the wall of the tube, pool them by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.



3. Use a micropipet with a fresh tip to add 2.5 µL of your DNA (from Part I) directly into the primer/loading dye mix. Ensure that no DNA remains in the tip after pipetting. + CENT.
4. Store your sample on ice until your class is ready to begin thermal cycling.
5. Place your PCR tube, along with those of other students, in a thermal cycler that has been programmed for 30 cycles of the following profile:

Denaturing step:	94°C	30 seconds
Annealing step:	58°C	30 seconds
Extending step:	72°C	30 seconds

The profile may be linked to a 4°C hold program after the 30 cycles are completed.

6. After thermal cycling, store the amplified DNA on ice or at –20°C until you are ready to continue with Part III.

III. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

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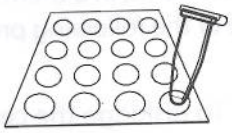

Prof = MTDNA

3. Use a micropipet with a fresh tip to add 2.5 µL of your DNA (from Part I) directly into the primer/loading dye mix. Ensure that no DNA remains in the tip after pipetting. + CENT.
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